temperatures above 40°C– 45°C while some grow in hot springs, (b) *Mesophiles* which normally grow in the temperature range of 25°C–40°C though the optimum temperature is 37°C for most microbes, and (c) *Psychrophiles* (*Cryophiles*) which actively grow below 20°C. They are cold tolerant and are often found in arctic and antarctic regions.

Thermal resistance of bacterial spore can also be calculated, since bacterial spores are more resistant to temperatures than the vegetative cells, where D_{250} is the time in minutes referred to *one log cycle* (90%) reduction in population of a given microbe (spores) in a given medium at a process temperature of 250°F.

For *Clostridium* B., D_{250} = 0.21 minute

From 1 spore to 10^{-12} spores = 12 log cycles.

Thus if 1 log cycle = 0.21 minutes at 250°F, then for 12 log cycle, time required = 0.21 × 12 = 2.52 minutes at 250°F.

But for vegetative cells it takes 1 minute at 160°F.

(iii) *pH.* It influences microbial growth through the action on enzyme activity and cell permeability. Normal bacterial growth can occur at a pH range of 6–8, while a few can tolerate it below 4, e.g. for *E. coli*, it is 4–7; for *Thiobacillus*, 1–4, while for nitrate bacteria, it ranges from 6.5–9.

(iv) *Osmotic pressure (OP).* At higher osmotic potential, microbes can take water by osmosis, while at lower OP, water comes out of cells (*exosmosis*). This feature is exploited in food preservation with higher salt or sugar use. Some species can grow at a very low osmotic potential and are called *halophiles*.

Control of bacterial growth

The growth of bacteria can be controlled either by physical or by chemical means. When a germicidal agent kills a bacterium by way of lysis of cell membrane it is called *bacteriocidal*. Otherwise, if it only prevents multiplication (though still viable), it is called *bacteriostatic*.

(i) ***Physical agents.*** Major physical agents include temperature (heat), uv-radiation, ultrasonic waves, etc.

Temperature above 120°C or below –20°C is detrimental. Thermal death time refers to the shortest period to kill bacterial cells/spores in a suspension (Fig. 3.17). For *E. coli*, it is 20–30 mins. at 57°C, whereas for *Salmonella* it is 4 mins. at 60°C.

UV-radiation. The germicidal effectiveness of ultraviolet radiation lies at 2600 Å. It disrupts the protoplasm and the enzyme system.

Ultrasonic waves. Vibration of the ultrasound (>20000 Hz) causes a sort of cavity in the water around the microbial cell and thus puts a mechanical pressure causing decomposition of protein and disruption of the membrane.

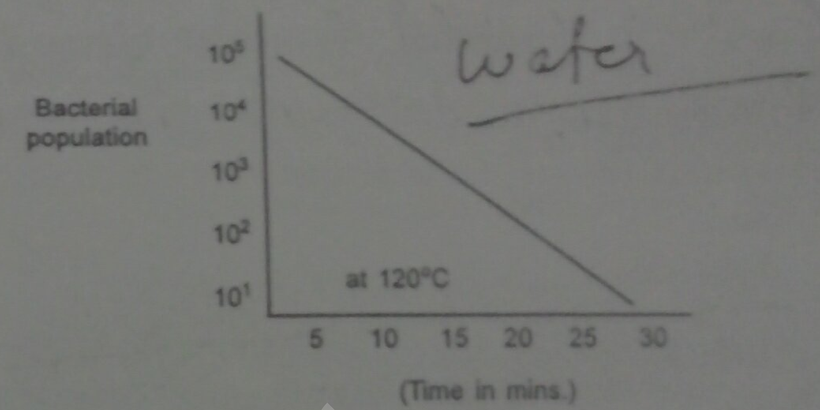

FIG. 3.17 Thermal death curve.

(ii) ***Chemical agents.*** Apart from the different antibiotic drugs, a large number of chemical compounds have the ability to inhibit bacterial growth. Some of them are phenol and its derivatives, acids and alkalies, salts like NaCl which change the cellular ionic balance, detergents, chlorine, heavy metals and organic compounds like PAB (*p*-aminobenzoate) and PAS (*p*-aminosalicylic acid), etc.

Phenol is a well-known bacteriocidal compound. It kills bacteria by damaging the membrane and denaturating the protein. It was first used by Joseph Lister in 1880.

The effectivity of a chemical agent is assessed by a comparative test with reference to phenol and this is done by determining the *phenol coefficient* (P.C.) of the compound, which is the ratio of the minimal sterilizing concentration of phenol to that of the compound. A germicide is generally recommended for use at 5 times this concentration.

$$\text{P.C.} = \frac{\text{Reciprocal of the dilution of the agent}}{\text{Reciprocal of the dilution of the phenol}} = \frac{X}{P}$$

Potassium permanganate is not a very powerful disinfectant, unlike chlorine. But HgCl at 1 ppm can control microbial growth.

Microbial antagonism may also deter the growth among different microbes. It is effected through the production of toxins. The toxin *colicin*, produced by the plasmid of *E. coli* in the gut of man, can control the development of *Salmonella* and *Shigella*.

3.6 BACTERIOLOGY OF WATER AND SEWAGE

Microorganisms, particularly bacteria, play an important role in aquatic system by the decomposition of organic matter. Hence water rich in organic matter encourage the growth of microbes, some of which could be pathogenic and

cause various diseases in both man and animal. Some of the water borne diseases are cholera, typhoid, paratyphoid, dysentery, bacillary dysentery, gastroenteritis etc., which sometimes turn into epidemics and cause havoc. The organisms responsible are *Bacillus, Vibrio, Salmonella, Klebsiella, Shigella, Clostridium, Streptococcus*, and *Escherichia* (Bonde, 1977).

Microbial flora in potable water primarily originate from untreated or partially treated sewage, tannaries, and food processing industries. Fortunately, not all of them are pathogenic, and even some pathogenic ones do not survive long, yet we have so many water borne diseases that sometimes turn into epidemics.

The degree of microbial pollution also varies with the type of natural water which is primarily classified as (i) *atmospheric water* from precipitation; (ii) *surface water* derived from rain and run-off; (iii) *stored water* in tanks and pools; and (iv) *ground water* much below the upper soil layers. Of them, the ground water is least polluted by bacteria due to the filtering action of soil layers and the absence of organic matter.

The potable or drinking water must be free from taste, odour and toxic chemicals apart from the pathogenic organisms.

3.6.1 Bacteriological Examination of Water

It is periodically done to detect the pollution from domestic sewage containing a number of pathogenic and non-pathogenic forms and to eliminate their possibility of transmission.

More often the examination is based on non-pathogenic forms and that too not on a single type, but from a group of them commonly called *Coliform*, and whether they are present or absent in the test water sample.

It can be quantified and is often done from the approximate numerical determination, MPN, i.e. most probable number.

3.6.2 Indicator or Index Organism

The most important groups of non-pathogenic enteric bacteria in sewage-polluted water are *Escherichia coli* (rod-shaped) and faecal streptrococci, *Streptococcus faecalis* (round cells). They are selected as indicators due to their constant presence in such type of polluted water along with the pathogenic ones and themselves are non-pathogenic. Other index organisms are *Clostridium perfingens* and *Biefidobacterium bifidus*. They can be selectively identified by simple cultivation methods (Allen and Geldreich, 1975; Gaudy and Gaudy, 1981).

3.6.3 Why Coliform?

Detection and estimation of pathogenic bacteria in water is tedious, time consuming and laborious too, whereas the *coliform* group is easily tested and

enumerated (Cabelli, 1982). It gives indication that the pollution is of faecal origin and accordingly, suitable steps can be undertaken within a short period. Moreover, the members of this group remain viable for a longer time and allow sufficient time for testing. But faecal streptococci survive for lesser time than *E. coli*. So their presence in high number in polluted water indicates that the pollution is of recent origin (Wilhm and Dorris, 1968; Train, 1979; Suess, 1982).

The advantage of such microbial tests is that they help in distinguishing pollution of faecal origin from the non-faecal one. Raw sewage contains approximately 10^7 *E. coli*/100 ml. On the basis of bacterial estimations, some *microbial standards* of water quality from different sources have been formulated, viz.

Highly polluted	≥	10,000	bacteria/litre
Moderately polluted	≥	1,000	bacteria/litre
Slightly polluted	≥	100	bacteria/litre
Water of satisfactory quality	≥	10	bacteria/litre
Pure drinking water	<	3	bacteria/litre

3.6.4 Routine Bacteriological Analysis

Proper sample collection in aseptic conditions, dechlorination with sodium thiosulphate and their testing in laboratories within shortest possible time is absolutely essential, although samples can be preserved at 4°C for 6 hours. But in no case the sample can be kept for more than 24 hours to avoid rapid changes in bacterial content. The methods of analysis comprise various tests that indicate the presence of coliforms and pathogens.

(a) *Standard plate count (SPC)*

It is done to enumerate total viable population and not to detect either coliform or other pathogenic forms present therein. This is primarily to check the efficiency of water treatment methods, and is done periodically. A bacterial spore is assumed to form a colony. It is expressed in numbers per ml.

$$SPC = m \times 10^n$$

where m is the number of colonies and n is the dilution factor.

(b) *Tests for coliforms*

There are two standard methods for estimation of total coliforms in water sample, viz. multiple tube fermentation (MTF) test and membrane filter (MF) technique. Both the techniques are in use and each one of them is recognized as a reliable method.

Multiple tube fermentation. It is essentially an acid fermentation system, where fermentation of lactose broth is tested at 35–37°C for 24 and 48 hours in a series of test tubes (Fig. 3.18). It actually involves three sequential tests (Pelczar *et al.*, 1986).

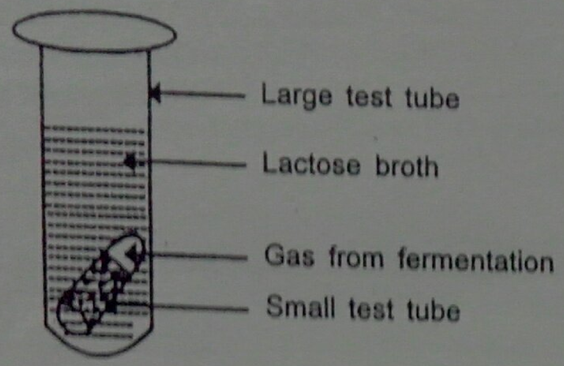

FIG. 3.18 MTF test.

1. Presumptive test. The source sample is diluted several times as per the expected bacterial population and allowed to incubate in the growth medium for 24 hours. The acid and gas production is checked after the scheduled period. If gas is formed, then the test is positive. If there is no gas formation, the test is negative.

2. Confirmed test. If no gas is formed in the previous test, it is again incubated for another 24 hours. If gas is formed now, it confirms the presence of coliform. On the other hand, if there is no gas formation even now, the test is considered negative, i.e. it can be presumed that there is no coliform in the tested sample. It is a confirmatory test for the presence of coliform, because gas production in the previous test could also be due to the presence of organisms other than coliform.

3. Completed test. This test ascertains the presence of coliform in the sample. Here a portion of the sample from the positive test is grown on Endo or EMB (eosin and methylene blue) agar plates and is incubated for 24 hours at 35°C. In this medium, *E. coli* will produce blue-black colonies with a greenish metallic sheen; whereas other members of the group will produce pale pink colonies without a metallic sheen.

Membrane filtration technique. The technique was first suggested by Goetz and Tsureishi in 1951 and named originally as *molecular filter technique.*

Membrane filter (MF) is made of cellulose acetate ester with a pore size of 0.3 to 0.5 μm. The filtration unit has the effective diameter of 10 nm. The membrane is first sterilized at 80°C for 20 minutes in distilled water and then put on the filtration unit aseptically, which is fixed to a vacuum pump. (Fig. 3.19). If necessary, a special type of syringe is also used for proper placement of the membrane (Stainer *et al.*, 1990).

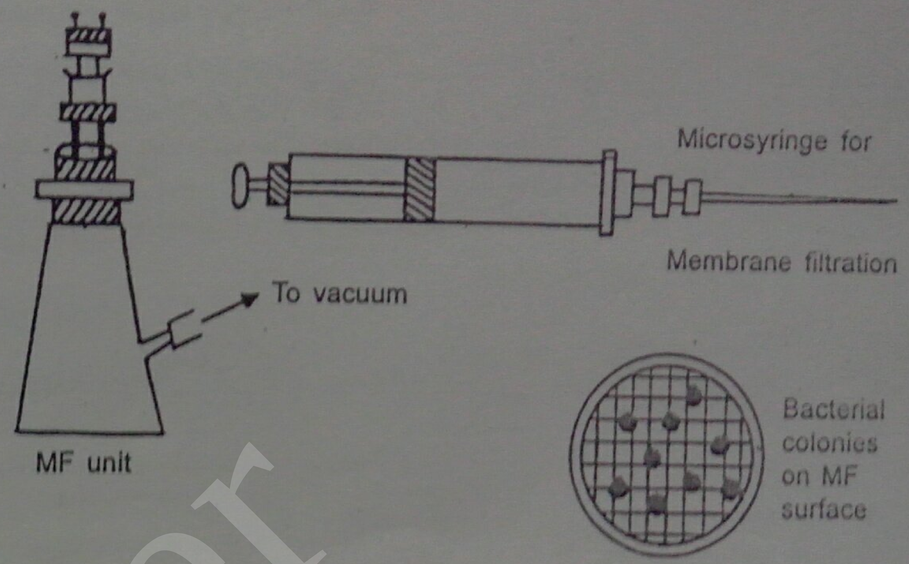

FIG. 3.19 Membrane filtration.

When the water sample is poured, the bacteria are held on the membrane surface (sample taken may be 0.1 to 1 ml of polluted water). The filter with the trapped bacteria is then dried and stained with a red dye called *erythrosine* for 30–60 mins. The filter is now placed on another filter saturated with distilled water and repeated to remove the excess stain. Finally, slight pink colour remains and the filter is microscopically examined and the microbes counted.

The result is expressed as

$$X = K \frac{N}{V}$$

where,

X = total no. of microbes
V = volume of water examined (usually 1 ml)
N = average no. of microbes in one microscopic field of view
K = coefficient of microscope, i.e. ratio of the size of the field (area of eye piece grid) to the area of the membrane filter.

Large sampling is, however, necessary to have a statistically significant result.

The advantage of MF over MTF technique is that it can be done rapidly and on the spot (field) using a hand pump. But it cannot be used in case the water is turbid and heavily polluted, as the filter gets clogged.

The degree of pollution is generally expressed in terms of (a) *Colititer* which is the smallest volume of water (in ml) containing one *E. coli*; and (b) *Coli index* that is the number of *E. coli* present in 1000 ml of water.

However, both MTF and MF methods are subject to false-positive and false-negative results (Border and Winter, 1978). Hence a rapid *colilert system*

(defined substrate technology) has been developed for the detection of total coliform and *E. coli* in water. This method can simultaneously determine the presence of total coliforms with yellow colour as indicator and production of fluorescence in the same tube demonstrates the presence of *E. coli* (Vaheri *et al.*, 1991). It is based on the concept that for each target microbe, there is a substrate for a specific enzyme. When the microbe digests the substrate a *chromogen* is produced. The substrate for total coliform in this case is ONPG (o-nitrophenyl β-D-galactopyrannoside), which turns yellow. While for *E. coli*, the substrate is MUG (4-methylumbelliferyl β-D-glucuronide), which produces fluorescence.

3.6.5 Methods for Differentiating Faecal from Non-faecal Coliforms

Among the enteric bacteria that can ferment lactose by producing acid and gas (i.e. coliform), all may not be obligate (completely) intestinal parasites like *E. coli*. For example, *Enterobacter* (*Aerobacter*) *aerogenes*, though can ferment lactose, is also found in nature in decaying plant materials. Therefore its presence in water may not necessarily indicate a faecal contamination. Thus it is essential to determine whether the coliform is of faecal or non-faecal origin.

Some of the preliminary differences between the two organisms are:

Characters	*E. coli*	*Ent. aerogenes*
(i) Colony size	Smaller (2–3 mm)	Larger (4–6 mm)
(ii) Colony surface	With metallic sheen	Without metallic sheen
(iii) Colony confluence	Discrete	Tend to coalesce
(iv) Colony elevation	Concave	Convex
(v) View under reflected light	Dark	Light brown
(vi) Incubation at 44°C for 24 hrs.	It can grow	It cannot grow

However, to have a final distinction between the two types of organisms and to conclude the faecal or non-faecal nature of the pollution, a series of physiological/biochemical tests are performed which are collectively known as IMViC test.

IMViC test

It consists of a series of four tests to rapidly identify *E. coli* (faecal) and

Enterobacter aerogenes (non-faecal). The letters in IMViC have different connotations which are as follows:

'*I*' represents Indole test
'*M*' is the Methyl red test
'*V*' stands for Voges-Proskauer test, and
'*C*' is the citrate test, while '*i*' is used only for phonetic purpose and has no significance with the test systems.

Indole test

It is a test based on protein catabolism, where indole is produced from tryptophan. It actually indicates whether the enzyme tryptophanase is present or not in the test organism.

Tryptophan + O_2 ⟶ Indole + Pyruvic acid
(Kovac's Reagent used is p-dimenthyl aminobenzaldehyde) ↓ Kovac's reagent gives Bright red colour indicating indole production

Methyl red test

It is a test for acid production from glucose. It is actually a measure of pH when sugar is broken down to acid, and at pH lower than 4.5, the red colour produced is a sufficient indicator of acid formation.

Voges–Proskauer test

It is a colour test of acetoin production from glucose, which is an intermediate stage in the production of butanediol from pyruvic acid. Acetyl methyl carbinol (AMC) in presence of 40% KOH and air is further oxidized and gives the characteristic pink colour within 2–4 hrs, which is a positive test for *Enterobacter aerogenes*.

Citrate test

It attempts to ascertain the ability to utilize citrate from sodium citrate as the sole carbon source. *E. coli* has not got the requisite enzyme citrate permease and cannot utilize the same, whereas *Ent. aerogenes* can assimilate it and grow so that turbidity develops during the culture.

Thus IMViC test differentiates the two organisms in the following manner:

Organism	*Tests*				
	I	*M*	*V*	*C*	*Pollution source*
E. coli	+	+	–	–	Faecal
Ent. aerogenes	–	–	+	+	Non-faecal

3.6.6 Most Probable Number (MPN) Estimation

It is the statistical expression of estimating the number of bacterial cells in a culture or a water sample. It gives an approximate estimation of the number of coliform bacteria present per ml in a given decimal serial dilution in the MTF (multiple tube fermentation) test (Fig. 3.20).

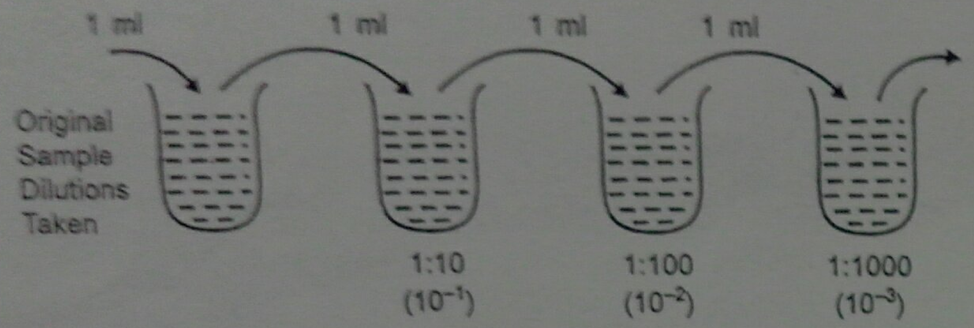

FIG. 3.20 Serial dilutions of sample.

The value is arrived at by assuming that the smallest gas-forming portion in a geometric series contains only one coliform organism. Therefore, reciprocal of the volume expressed in ml will equal the number of organisms per ml. It often happens that from a combination of positive and negative results, one has to draw an approximate value.

If the fermentation tube containing 0.1 ml of the same gives positive result, while the tube with 0.01 ml does not, then the MPN is considered as 10 per ml. In this, a statistical estimation is made and compared with MPN index value table (APHA, 1971). In fact, a sanitary bacteriologist is not specifically interested in exact number, but the approximate one to take effective control measures.

3.7 FUNGI

Fungi (sing. fungus) are a class of eukaryotic, spore-bearing non-chlorophyllous organisms and include molds and yeasts. Molds are those which have multicellular filamentous bodies and yeasts are unicellular. They have a wide range of appearence from small microscopic filaments to large macroscopic structures (Fig. 3.21). The filaments (thread like structure) known as *hyphae* (sing. hypha), which form a wooly mass called mycelium. These filaments may be made up of several cells put end to end, or within one long thread there may be many nuclei, when it is called *coenocytic*. The mycelia (sing. mycelium) may coalesce and form a regular macroscopic vegetative structure called *fruit-body* as in higher groups of fungi. The study of this group of organisms is termed *mycology* (*mykes* = mushrooms).

Chief characteristics

1. Lack of chlorophyll (green pigments)
2. Cell wall is made of *chitin*, instead of cellulose.
3. Food reserve is *glycogen* (like animals) and *not* starch as in plants.
4. Nuclei within cells are very small.
5. Major mode of reproduction is asexual by *spore* formation. Some may reproduce sexually by conjugation.
6. They may be sub-terranean (grow under the soil), terrestrial or aquatic. Some grow as parasites on plants or on organic food stuff and make them rot (as on breads and fruits like oranges and lemons). There are also pathogenic forms causing diseases in human beings, plants and animals.

Although fungi are economically important for some industries like pharmaceutical yielding drugs (alkaloids, vitamins and antibiotics) and breweries in the alcohol fermentation, they cause heavy loss as parasites of crop plants and forest trees, and in rotting food materials. Some of these fungi are edible too, for example, mushrooms of the varieties called toad-stool, morel, morchella, etc. Edible mushroom cultivation has become an industry by itself. Some fungi grow in association with the roots of higher plants for mutual benefit and are called *mycorrhizae*.

From environmental standpoint, fungi can grow in aquatic reservoirs, sewage structures, air-borne spores of many fungi cause various skin diseases of man and the fungal toxins (mycotoxins) called *aflatoxins* produced by some fungi (*Aspergillus, Fusarium*) are very much harmful (Ross, 1979).

3.7.1 Classification

Fungi are broadly divided into three groups:

Lower fungi or phycomycetes

They are alga-like filamentous, primitive and microscopic. The mycelium is non-septate (i.e. not partitioned by wall), i.e. *coenocytic*. No fruit body is produced. Spores are produced endogenously (inside the hyphae). Aquatic fungus *Saprolegnia* belongs to this group having a long, much branched multinucleate mycelium.

Mucor and *Rhizopus*, which are called bread molds, also belong to this group. *Rhizopus arrhizus* mycelium is now frequently used to recover minerals from mine waste effluent through adsorption on its biomass.

Higher fungi

They have definite fruit bodies made of compact mycelia, which have septate hyphae with partitions formed in them, and are evolutionary advanced. There are two sub-groups.