CC9: Food Microbiology.

Unit 7: CULTIVATION OF MICROORGANISMS – PART 2

By

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Dear viewers, welcome to second part on cultivation of Microorganisms .This part will be covered under the following sections

Methods of enumeration-- qualitative and quantitative
Cultivation medium for microorganisms
Factors affecting microbial growth

Microbial analysis is useful to assess the safety and quality of food. Effective food control systems are essential to protect the health and safety of consumers. Microbial analysis is needed to maintain the quality of safety, quality of wholesomeness, to know the number of microorganism relate to product, to assess overall quality shelf life and safety of food. In addition, it is needed during handling practice, processing, fermentation, and to give a standard specification while maintaining the regulation and to identify presence of spoilage in food.

Methods of Enumeration--- qualitative and quantitative

1 <u>Direct methods:</u> Direct methods of enumeration involve counting the number of microorganisms directly, for example by counting the number of colonies on an agar plate or by using a microscope to count the number of cells observed.

a) Direct count of microorganisms (DMC) using a hemocytometer: A hemocytometer is a counting chamber as shown in **Fig.1** It is a thick glass slide containing a well in the central section. On the bottom of the well a grid is etched containing squares of known area. Each square is 0.04 mm^2. A cover slip is placed over this well forming a chamber of known depth (0.1mm). Thus, the volume of liquid that each square can hold is 0.004 mm3 (0.04 × 0.1= 0.004mm3). The sample to be counted is placed in the well by placing a drop of the sample at the edge of the cover slip so that it runs into the well and over the grid. The hemocytometer is then viewed using a microscope. The number of micro-organisms in several squares is counted and the average number of micro-organisms is calculated. This number of micro-organisms is then used to calculate the number of micro-organisms in the original sample.**Fig.1 depicts Direct microscopic count using hemocytometer**

b) Wet mount method: Wet mount is a method for observing microbial samples under microscope. Here a desired liquid samples is placed on the slide (Fig.2.). Thereafter, a cover slip is placed without forming any air bubbles. The fluid spreads out in a thin layer between cover slip and slide. The mount is now examined under the microscope at appropriate magnifications (e.g., 10x100 X). This method is commonly used to view microscopic organisms that grow on liquid media, especially when studying their movement and behavior. **Fig. 2 shows Procedure for wet mount method**

c) Dry mounts: Microorganisms, particularly bacteria, being too small need their permanent preparations which is made by drying and fixing them on clean slide with or without staining. For preparing a dry mount, a drop of distilled water with a small amount of culture is spread as a thin smear on a clean slide. The smear is allowed to dry and then ‘fixed’ by passing it through a flame two to three times with the smeared slide away from the flame. If desired, this dried

and fixed culture may be stained and dried again for observation under the microscope

d) Hanging drop mount: It is used to observe the motility or germination or fission of microorganisms **(Fig.3.).** In this method a cavity slide, which has a circular concavity in the centre, is used. The periphery of the concavity on the cavity slide is smeared with Vaseline. A drop of liquid microbial culture is placed in the center of the cover glass for liquid culture. If the culture is grown on a solid media, it is mixed with a drop of distilled water before placing on the cover glass. The cover glass is inverted over the concavity so that the drop hangs freely and the edge of cover glass adheres tightly to the Vaseline coated periphery of the concavity. The microorganisms present in the hanging drop are now observed for their type of mobility under the microscope **Fig. 3 Hanging drop method**

e) Simple stain: It is a quick procedure to determine the presence and to observe the morphology of bacteria in food samples. A simple staining technique involves the application of one stain. This helps in observing the cell shape and arrangement. However some samples do not stain with simple stains. e.g bacterial spores.

f) Differential stain: Differential staining involves the usage of 2 or more stains. Sometimes it also involves heating. Such staining techniques helps in differentiating between different parts of a cell, e.g. areas of fat storage. It can also differentiate the various groups of bacteria, e.g. between Gram-positive and Gram-negative bacteria **(Fig.4).** The reaction of bacteria to Gram's staining method is a consequence of differences in the chemical structure of the bacterial cell wall and is a key feature in their identification. The basis of Gram's staining method is the ability of the cell to get stained with crystal violet to retain the colour when treated with a differentiating agent, usually alcohol (although

professionals sometimes use acetone). They are further stained in the contrasting colour of a counter stain, usually pink/red. Bacteria that retain the violet/purple colour are called Gram-positive. Those that loose the violet/purple colour but take the pink/red are called Gram negative. Fig**.4 Gram staining procedure**

Please Note: Always use a young culture (18-24hours old). Older cultures of Gram-positive bacteria tend to lose the ability to retain the crystal violet-iodine complex and appear to be Gram-negative. Some bacteria are naturally weakly Gram-positive. The amount of alcohol treatment (the differential stage) must be judged carefully, because, over-treatment washes the crystal violet iodine complex from Gram-positive bacteria and they will appear to be Gram-negative.

2 Indirect methods: Indirect count is made by growing micro-organisms in liquid broth. As micro-organisms grow, the broth becomes cloudy or turbid. The turbidity is measured using a device i.e turbid meter or colorimeter or spectrophotometer.

a) Aerobic plate count (APC): Aerobic Plate Count (APC) is used as an indicator of bacterial populations on a sample. It is also called the aerobic colony count, standard plate count, Mesophilic count or Total Plate Count. The test is based on an assumption that each cell forms a visible colony when mixed with agar containing the appropriate nutrients. It is not a measure of the entire bacterial population but it is a generic test for organisms that grow aerobically at mesophilic temperatures (25 to 40 °C). The count is expressed as colony forming unit (CFU)/gm or ml.

APC does not differentiate types of bacteria. APC can be used to gauge sanitary quality, organoleptic acceptability, adherence to good manufacturing practices,

and to a lesser extent, as an indicator of safety. APC may also provide information regarding shelf life or impending organoleptic change in a food.

b) Standard Plate Count or Plate Loop Count (SPC) Plate loop count (PLC): Standard Plate Count or Plate Loop Count (SPC or PLC) is the measure of the total number of aerobic bacteria in the milk. The most common causes of a high SPC could be unhygenic milking equipment, poor cooling, and poor udder preparations. Mastitic cows can be responsible for high counts. The regulatory limit for SPC is 100,000 bacteria/ml of milk.

c) Spiral plate count (SPLC): The spiral plate count (SPLC) method is used for counting microorganisms in milk, food and cosmetics. In this method, a mechanical plater inoculates a rotating agar plate with liquid sample. The sample volume dispensed decrease as the dispensing stylus moves away from the center to the edge of the rotating plate. The microbial concentration is determined by counting the colonies on a part of the petri dish where they are easily countable and dividing this count by the appropriate volume. One inoculation determines microbial densities between 500 and 500,000 microorganisms/ml. Additional dilutions may be made for suspected high microbial concentrations.

d) Membrane filtration (MF): Membranes with a pore size that will retain bacteria (generally 0.45 um) but allow water or diluent to pass are used (Fig.5). Following the collection of bacteria upon filtering a given volume, the membrane is placed on an agar plate or an absorbent pad saturated with the culture medium of choice and incubated appropriately. Following growth, the colonies are enumerated. Alternatively, a DMC can be made. In this case, the organisms collected on the membrane are viewed and counted microscopically following appropriate staining, washing, and treatment of the membrane to render it transparent. These methods are especially suited for samples that

contain low numbers of bacteria. The use of fluorescent dyes and epifluorescent microscopes to enumerate bacteria in waters has been employed rather widely since the early 1970s. Cellulose filters were among the earliest used; however, polycarbonate Nucleopore filters offer the advantage of retaining all bacteria on top of the filter. The advantage and disadvantage for membrane filtration is given in **table 1. Fig.5 Estimation of cell numbers by membrane filtration**

Table. **1 Advantage and disadvantages of membrane filtration**

Technique	Advantages	Disadvantages
Membrane filtration	-Flexible sample volume range enabling the use of large sample volume and therefore increased sensitivity -Water soluble impurities interfering with the growth of target organisms separated from the sample in the filtration step -Quantitative result and good precision if the number of colonies grown adequate -Further cultivation steps not always needed, which lowers the cost and time needed for the analysis -When confirmation is needed isolation from well separated colonies on membrane is easy	-Quality of membrane varies -Solid particles and chemicals absorbed from samples to the membrane during filtration may interfere with the growth of the target organism -Not applicable to turbid samples -Scoring of typical colonies not always easy -Can be used for only samples with low microbial counts

e) Most probable number (MPN): The method was introduced by McCrady in 1915. This is a qualitative method for determination of the presence of

coliforms in potable water. The selective medium used is MacConkey's medium which contains a bile salt inhibitory for growth of non-intestinal lactose fermenting bacteria. Since the method is statistical in nature, MPN results are generally higher than SPC.

f) Dye reduction test: Dye reduction test involve the use of redox dyes like methylene blue to determine the quality of milk. Methylene blue is reduced and loses its color in the presence of actively growing bacteria. The time taken for the reduction of methylene blue is inversely proportional to the number of viable bacteria. The shorter the methylene blue reduction time higher is the microbial count and poorer is the quality of the milk.

3 Special methods for the cultivation of microorganism

The artificial culturing of microorganisms requires a supply of the necessary nutrients, along with the provision of appropriate conditions such as temperature, pH and oxygen concentration. All microorganisms require a good supply of nutrients. Macronutrients like carbon, nitrogen, oxygen, hydrogen, sulphur and phosphorus are required by all the organisms.these are required for all the biological activities like growth, reproduction etc. Micronutrients like magnesium, potassium, sodium, calcium and iron (in their ionised forms) are required in lesser quantities. Micronutrients are all metal ions, and frequently serve as cofactors for enzymes.

Cultivation medium for microorganisms

For cultivation of microorganisms we need to provide nutrients necessary for the organisms of interest. There are different ways to classify media:

a) Liquid medium: When the required nutrients are provided in the form of a liquid, it is called a broth. The liquids are usually placed in a test tube or a flask.

b) **Solid medium**: Liquid media that has been solidified by the addition of agar (usually 1.5 % w/v). Agar is a complex polysaccharide obtained from sea algae. The solid media can be placed in Petri dishes (agar plates) or test tubes with a large surface area (agar slants).

Media are classified based on the nutrient as follows:

1) Nutrient media: Are specific chemical formulations that contain all the nutrients and minerals that many microorganisms needs for normal growth.

2) Defined medium: A defined medium is one whose precise chemical composition is known.

3) Undefined medium: An undefined or complex medium is one whose precise chemical compositions are not known. An undefined or complex medium may have a variable composition due to the inclusion of a component such as blood, yeast and meet extract or tap water.

4) Selective medium: A selective medium is one that favors' the growth of particular organism or group of organisms. It often suppress the growth of others organisms. An enrichment culture uses a selective media component to encourage the growth of an organism present in low numbers. For example, mannitol salt agar is selective for Staphylococci because most other bacteria cannot grow in its high-salt environment. Another selective medium is brilliant green agar, a medium that inhibits Gram-positive bacteria while permitting Gram-negative organisms such as Salmonella species to grow.

5) Differential medium: A differential medium allows colonies of a particular organism to be differentiated form others growing in the same culture. These media provide environments in which different bacteria can be distinguished from one another. For instance, violet red bile agar is used to distinguish coliform bacteria such as *Escherichia coli* from non-coliform

organisms. The coliform bacteria appear as bright pink colonies in this media, while non-coli forms appear a light pink or clear.

Factors affecting microbial growth

Microorganisms require favorable physical parameter like temperature, pH, aeration etc.

a) Temperature: Microorganism is able to grow over a wide range of temperature from freezing to above boiling point. Every microorganism has minimum and maximum growth temperature. Growth is slower at lower temperature because enzymes work less efficiently and also because lipids tend to harden and there is a loss of membrane fluidity. The optimum temperature is generally closer to the maximum growth temperature than the minimum (Fig. 6).

Fig 6. Effect of temperature on microbial growth rate.

Microorganism can be categorized based on the temperature at which they grow (Fig 7)

(i) **Mesophilies** : Microorganism has optimal growth around 20 to 45 ℃.

Example: *Staphylococcus aureus, Salmonella, Listeria, Pesudomonas, E.coli*, *Penicillium* spp, *Aspergillus* spp. *Mucro* spp, *Rhizopus* spp.

(ii) **Thermophiles:** Those microorganisms capable of growth within a range of about 40 to 80 ℃, with optima around 50 to 60 ℃ They are adapted to not only surviving, but thriving at much higher temperature.

Example: *Hydrogenobaculum, Thiomonas, Acidimicrobium, Humicola, Thermoascus.*

(iii) **Extreme theromophiles or Hyper thermophiles**: These are microorganism which have optimum value and can tolerate temperature excess 100 ℃.

Example: *Thermococcus barophilus, Thermus aquaticus, Thermoccus itorali*

(iv)**Psychrophiles**: Physchrophiles occupy the other extreme of the temperature range; they can grow at 0 ℃, with optimal growth occurring at 15 ℃ or below.

Example *Arthrobacter* spp. *Psychrobacter* spp. *Halomonas* spp, *Hyphomonas* spp, *Sphingomonas* spp. *Aureobasidium pullulans, Phoma* spp. *Chrysosporium*

(v) **Psychrotrophs:** They can also grow at 0 ℃, have much higher temperature optimum (20-30 ℃).

Fig.7 Classification of microorganisms on basis of preferred growth temperature

b) pH

Microorganisms are strongly influenced by pH of their surroundings. The pH range (between minimum and maximum values) is greater in fungi than in bacteria. Based on their tolerant and optimum growth they can be acidophilic, neutrophile, alkalophile **(Fig. 8)**

Fig. 8 Type of microorganisms based on the pH requirement for their growth.

c) Oxygen

Earth atmosphere consist oxygen (20%) and most of life forms are dependent upon it for survival and growth. Various types of oxygen requirement by microbes is give in **Fig 9**

(i) **Aerobes:** Microorganism which require oxygen for growth.

Examples: Bacteria (*Alcaligenes, Pseudomonas, Lactobacillus, Leuconostoc, Micrococcus, Bacillus, Serratia marcescens, Flavobacterium*). Yeast (*Rhodotorula), Molds (Rhizopus, Aspergillus, Penicillium, Mucor, Cladosporium, Sporotrichum*)

(ii) A**naerobes**: They are able to survive in the absence of oxygen or grow in the absence of oxygen.

Examples: Bacteria (*Alcaligenes, Clostridium* spp)

(iii) **Obligate anaerobes:** Microorganism cannot tolerate oxygen at all requires culturing in special anaerobic chambers.

Example: *Clostridium botulinum, C. tetani, C. perfringens, Mycobacterium tuberculosis, Piromonas, Sphaeromonas*

(iv)**Facultative anaerobes:** They are able to grow like aerobes in the presence of oxygen, but have the added facility of being able to survive when conditions become anaerobic.

Example: *Staphylococcus spps, Listeria* spp, *Saccharomyces cervisiae*.

(v) **Aerotolerant** anaerobes: They are basically anaerobic, not inhibited by oxygen, which they do not utilize it.

Example: Thiobacillus, Thiococcus.

(vi)**Microaerophiles:** They require oxygen, but are only able to tolerate low concentration i.e 2-10%.

Example: *Campylobacter* spp, *Helicobacter pylori,*

Fig.9 Effect of oxygen on the growth of various types of microorganisms

d) Osmotic pressure:

Osmosis is the diffusion of water across a semipermeable membrane from a less concentrated solution to a more concentrated one, equalizing concentrations. The pressure required to make this happen is called the osmotic pressure. Microorganisms able to tolerate NaCl concentration of between 0.5 to 3.0 per cent are classified as osmotolerent microbes.

(i) **Haloduric:** They are salt –tolerate bacteria able to tolerate concentration ten times as high, but prefer lower concentration.

(ii) H**alophilic:** They are salt loving; forms are adapted to grow best in condition of high salinity such as those in Dead Sea.

Conclusion

There are a number of methods for enumeration of microorganism. Every methods used in the detection of microbial load has its own advantages and limitation and the application depends on the type of samples. In addition, it is also very important to know physical parameters required for cultivation and enumeration of microorganism. As microbial growth depends on parameters like pH, temperature, aeration etc.

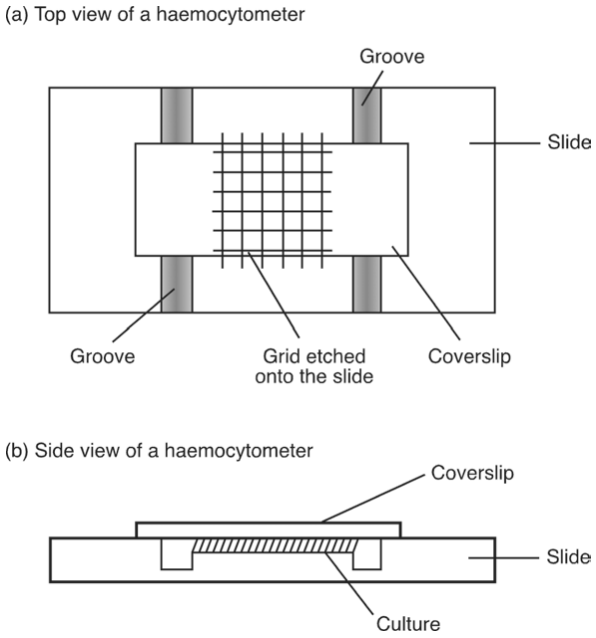


Fig.1 Direct microscopic count using hemocytometer

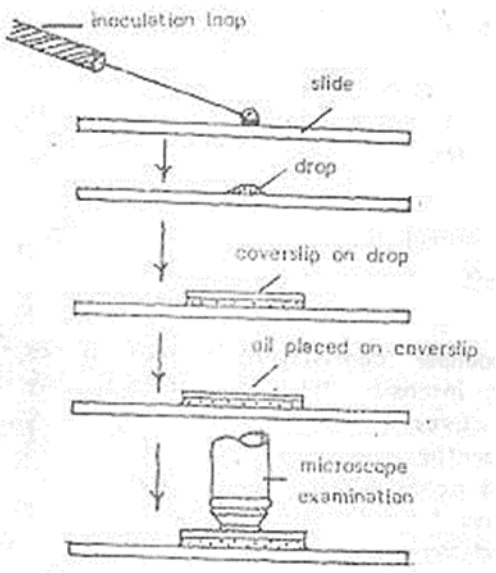


Fig. 2 Procedure for wet mount method

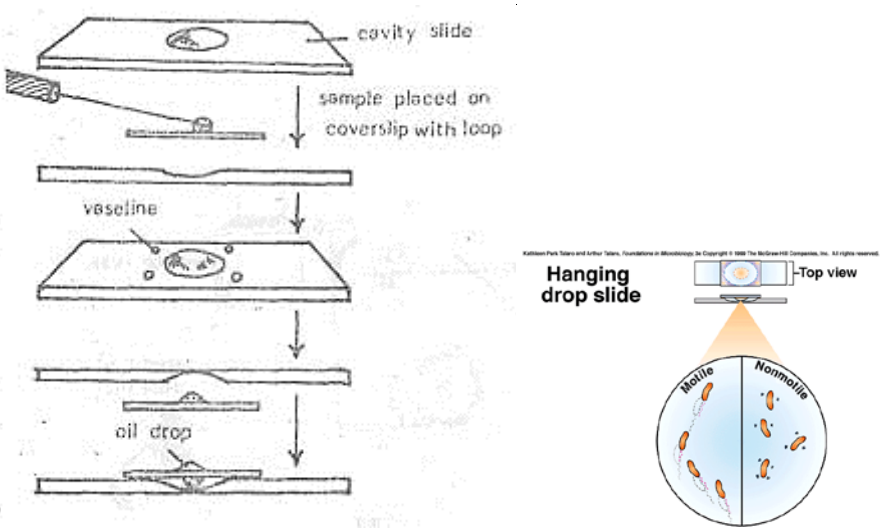


Fig.3 Hanging drop method

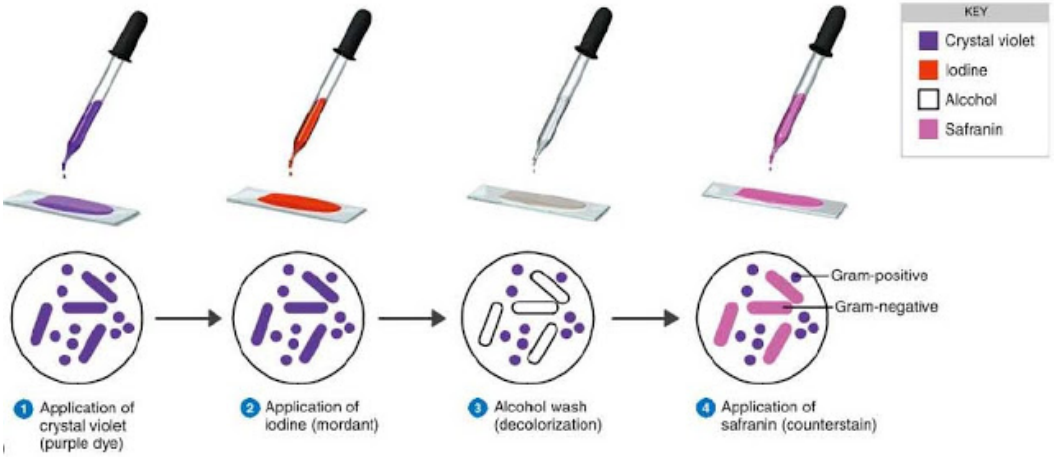


Fig. 4 Gram staining procedure

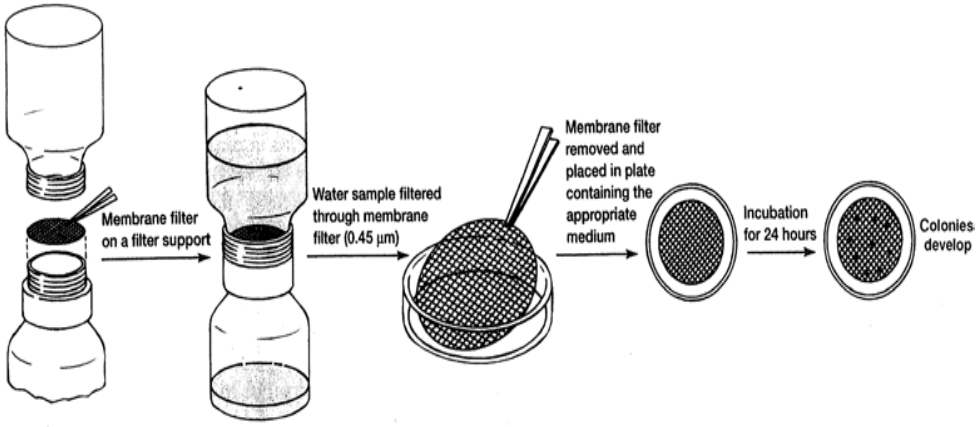


Fig.5 Estimation of cell numbers by membrane filtration

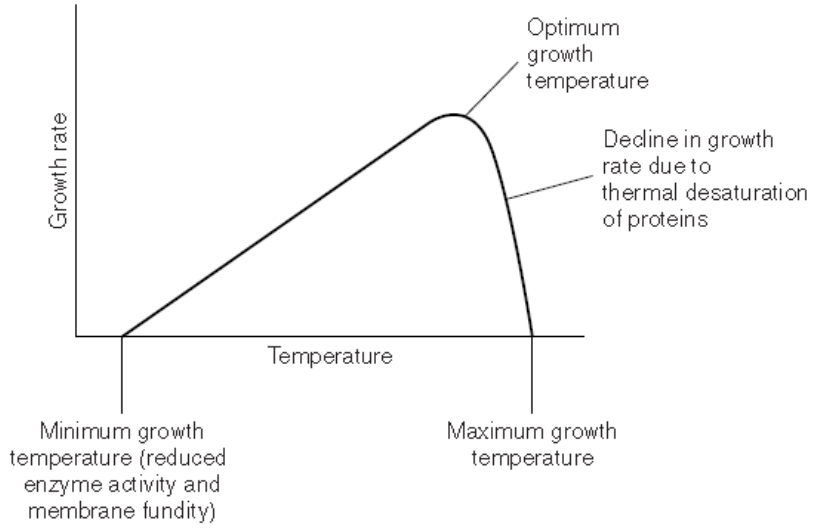


Fig. 6. Effect of temperature on microbial growth rate.

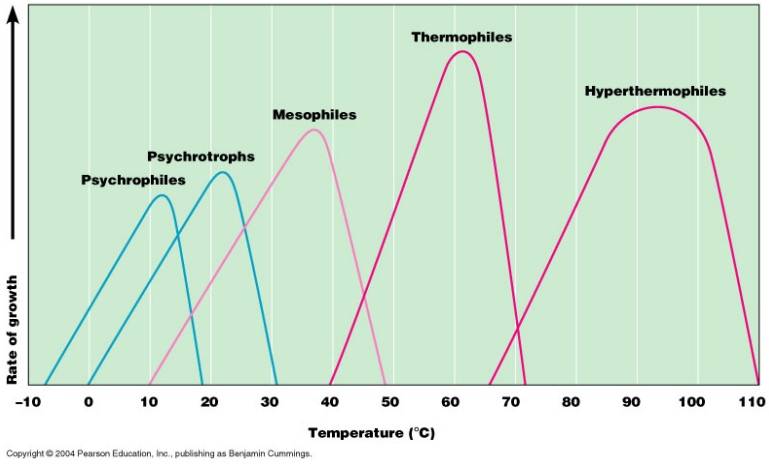


Fig. 7 Classification of microorganisms on the basis of their preferred temperature

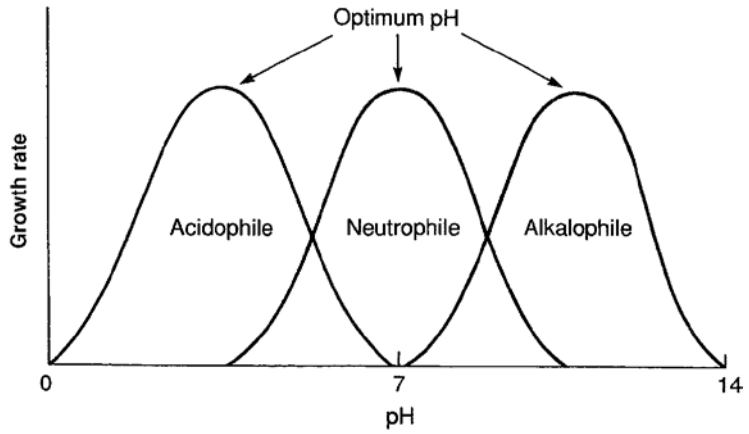
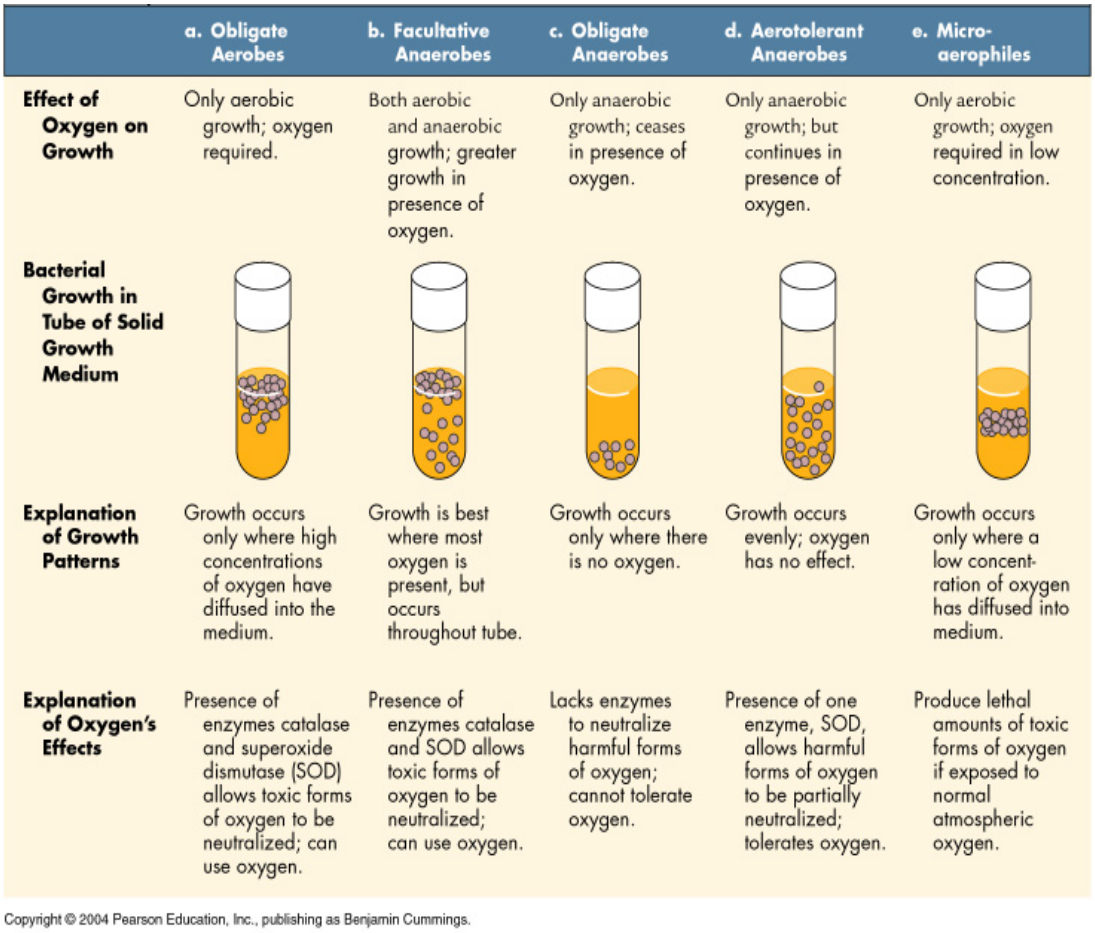


Fig. 8 Classification of microorganisms based on pH



	a. Obligate Aerobes	b. Facultative Anaerobes	c. Obligate Anaerobes	d. Aerotolerant Anaerobes	e. Micro-aerophiles
Effect of Oxygen on Growth	Only aerobic growth; oxygen required.	Both aerobic and anaerobic growth; greater growth in presence of oxygen.	Only anaerobic growth; ceases in presence of oxygen.	Only anaerobic growth; but continues in presence of oxygen.	Only aerobic growth; oxygen required in low concentration.
Bacterial Growth in Tube of Solid Growth Medium					
Explanation of Growth Patterns	Growth occurs only where high concentrations of oxygen have diffused into the medium.	Growth is best where most oxygen is present, but occurs throughout tube.	Growth occurs only where there is no oxygen.	Growth occurs evenly; oxygen has no effect.	Growth occurs only where a low concentration of oxygen has diffused into medium.
Explanation of Oxygen's Effects	Presence of enzymes catalase and superoxide dismutase (SOD) allows toxic forms of oxygen to be neutralized; can use oxygen.	Presence of enzymes catalase and SOD allows toxic forms of oxygen to be neutralized; can use oxygen.	Lacks enzymes to neutralize harmful forms of oxygen; cannot tolerate oxygen.	Presence of one enzyme, SOD, allows harmful forms of oxygen to be partially neutralized; tolerates oxygen.	Produce lethal amounts of toxic forms of oxygen if exposed to normal atmospheric oxygen.

Fig.9 Effect of oxygen on the growth of various types of microorganisms