



ALAGAPPA UNIVERSITY

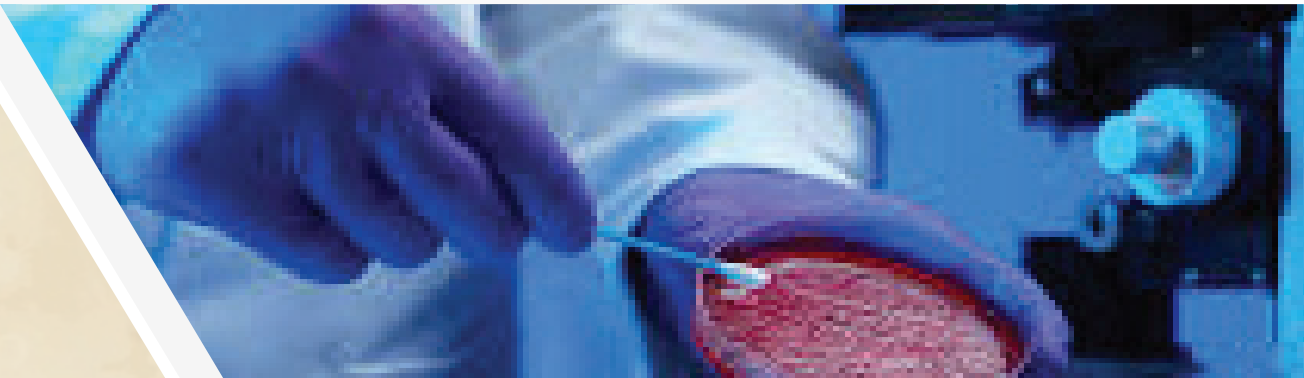
[Accredited with 'A+' Grade by NAAC (CGPA:3.64) in the Third Cycle
and Graded as Category-I University by MHRD-UGC]

KARAIKUDI – 630 003

DIRECTORATE OF DISTANCE EDUCATION



M.Sc. [Microbiology]
364 14



**LAB I – GENERAL MICROBIOLOGY, MICROBIAL
PHYSIOLOGY AND BIOCHEMISTRY**

I - Semester



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(A State University Established by the Government of Tamil Nadu)

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LAB I – GENERAL MICROBIOLOGY, MICROBIAL PHYSIOLOGY AND BIOCHEMISTRY

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Appendix: (I-IV)

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SYLLABI-BOOK MAPPING TABLE

LAB I – General Microbiology, Microbial Physiology and Biochemistry

Syllabi

General Microbiology

1. Principle and methods of sterilization.
2. Preparation of media: nutrient broth, nutrient agar plate, and soft agar.
3. Pure culture techniques: streak plate, spread plate and pour plate.
4. Motility determination – Hanging drop method.
5. Isolation and enumeration of bacteria from different environmental samples.
6. Enumeration of bacteria – viable count (plate count) and total count (Haemocytometer count).
7. Direct microscopic observation of fungal spores and mycelium.
8. Fungal slide culture.
9. Staining method: simple, negative, Gram's staining and spore staining.

Biochemistry

10. Measurement of growth rate and generation time by turbidometry method.
11. pH metry -Preparation of buffer.
12. Spectrophotometry- Wavelength scan.
13. Chromatography - Paper and Thin layer chromatography - separation of amino acid.

Microbial Physiology

14. Carbohydrates: Quantitative estimation of glucose, glycogen from bacterial and yeast cell.
 15. Protein- Quantitative estimation of protein from bacterial yeast cell.
 16. Enzyme- Estimation of alkaline phosphatase activity.
 17. Environmental factor- Effect of temperature and pH on bacterial growth.
 18. Physiological groupings of bacteria- Isolation of saccharophilic microorganisms (starch hydrolysis)- Proteolytic activity of microorganisms (casein and gelatin hydrolysis)- Lipolytic activity of microorganisms.
 19. Utilization of Unusual compounds- Microbial degradation of azodyes.
 20. Bioenergetics- Cytochrome oxidase assay - Catalase assay.
 21. Nitrogen metabolism- Nitrate reduction test.
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INTRODUCTION

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There are millions of different organisms that inhabit the planet Earth. The most common forms of organisms are those which can be seen without the aid of any magnifying devices. On the other hand, there are some organisms which are smallest of the small and the simplest of the simple.

Microbiology is that branch of biology which studies microorganisms and their effects on human beings and other living organisms. The study of microorganisms include the unicellular or cell-cluster microscopic organisms and is subdivided into physiology, bacteriology, virology, mycology, parasitology, and many more fields. The importance of microbiology in medical science cannot be underestimated. It is useful in waste management, medicine, research, mining, food production as well as biotechnology.

Microbial Physiology is the study of how microbial cell structures, their growth and metabolism function in living organisms. It covers the study of viruses, bacteria, fungi and parasites. The study of microbial cell functions includes the study of bacterial growth, microbial growth, microbial metabolism and microbial cell structure. Microbial physiology is important in the field of metabolic engineering and also functional genomics. Fundamentally, microbial physiology is an enormous discipline encompassing the study of thousands of different microorganisms.

Biochemistry involves the study of the chemical components and describes the structures and functions of cellular elements, which includes proteins, carbohydrates, lipids, nucleic acids and several other biomolecules. It also studies the chemical properties of these cellular, and in particular the chemistry of enzyme-catalysed reactions. Today almost each area of the life sciences is involved in microbiological and biochemical research. These researchers aim at understanding how biological molecules lead to the processes occurring within living cells. This mechanism is greatly related to the study and understanding of whole organisms. Practical applications of biochemistry are virtually boundless. Pharmaceuticals and genetics are the two of the biggest fields of work that require the knowledge of biochemistry, genetics, molecular biology and biophysics.

This book, *General Microbiology, Microbial Physiology and Biochemistry*, deals with the practical aspects of qualitative and quantitative analysis of the techniques in the laboratory. Characteristically, a qualitative analysis helps in determining the methods of sterilization, preparation of media, pure culture techniques, motility determination, isolation and enumeration of bacteria from different environmental samples (the isolation, identification and sterilization of bacteria), staining method, pH analysis, spectrophotometry, chromatography; while the quantitative estimation helps in determining the quantitative analysis of glucose, glycogen from bacterial and yeast cells, protein, etc.

PRACTICE AND SAFETY RULES IN LAB

*LAB I – General
Microbiology, Microbial
Physiology and
Biochemistry*

1. First clean your hand (by washing with soap).
2. No eatable allowed in Lab.
3. Wear a Lab-coat and safety glasses.
4. Wear appropriate shoes (sandals are not allowed) in the Lab.
5. Place needed items on the floor near your feet, but not in the aisle.
6. Label/mark everything clearly.
8. Do not open Petri dishes in the Lab unless absolutely necessary.
9. Please keep off Bunsen burners when it is not in use.
10. Treat all microorganisms as potential pathogens. Use appropriate care and do not take cultures out of the Lab.
11. Wear disposable gloves when working with potentially infectious microbes or samples.
12. When you are working in the Lab and dealing with equipments then first sterilize equipments and materials.
13. Consider everything a biohazard. Do not pour anything down the sink.
14. Dispose of all solid waste materials in a biohazard bag.
15. Dispose of broken glass in the broken glass container.
16. Dispose of razor blades, syringe needles, and sharp metal objects in the 'sharps' container.
17. Report spills and accidents immediately to your Lab Incharge teacher or supervisor.
18. Report all injuries or accidents immediately to your Lab Incharge teacher or supervisor.
19. Put the burners to the rear of the cabinet to handle and reduce the air turbulence.
20. Never point a test tube being heated at another student or yourself.
21. Do not taste or smell chemicals.
22. Long hair must be tied back to avoid catching fire.

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GENERAL MICROBIOLOGY

Experiment 1: Principle and Methods of Sterilization

Sterilisation: it implies the whole destruction of all the small scale life forms including spores, from a protest or condition. It is typically cultivated by warmth or filtration yet synthetics or radiation can be utilized.

Sterilization versus Disinfection

Sterilization is recognized from cleansing in that it executes, deactivates or dispenses with a wide range of life or common administrators.

Sterilization implies expressly to the removal of hurtful organic specialists. In every practical sense, purging may be required in prosperity essential conditions, for instance, wellbeing lopes while sterilization is a strategy you use in your kitchen or bathroom.

Methods of Sterilization

(1) Dry Heat and Moist

Moist: To misshape and pulverize the structure of life forms with warmth. The mix of steam and weight, the temperature is typically 121°C and 15 lbs. per inch for thirty minutes is all the more viably work with warmth..

Dry Heat: Kills microorganism or other common administrators utilizing oxidation strategies. Most safe of the spores requires a temperature about 120 °C to 125 °C for around two hours.

Autoclave/Pressure Cooker

An autoclave/weight cooker disinfects utilizing high steam weight with warmth which is made by high temperature bubbling water and weight. The steam vibrations devastate the microorganisms

(2) Ionizing Radiation (Like X-Rays)

It is additionally an effective method for sterilization, acting to discharge electrons from atoms. Since numerous transmissible specialists require DNA and RNA for their engendering, separating the nucleic corrosive spine can be a helpful way to lessen their transmission. While this strategy has demonstrated viable for safe purification, one must give careful consideration to an ensuing evacuation of these mixes, as bacterial and parasitic spores oppose most light strategies.

Ultra-Violet C (UVC) Lamp

The UVC lamps (280 – 100 nm) are used for sterilization, they damage cells organelles and destroyed the DNA sequencing.

Experiment 2: Preparation of Media

The survival and advancement of microorganisms depend upon available and a positive improvement condition. Culture media are the enhancement game plans used in research offices to create microorganisms. For the fruitful culture of a given microorganism it is critical to fathom its dietary necessities and after that supply it with its fundamental enhancements in the most ideal shape and extents in a culture medium. The general formation of a medium is according to the accompanying–

1. H-Donors and Acceptors (approximately 1-15 g/L)
2. C-source (approximately 1-20 g/L)
3. N-source (approximately 0,2-2 g/L)
4. Inorganic Nutrients (Such as, S, P, (50mg/L)
5. Trace Elements (0,1-1 µg/L)
6. Growth Factors (aminoacids, purines, pyrimidines, occasionally 50 mg/L, vitamins occasionally 0,1-1 mg/L)
7. Solidifying Agent (Such as, agar 10-20 g/L)
8. Solvent (usually distilled water)
9. Buffers

As indicated by the consistency three sorts of media are utilized:- **liquid** or **broth** media, **semisolid** media, and **solid** media. The real contrast among them is that solid and semisolid media contain a solidifying or gelling agent [such as agar, gelatine], though a liquid medium does not.

• **Liquid Media:** Supplement juices, tryptic soy soup or glucose juices can be used in examinations of advancement and digestion in which it is important to have homogenous media conditions, to seek after optical thickness, and to allow early looking at for examination of substrates and metabolic items. Cylinders and flagons with fluid societies can be hatched with either static or shaken brooding.

• **Semisolid Media** can also be used in fermentation studies in determining bacterial motility, and in promoting anaerobic development.

• **Solid Media:** In solid media nutrient agar are used

Reason for Solid Agar Used-

- (1) For growing microorganisms on surface to observe colony morphology
- (2) For pure culture isolation
- (3) to observe specific biochemical

Strong media put in to test cylinder or Petri dish.

In the event that the medium put in the test tube is permitted to solidify in an inclined position, the cylinder is said an agar incline.

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On the off chance that the cylinder is permitted to solidify in an upstanding position, the cylinder is said an agar profound cylinder.

In the event that the agar is put into a Petri dish, the plate is said an agar plate.

Media are classified by their structure or Mixture of the substances-

Synthestic Media: If a medium is developed of known quantity and quality with pure chemicals these media is known as synthetic or chemical media.

Non-Synthestic Media: If a medium is developed from complex materials which is rich in vitamins and nutrients and chemical composition is not well defined is known as Non synthetic media.

Classification of media on the basis of their functions

- **All-Purpose Medium** Like- Tryptic Soy Agar, supports the growth of most bacteria cultured in the laboratory. They do not have any special component mixture..
- **Selective Media** this type of media allow only special type of microorganism only,
- **Differential Media** allow identification of microorganisms usually through the (visible) physiological reactions unique to those bacteria. The most practical media are those that both select for and differentiate common pathogens.
- **Enrichment Media**-In this media special component are added to allow the micro-organism to grow and its help to study the metabolic activity of these organisms. Blood agar is a great example of enriched media

Preparation of Media

Preparation of Tryptic Soy Broth: For creating 1lt media we have required 30g of the dried out medium in 1.000 ml refined water. Blend altogether in a 2 liter Erlenmeyer carafe. Apportion and clean for 20 minutes at 121°C [15 lbs pressure]. As noticed, the measure of powder for 1.000 ml of water will be demonstrated. In the event of planning of media from an equation pons 500 ml of refined water into a 2000ml Erlenmeyer flagon. At that point measure sufficient measure of media parts and disintegrate totally in the water in the request of the equation. Toward the end rins the cup with the staying 500 ml water. Blend the medium completely, change the pH and disinfect.

The majority of the activities we need to do manual and include the utilization of sterile media culture tubes. These cylinders must be topped so as to keep up media sterility. This can be practiced by utilizing cotton plugs, plastic froth fittings or plastic or metal tops. These tops keep societies free of defilement while permitting air into the way of life tube, and limiting vanishing in the meantime. It is once in a while alluring to utilize screw top culture tubes. This is particularly evident when the way of life, for example, on account of inclinations, might be fixed and put away for extensive stretches. Culture stock can be abstained from the pipetting machine, a programmed syringe, or an ordinary pipette.

Agar profound cylinders can be put away after sterilization for use in the arrangement of Petri plates when required. Some agar deeps might be put away at room temperature for a few days before use. In the event that more drawn out times of capacity are required, they ought to be put in the cooler in request to avoid drying of the agar. At the point when Petri plates are required, the agar deeps are dissolved either in a bubbling water shower or by bringing them to 121°C in an autoclave for 30 to 60 seconds, and after that discharging the steam under moderate fumes. After the agar has dissolved, the pours are exchanged to a 48 to 50°C water shower and kept there for somewhere around 5 to 10 minutes before use. The agar deeps ought to be cooled to around 50°C before they are utilized to limit the measure of steam buildup on the Petri plate covers after the agar has been poured. Agar does not cement until the point when its temperature drops to around 42°C. At the point when the deeps have achieved 50°C, one is cleaned up and the outside is dried with a paper towel. Its top is evacuated and the best is quickly blazed utilizing a Bunsen burner. The agar is quickly filled a sterile Petri plate while holding the best cautiously over the Petri plate base so as to dodge sully. Supplant the best, enable the agar to cool and solidify, and store the Petri plates in a reversed position.

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Experiment 3: Pure Culture Techniques: Streak Plate, Spread Plate and Pour Plate

Pure Culture Techniques-Streak Plate

The strategies was structure and created by Loeffler and Gaffkey. Streak plate technique is used for the detachment into unadulterated culture of the living beings (generally microbes), from blended populace. The inoculum is streaked over the agar surface in such a route in this way, to the point that it ‘scatter’ the microscopic organisms. Some individual bacterial cells are disengaged and especially isolated from each other. As the first example is weakened by streaking it over progressive quadrants, the quantity of life forms diminishes. As a rule by the third or fourth quadrant just a couple of living beings are exchanged which will give discrete Colony Forming Units (CFUs).

Principle

The example is weakened by streaking it over the surface of the agar plate. While streaking in dynamic regions of the plate, the example is weakened to the point where there is just a single bacterial cell kept each couple of millimeters on the surface of the agar plate. At the point when these solitary bacterial cells separation and offer ascent to a large number of new bacterial cells, a segregated settlement is shaped. Unadulterated societies can be gotten by picking very much disconnected provinces and re-marking these on new agar plates.

Materials Required

A source of bacteria (stock culture, previously streaked agar plate or any other inoculum)

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Inoculation Loop

A Striker/Lighter

Bunsen Burner

Lysol (10%v/v)

Agar Plate (Nutrient agar or any other agar medium)

Paper Towels

The streak plate procedure depends upon spatial segment of single cells. The mixed microbial culture is traded to the edge of an agar plate and after that a movement of parallel non-covering streaks are made in some unequivocal precedent over the surface of the enhancement medium with the help of inoculating circle. As the microorganisms are rubbed off the hover on to the medium, there is a perpetual decline in the amount of living beings till the last cells to be rubbed off the circle are adequately far isolated to outline confined/discrete states. The restricted state can be picked and lay reaked on enhancement agar plate to get an unadulterated culture. Streak plate strategy is the most regularly utilized technique to disconnect unadulterated societies. As talked about above, guideline of streak plate technique is constant weakening of the microorganisms, bringing about partition of individual cells. These cells at that point shape confined provinces. Continued picking what's more, restreaking of confined settlement at last outcomes in an unadulterated culture. Diverse examples can be utilized for streaking. Basic ones are quadrant (four way streaks) and full plate streak strategies. There are different variations of streaking design moreover. The system capacities honorably when the living being to be isolated is accessible in enormous entireties in a mix. In any case, when the proportion of needed living being is less, its measurement should be extended by using express improvement culture before performing streaking.

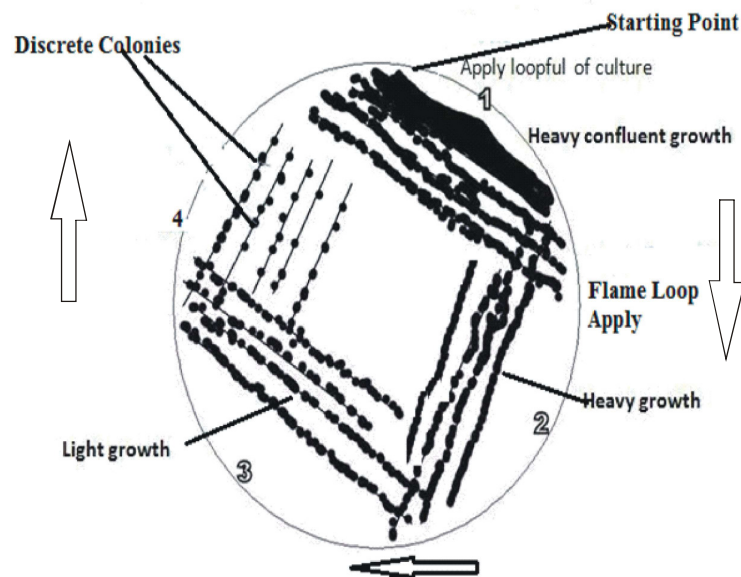


Fig. 1: Four Way Streak Plate Inoculation

Four Way Streak Plate inoculation

- Sterilize the inoculating loop in the bunsen burner by putting the loop into the flame until it is red hot. Allow it to cool.
- Pick an isolated colony from the agar plate culture and spread it over the first quadrant (approximately 1/4 of the plate) using close parallel streaks or Insert your loop into the tube/culture bottle and remove some inoculum.
- Immediately streak the inoculating loop very gently over a quarter of the plate using a back and forth motion (see Area 1 in the Figure 1).
- Flame the loop again and enable it to cool. Returning to the edge of Area 1 that you simply streaked, expand the streaks into the second quarter of the plate (Area 2).
- Flame the loop again and enable it to cool. Returning to the edge of Area 2 that you simply streaked, expand the streaks into the the third quarter of the plate (Area 3).
- Flame the loop again and enable it to cool. Returning to the edge of Area 3 that you simply streaked, expand the streaks into the center fourth of the plate (Area 4).
- Flame your loop once more.

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Spread Plate Techniques

In this technique, diluted microbial suspension containing around 30 to 300 states is spread consistently on the agar surface with a sterile bent rod (spreader). By continued picking and restreaking of an all around confined province, an unadulterated culture can be acquired. The scattered cells form into detached settlements. The strategy can likewise be utilized for quantisation of microbial number in the sample.

Procedure

In spread plate system, a little volume of the weakened example (about 0.1 ml) is exchanged to the focal point of a pre-poured set agar plate and after that spread consistently over the surface of the medium with a sterile L-formed glass pole or spreader. In the wake of bring forth, the distributed cells outline withdrew settlements on agar surface, the amount of which is used to discover the proportion of microorganisms in a given example. It is critical that the surface of the plate be dry so the way of life that is spread absorbs

Pour Plate Techniques

Disengaged provinces can likewise be acquired by pour plate strategy. The method incorporates mixing of little volume of microbial suspension with fluid enhancement agar at 45°C and emptying quickly into sterile petri plate. The microbial suspension ought to be weakened adequately to get discrete provinces on plating. The pour plate system includes including determined sum (0.1 ml or 1.0 ml) of the weakening

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to the sterile petri plate. Twenty to twenty five ml of supplement agar medium kept melted in a water shower at 45°–50°C is then added to the sterile plate and blended with the weakening legitimately by delicate turn of plate in a roundabout movement on the table best. This outcomes in equivalent conveyance of creature (Micro living being). When the agar has thick, every cell is settled set up and frames a particular state on brooding. Provinces seemed both inside the supplement agar, and additionally, on the surface of agar plate. Microbial cells get settled on hardening of agar and structures singular states on brooding. Provinces are available both on the agar surface and installed in the supplement medium. Provinces developing at first glance can be utilized to immunize new vehicle for unadulterated societies. The technique can likewise be utilized for microbial cells specification in the first example.

Experiment 4: Motility Determination – Hanging Drop Method

Hanging drop plan is an unprecedented sort of wet mount (in which a drop of medium containing the living beings is put on a magnifying lens slide), routinely is used in dull light to watch the motility of microorganisms.

In this strategy a drop of culture is determined to a coverslip that is encompassed with oil jam (or some other sticky material). The coverslip and drop are then rearranged over the well of a melancholy slide. The drop dangles from the coverslip, and the oil jam shapes seal that envisions vanishing. This readiness gives incredible viewpoints of microbial motility.

Materials Required

- Glass Slides and Paraffin Ring
- Paraffin Wax
- Loop
- Coverslip
- Microscope
- Bunsen Burner
- Young Broth Culture of Motile Bacteria

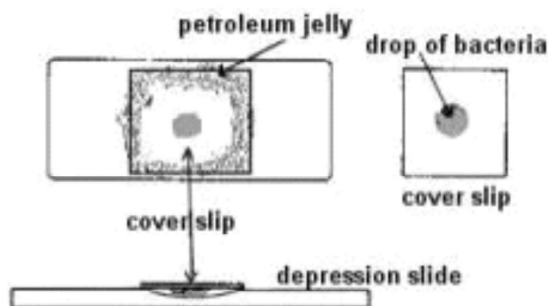


Fig. 1: Prepration of Hanging Drop Method

Procedure

Hanging drop slides are useful in watching the general condition of living minute living beings and the course of action of bacterial cells when they relate together. Living creatures are found in a drop that is suspended under a cover glass in an internal distress slide. The slide for a hanging drop is ground with an internal well in the center; the cover glass holds a drop of the suspension. Right when the cover glass is changed over the well of the slide, the drop swings from the glass in the vacant concavity of the slide. Since the drop exists in an encased glass chamber, drying out happens bit by bit. A ring of Vaseline around the edge of the cover slip shields the slide from drying out.

- Take a clean glass slide and apply paraffin ring, adhesive tape ring to make circular concavity.
- Hold a clean coverslip by its edges and carefully dab Vaseline on its corners using a toothpick.
- Place a loopful of the broth culture to be tested in the center of the prepared coverslip.
- Turn the prepared glass slide or concavity slide upside down (concavity down) over the drop on the coverslip so that the vaseline seals the coverslip to the slide around the concavity.
- Turn the slide over so the coverslip is on top and the drop can be observed hanging from the coverslip over the concavity.
- Place the preparation in the microscope slide holder and align it using the naked eye so an edge of the drop is under the low power objectives.
- Turn the objective to its lowest position using the coarse adjustment and close the diaphragm.
- Look through the eyepiece and raise the objective slowly using the coarse adjustment knob until the edge of the drop is observed as an irregular line crossing the field.
- Move the slide to make that line pass through the center of the field.
- Observe the slide through the eyepiece and adjust the fine adjustment until the edge of the drop can be seen as a thick, usually dark line.
- Focus the edge of the drop carefully and look at each side of that line for very small objects that are the bacteria. The cells will look either like dark or slightly greenish, very small rods or spheres. Remember the high dry objective magnifies a little less than half as much as the oil immersion objective.
- Adjust the light using the diaphragm lever to maximize the visibility of the cells.
- Observe the cells noting their morphology and grouping and determine whether true motility can be observed.

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- Brownian movement should be visible on slides of all the organisms, but there should also show true motility.
- Wash the depression slide and after soaking in lysol buckets or discard the prepared glass slide.

Experiment 5: Isolation and Enumeration of Bacteria from Different Environmental Samples

Principles

One approach to identify the quantity of microbes present in a dirt example is to use weakening and plating strategy. This system uses agar as a vehicle for bacterial development, a procedure named, ‘culturable innovation’. Because of the huge quantities of microbes found inside soils, a little example of soil is sequentially weakened in water, preceding being plated on agar inside a Petri plate. Regularly, a little measure of soil contained inside 0.1 to 1 mL of the weakened soil suspension is ‘spread’ over the surface of the agar plate. The plates contain agar, which is liquid when hot, however strong when cool. Notwithstanding the agar, supplements, for example, peptone yeast or an item financially accessible as R₂A, are added to the medium to take into consideration the development of heterotrophic microbes.

Weakening and plating is a cheap and moderately basic innovation for the count of soil microbes. Be that as it may, there are a few downsides to the strategy. Some basic blunders and suppositions related with weakening and plating measures are as per the following:

- It is expected that each and every dirt bacterium offers ascend to a settlement, however in all actuality a state may emerge from a cluster of cells, bringing about an underestimation of genuine culturable check.
- Amid sequential weakening of the dirt, soil particles can settle out (tumble to the base), so the genuine aliquot of soil is not passed on into the following weakening.
- Many soil organisms are practical however non-culturable. Moderate developing microbes may not result in unmistakable settlements inside a sensible time period (1-2 weeks).

Additionally, anaerobic microscopic organisms do not develop under oxygen consuming conditions, and microbes that do develop are chosen for by the supplements added to the medium. Along these lines R₂A chooses for heterotrophic microbes, while natural sulfur chooses for autotrophic sulfur oxidizers. In general, it is assessed that just 0.1 to 1% of all dirt microscopic organisms can be refined. Along these lines, weakening and plating of soil microscopic organisms represents culturable microbes and thinks little of the genuine reasonable soil populace by one to two requests of greatness. A case of heterotrophic bacterial settlements that came about because of soil weakening and plating is appeared in Figure 1. Note that roughly 1 million bacterial cells are required for a settlement to be unmistakable to the exposed eye.

This trial exhibits the weakening and spread plating procedure used to specify the quantity of microbes inside a dirt example. In particular, two media are utilized: one intended for all microbes, and the other that chooses for actinomycetes. When the bacterial provinces have developed on the agar plates, disengage the unadulterated societies of chose states by utilizing a streak plate system. Such unadulterated societies would then be able to be additionally broke down and portrayed for explicit characteristics and capacities.

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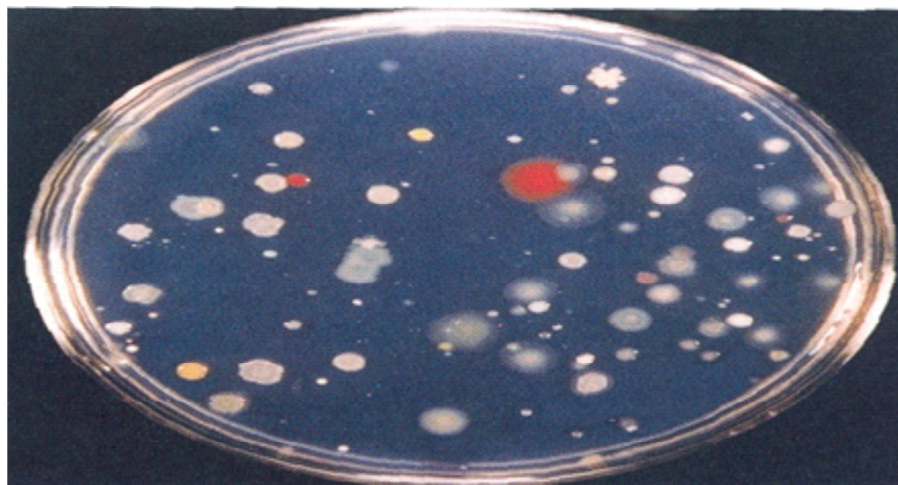


Fig. 1: Bacterial Settlement

Procedure

1. Preparation of Soil Dilutions

1. To begin the procedure, weigh out 10 g of soil sample and add to 95 mL of deionized water. Shake the suspension well, and label as 'A'.
2. Before the soil settles, remove 1 mL of the suspension with a sterile pipette and transfer it to a 9-mL deionized water blank. Vortex thoroughly, and label as 'B'.
3. Repeat this dilution step three times, each time with 1 mL of the previous suspension and a 9 mL deionized water blank. Label these sequentially as tubes C, D, and E. This results in serial dilutions of 10^{-1} through 10^{-5} grams of soil per mL

2. Making Spread Plates for Bacterial Culture

1. To grow bacterial colonies, take three pre-prepared peptone-yeast agar plates and label them as C, D, and E. Vortex samples C, D, and E, and pipette 0.1 mL onto each plate. This increases the dilution value further, by a factor of ten ($C = 10^{-3}$, $D = 10^{-4}$, $E = 10^{-5}$).
2. Next, dip a glass spreader into ethanol. Place the spreader in a flame for a few seconds to ignite and burn off the ethanol. This will sterilize the spreader.

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3. Hold the spreader above the first plate until the flame is extinguished. Open the plate quickly, holding the lid close by. Touch the spreader to the agar away from the inoculum (Inoculum = Cells used to begin a culture) to cool, and then spread the drop of inoculum around the surface of the agar until traces of free liquid disappear. Replace the plate lid.
4. Re-flame the spreader and repeat the process with the next plate, working quickly so as not to contaminate the agar with airborne organisms
5. Incubate the bacteria plates at room temperature for 1 week. Make sure the plates are inverted during the incubation to prevent drops of moisture from condensation from falling onto the agar surface.

3. Making Spread Plates for Actinomycetes

1. To grow actinomycetes, take three pre-prepared glycerol-casein plates and label them as B, C, and D. Using the techniques shown previously, spread plate 0.1 mL from the suspensions B, C, and D. The lower dilutions are used because actinomycetes are typically present as 1/10th of the bacterial population ($B = 10^{-2}$, $C = 10^{-3}$, $D = 10^{-4}$).
2. Incubate the actinomycete plates (inverted) at room temperature for 2 weeks.

4. Bacterial and Actinomycete Counts

1. After incubation, examine all of the bacteria plates carefully, and note differences in colony size and shape. When grown on agar, bacteria produce slimy colonies ranging from colorless to bright orange, yellow, or pink. In contrast, actinomycete colonies are chalky, firm, leathery, and will break under pressure, where other bacterial colonies will smear. This allows colonies to be distinguished by touch with a sterile loop.
2. Count and record the number of bacterial colonies, including any actinomycetes. Only count plates with 30-200 colonies per plate.

5. Isolation of Pure Cultures

1. Select individual bacterial colonies from any of the plates. More colonies can be selected if there is particular interest in the soil. Use a high dilution plate, as it tends to have pure colonies that are separated well. Choose only colonies that are well-separated from neighboring colonies and look morphologically distinct from each other.
2. Sterilize the loop by dipping it in alcohol and flaming it. Quickly open the Petri dish of interest, and touch the loop to a bare spot in the agar to cool it. Then, remove a small amount of a colony of interest onto the loop.

3. Taking a fresh peptone-yeast plate, make a streak a few centimeters long on one side. Sterilize and cool again, then make a streak that crosses the initial streak only on the first pass. Repeat this process twice more in the same manner. This streaking “dilution” results in cells on the loop being separated from one another. Place the plate in a dark area to incubate at room temperature for two weeks.

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Experiment 6: Enumeration of Bacteria - Viable Count (Plate Count) and Total Count (Haemocytometer Count)

The quantity of practical ‘germs’ in a culture can be found out by deciding the quantity of colony forming units CFU with the colony counting technique. Somewhere in the range of 20 and 200 CFU can be relied on a regular Petri plate. Microbial societies of high thickness must be weakened before they are plated. A known volume [0,1ml] of these weakenings is plated onto appropriate development medium in the Petri dish. After the plates have been brooded up to seven days, the normal number of provinces on plates is resolved. The quantity of suitable microorganisms per milliliter (or g) of the underlying society (test) can be determined from the normal CFU’s and the realized weakening element. The practical tally is constantly not exactly the aggregate cell check since it quantifies just cells fit for partitioning. The real detriments of the colony plating technique are : (i) The brooding time frame is protracted, (ii) Sterile media , pipets and plates are required, (iii) Examining and weakening mistakes happen.

Two methods of this examinations are differentiated:

- (1) In the **pour–plate method**, an example from a precise weakening of organisms/test is pipetted onto a Petri - dish, at that point agar medium is poured over the fluid and blended.
- (2) In the **spread–plate strategy**, for the most part 0.1ml of the weakened example is pipetted onto the surface of a hardened agar medium and spread with a disinfected, bowed, glass bar.

The hypothesis behind the technique of CFU builds up that a solitary organism can develop and turn into a colony through division. These provinces are plainly unique in relation to one another, both infinitesimally and visibly. This technique enables the client to know what number of CFU’s are available per mL in the example. In this manner, it empowers us to know the microbiological stack and the extent of the contamination in people and creatures, or the level of defilement in tests of water, vegetables, soil or leafy foods mechanical items and theequipment.

Quantifying Bacteria by Spread Plate

The quantity of microscopic organisms in an answer can be promptly measured by utilizing the spread plate technique. In this technique, the example is properly weakened and a little aliquot is exchanged to an agar plate. The microscopic organisms are then circulated uniformly over the surface by a exceptional streaking technique. After states are developed, they are tallied and the quantity of microscopic

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organisms in the first example is determined. The end purpose of our examination is the quantity of colony forming units per mL (CFU/mL) since we are counting the quantity of provinces as opposed to the genuine number of microorganisms. CFU/mL is all things considered a more valuable assurance than counting every one of the microorganisms under a magnifying instrument, since in numerous bacterial populaces, a critical number will be dead cells and along these lines of nointerest.

Diluting the Bacteria: Microorganisms usually grow up to densities around 10⁹ CFU/mL, despite the fact that the greatest densities shift immensely relying upon the types of microorganisms and the media they are developing in. Thusly, to get promptly countable quantities of microorganisms, we need to make an extensive variety of weakenings and examine every one of them with the objective of having one or two weakenings with countable numbers. We do this by making sequential 10-overlap weakenings of the microbes that cover the whole plausible scope of focuses. At that point we exchange 0.1 mL of every weakening to an agar plate, which essentially makes another 10-overlap weakening, since the last unit is CFU/mL and we just streak 0.1 mL.

Inoculating the Plate: Streaking in this technique is finished utilizing a twisted glass pole. 0.1 mL of bacterial suspension is set in the focal point of the plate utilizing a sterile pipette. The glass pole is disinfected by first dunking it into a 70% liquor arrangement and afterward going it rapidly through the Bunsen burner fire. The consuming liquor cleans the pole at a cooler temperature than holding the pole in the burner fire, in this way decreasing the possibility of you consuming your fingers.

At the point when all the liquor has consumed off and the pole has air-cooled, mark the pole forward and backward over the plate working all over a few times. Not at all like streaking for disconnection, you need to backtrack commonly so as to convey the microbes as equally as could be expected under the circumstances. Turn the plate 90 degrees and rehash the side to side, here and there streaking. Turn the plate 45 degrees furthermore, mark a third time. Try not to sanitize the glass bar between plate turnings. Cover the plate furthermore, hold up a few minutes previously flipping around it for brooding. This will permit the stock to drench into the plate so the microscopic organisms won't dribble onto the plate cover.

Counting Bacteria: Colonies are most readily counted using a plate counter. The plate counter has a light source and a magnifying glass making colonies easier to see. If at all possible, you don't want to count plates with more than 300 or less than 30 colonies. In the former case, the colonies, run together, and, in the latter, there are too few to allow statistically accurate counts. Once you count the colonies, multiply by the appropriate dilution factor to determine the number of CFU/ml in the original sample.

Total Counts

Direct Counts: Microscopic determination of the numbers of microorganisms which generally give the highest estimates. Usually applied with staining, in particular fluorescent staining is used with environmental samples.

Acridine Orange: Binds to nucleic acids and fluoresce either green or orange

DAPI: Fluoresce bright blue

INT: Stains respiring microorganisms (viable)

Counting Chamber: Counting numbers cells in known volumes, for example haemocytometer

Preparing Hemocytometer

1. If using a glass hemocytometer and coverslip, clean with alcohol before use. Moisten the coverslip with water and affix to the hemocytometer. The presence of Newton's refraction rings under the coverslip indicates proper adhesion.
2. If using a disposable hemocytometer (for example, INCYTO DHC-N01), simply remove from the packet before use.

Preparing Cell Suspension

1. Gently swirl the flask to ensure the cells are evenly distributed.
2. Before the cells have a chance to settle, take out 0.5 mL of cell suspension using a 5 mL sterile pipette and place in an Eppendorf tube.
3. Take 100 μL of cells into a new Eppendorf tube and add 400 μL 0.4% Trypan Blue (final concentration 0.32%). Mix gently.

Counting

1. Using a pipette, take 100 μL of Trypan Blue-treated cell suspension and apply to the hemocytometer. If using a glass hemocytometer, very gently fill both chambers underneath the coverslip, allowing the cell suspension to be drawn out by capillary action. If using a disposable hemocytometer, pipette the cell suspension into the well of the counting chamber, allowing capillary action to draw it inside.
2. Using a microscope, focus on the grid lines of the hemocytometer with a 10X objective.
3. Using a hand tally counter, count the live, unstained cells (live cells do not take up Trypan Blue) in one set of 16 squares (Figure 1). When counting, employ a system whereby cells are only counted when they are set within a square or on the right-hand or bottom boundary line. Following the same guidelines, dead cells stained with Trypan Blue can also be counted for a viability estimate if required.

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4. Move the hemocytometer to the next set of 16 corner squares and carry on counting until all 4 sets of 16 corners are counted.

Viability

To Calculate the Number of Viable Cells/mL:

1. Take the average cell count from each of the sets of 16 corner squares.
2. Multiply by 10,000 (10^4).
3. Multiply by 5 to correct for the 1:5 dilution from the Trypan Blue addition.

The final value is the number of viable cells/mL in the original cell suspension.

Experiment 7: Direct Microscopic Observation of Fungal Spores and Mycelium (Fungal Slide Culture)

Materials Required:

- Culture: 7-10 Day Old Fungal Culture
- Media: Sabouraud Agar
- Preparation of Sabouraud Agar (pH-5.6)
- Sabouraud Agar Supplemented with Aureomycin
- Peptone-10g/liter
- Dextrose-40g/liter
- Agar-15g/liter

Equipments

- Sterile Petri Dish
- Filter Paper (9cm diameter)
- U-shaped Glass Rod
- Microscope Slides and Coverslips (Sterile)
- Sabouraud's Plate with Mixed Culture of Fungi
- Sterile Sabouraud's Agar Plate
- Lactophenol Cotton Blue Stain
- Glass Capillary Tube
- Scalpel
- Inoculating Needle
- Sterile Distilled Water
- 95% Ethanol
- Forceps

Procedure

Slide Culture Preparation

Aseptically, with a couple of forceps, put a sheet of sterile channel paper in a Petri dish.

Place a sterile U-molded glass pole on the channel paper. (Bar can be disinfected by blazing, whenever held by forceps.) Pour enough sterile water (around 4 ml) on channel paper to totally soak it.

With forceps, put a sterile slide on the U-molded pole Gently fire a surgical tool to disinfect, and cut a 5 mm square of the medium from the plate of Sabouraud's agar or Emmons' medium. Get the square of agar by embeddings the surgical tool and cautiously exchange this square aseptically to the focal point of the slide. Immunize four sides of the agar square with spores or mycelial parts of the growth to be analyzed. Make sure to fire and cool the circle before getting spores. Aseptically, put a sterile cover glass on the upper surface of the agar solid shape.

Place the cover on the Petri dish and brood at room temperature for 48 hours. Following 48 hours, look at the slide under low power. On the off chance that development has happened there will be development of hyphae and generation of spores. In the event that development is deficient and spores are not apparent, enable the shape to develop for another 24–48 hours before making the recolored slides.

Experiment 8: Staining Method: Simple, Negative, Gram's Staining and Spore Staining

Simple Staining Method

Stains join to something due to charge contrasts between the question and the stain. Diverse stains can show up as an alternate shading since they contain distinctive chromophore gatherings, which shift in the wavelength of light they retain. When all is said in done there are two primary stain types. Decidedly charged stains have a positive chromophore. The second sort, contrarily charged stains, has a chromophore that conveys a negative charge. Emphatically charged stains are phenomenal in restricting contrarily charged structures, for example, bacterial cell dividers and, in the event that they can enter the cell, numerous macromolecular structures, for example, DNA and proteins. Cationic (essential) stains have a positive accuse related of them while anionic (acidic) stains convey a negative charge. Instances of cationic stains incorporate precious stone violet, safranin, fundamental fuschin, and methylene blue. Instances of anionic stains incorporate eosin, nigrosin, and congo red. Corrosive colors are regularly used to recolor the slide foundation, which leaves the organism straightforward. Hence, in the field of view the organism will show up as clear specks against a dark foundation. Stains require a short presentation time to their objective pursued by a concise, light flush with deionized (DI) water. This evacuates any overabundance recolor and permits better review of the cells that convey the stain.

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Materials Required

- Safranin and Crystal Violet
- Overnight Bacterial Cultures of *S. aureus* and *B. megaterium*
- Glass Slide
- Nichrome Loop

Procedure

Place a loop-full of bacteria from an overnight culture onto the center of a glass slide. Heat fix cells by placing the slide on top of the bacticinerator until it appears dry. Use a forceps to handle the slide.

Gram's Staining

A standout amongst the most imperative stains performed by both the youngster microbiologist and expert microbiologist is the Gram recolor. This stain is named after Hans Christian Gram who was the first to execute the technique. Despite the fact that Hans did not know it at the time, the Gram recolor permits separation between Gram-negatives and Gram-positives. This is to a great extent because of the structure of the cell divider and the nearness or nonappearance of an external layer that happens in Gram-negative microscopic organisms. After warmth settling a circle brimming with medium-term culture, the cells are prepared to be recolored. Initial an essential stain, called gem violet, is utilized as an essential stain. This cationic stain will hold fast to all living beings, since cells convey a general negative charge.

Subsequent to flushing, a stringent is connected called Gram's iodine, which advances maintenance of the essential stain.

The second wash is performed with EtOH. The EtOH capacities to complete a few things one of which is to recoil the pores in the peptidoglycan layer. This device the precious stone violet-iodine complex in the Gram-positive cell. The bigger pores and more slender peptidoglycan in Gram-negative life forms isn't changed to such a degree contrasted with the Gram-positive living beings. Nonetheless, the EtOH additionally expels the external film of the Gram-negative life forms. Basically, the EtOH wash adequately leaves the Gram-negative cells dreary. While the Gram positives are recolored purple, a counterstain is connected to recolor the dull Gram negatives. These will seem ruddy in shading.

Materials Required

- Overnight Cultures of *S. aureus*, *E. coli*, and an unknown
- Crystal Violet
- Gram's Iodine
- Safranin

- DI H₂O
- EtOH

Procedure

- On three different areas of the glass slide place *S. aureus*, the unknown, and *E. coli*. Do not add too many cells.
- Heat fix cells to glass slide.
- Add enough crystal violet to cover the specimens and wait for 1 minute.
- Wash with DI H₂O.
- Add Gram's Iodine and wait ~1 minute.
- Add a few drops of EtOH and tilt the slide onto the collection pan. Rinse.
- Counterstain with Safranin for 1 minute, rinse, and blot dry.

Negative's Staining

A few sorts of stains can be performed on microbes to decide their cell cosmetics. The negative stain depends on utilizing nigrosin, which leaves nonheat-settled cells dull against a dim foundation. There are a few focal points to utilizing a negative stain contrasted with a straightforward stain where cells are warm settled. One of the significant preferences is that cells are all the more effortlessly seen in their 'normal' state. This occasionally gives a superior perspective of cell estimate and their morphological course of action. For instance, spirochetes will regularly lose their shape when heatfixed. Furthermore, when cells are warm settled, they can be defenseless to lysing or harm, which abandons them hard to see. Consequently, cells that are exceptionally fragile can be seen much better utilizing a negative stain. The acidic stain nigrosin is utilized to leave the cells straightforward, since cells are encompassed by negative charges (following the familiar saying, 'opposites are drawn toward eachother'). In the negative stain, the acidic color is repulsed by the cell charges and leaves the cell flawless.

Materials Required

- Overnight Cultures of *Serratia Marcescens*
- Two Glass Slides
- Nigrosin

Procedure

- Place a drop of nigrosin to the left side of the slide.
- Place a loop-full of bacteria and mix with the nigrosin.
- Second slide, touch and drag the nigrosin across the first slide as shown in Figure 1.
- Allow the slide to air dry.

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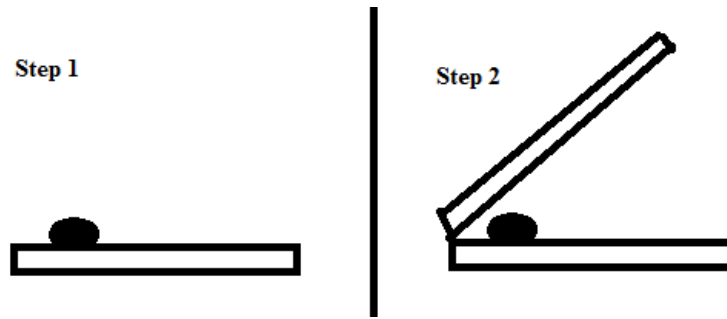


Fig. 1: Slide Preparation

Spore Staining

Microorganisms are omnipresent; in any case, conditions that are favorable for fast development are not constantly present. Under unforgiving conditions, for example, outrageous temperatures, a few microorganisms can deliver a spore inside their phones, apropos called an endospore. The endospore is a survival system that can hold on under extraordinary conditions. A few fascinating investigations have been done to decide the endospore divider structure. Similarly fascinating are the distinctive periods of endospore advancement. The vegetative cell communicates unmistakable arrangements of qualities to deliver the endospore in a process called sporogenesis. Endless supply of the endospore the vegetative cell can pass on, leaving a free spore (Refer Figure 1). Free spores are to a great degree solid and can endure bubbling and serious radiation. At the point when conditions helpful for development restore, the spore will sprout (germination) and another vegetative cell results. The vegetative cell can experience twofold splitting and result in a few additional microbes. In the event that unforgiving developing conditions restore, an endospore is created once more.

Two genera of microbes that have medicinal significance are *Clostridium* and *Bacillus*. *Clostridium tetani* creates an endotoxin that causes lockjaw. *C. perfringens* and *C. defficile* can cause gas gangrene and pseudomembranous colitis, individually.

Materials Required

- Overnight Culture of *Bacillus Megaterium*
- Hot Water Bath
- Malachite Green
- Safranin

Procedure

- Place a loopfull of *B. megaterium* on a glass slide.
- Heat-fix the specimen.
- Place the slide over the boiling water bath.
- Cover the specimen with a small piece of paper towel.

- Apply malachite green.
- Allow the heat to carry the malachite green into the endospore for 6-7 minutes. Be sure to reapply malachite green if the towel becomes dry.
- Rinse the slide with water and apply safranin as the counterstain.
- After 1 or 2 minutes, rinse and blot dry.

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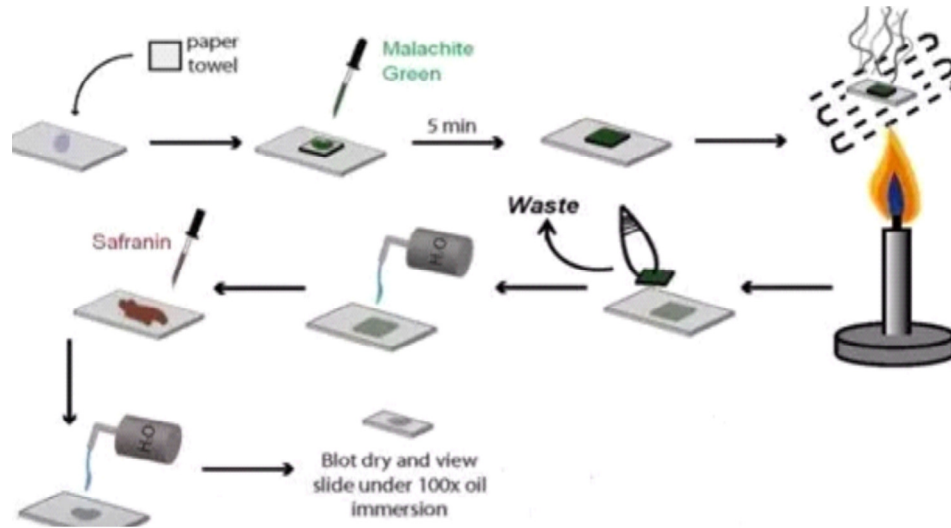


Fig. 1: Endospore Staining

(Figure from - <https://microbeonline.com/endospore-staining-principle-procedure-results>)

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BIOCHEMISTRY

Experiment 1: Measurement of Growth Rate and Generation Time by Turbidometry Method

Bacteria are as interesting as they are diverse. Despite the fact that small, these unicellular living things make immense commitments to numerous frameworks and cycles. From assisting separate nourishment in your digestive system; to making the molecular assist in all three of the carbon, phosphorus, and nitrogen cycles - these little microscopic organisms can achieve huge things. Obviously, microorganisms are show living beings for research. Because of their assorted variety, as well as in light of the fact that they are effortlessly contained and repeat rapidly. When utilizing microscopic organisms for research, it is essential to comprehend and follow rates of bacterial development.

Inoculant microscopic organisms in a regular research facility setting will in general continue through four particular development stages (Refer Figure 1):

- Lag Phase
- Exponential Phase
- Stationary Phase
- Death Phase

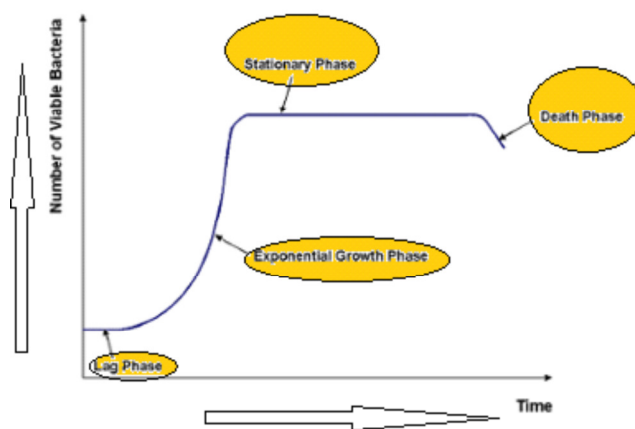


Fig. 1: Phases of Development Stages

The lapse of these phases provides data that can be compiled into a bacterial growth curve. During the lag phase, freshly cultured bacteria adjust to the media they've been placed in or on. As they figure out their environment and dietary alternatives, the cells increment chemical generation and cell measure as needs be. The cells have entered the exponential stage when they start to develop and partition at a consistent pace. This stage is likewise called the logarithmic stage since development happens quickly and the phone tally can venture into the billions. In that capacity, the quantity of cells is determined utilizing logarithmic capacities.

Metabolic procedures additionally happen at a steady rate and are impacted by conditions, for example, pH, temperature, and properties of the medium. The stationary stage is set apart by a level in development. Cell demise and expansion are generally equivalent. Supplements are progressively exhausted, poisons amass, and cell practicality diminishes. The demise stage happens when these conditions cause a more prominent rate of cell mortality than cell multiplication. The populace starts to decrease at and by exponential rate.

Bacterial development bends are critical for computing age time. Age time is the time it takes for two new cells to emerge from a unique cell. In the event that beginning with an inoculum with a known number of microorganisms, intermittent testing should be possible to figure the age time utilizing the condition:

$$GT = \frac{1}{3.3 \log \left(\frac{b}{B} \right)}$$

- GT is generation time.
- t is the time interval between measurements b and B .
- B is the initial population.
- b is the population after time t .

The two most common classroom methods to determine bacterial growth are the Standard Plate Count (SPC) technique and turbidimetric measurement. Examples of other methods include: microscopic count, membrane filter count, nitrogen determination, cellular weight determination, and biochemical activity measurement.

Plotting a Bacterial Growth Using Turbidimetric Determination

Turbidimetric assurance is valuable for plotting development bends of microorganisms in juices or fluid media. It is one of the most straightforward techniques used to break down patterns in development since it utilizes a spectrophotometer to follow changes in the Optical Thickness (OD) after some time. At the end of the day: as the quantity of cells in an example increment, the transmission of light through the example will diminish. The standard OD setting is 660 nanometers for yellow to dark colored stock examples, however can be balanced if the shading isn't in this range or if development is relied upon to be lower or more noteworthy than normal. Coming up next is a case of a standard methodology utilizing this technique. It uses spectrophotometric estimations like clockwork for up to three hours. Make sure to monitor time and record information properly.

- Prepare sterile broth or media that will be used for inoculation. When ready to use, make sure the temperature is not too hot. The media container should be comfortable to the touch.

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- Power on a spectrophotometer and enable it to heat up, ideally for a few minutes previously use.
- Set the wavelength of the spectrophotometer to 660 nm (or another suitable setting). Include 5 mL of uninoculated sterile media to a clean cuvette and clear the machine by setting it to 0 ABS with this example. This progression institutionalizes the turbidity of the media with no cells in it, so further computations can quantitate development because of changes in the turbidity.
- Inoculate the essential media from which tests will be taken.
- Following vaccinating, take a 5 mL test of the immunized media and pipette it into a clean cuvette. Place it in the blanked spectrophotometer, and record the OD perusing. This perusing ought to be recorded at time “0”.
- Rehash the past advance at 15 minute interims until the absorbance never again increments.
- Plot the readings on a chart with Time as the X-axis and OD as the Y-axis.

Turbidimetric assurance is exceptionally useful for plotting a standard development bend; it enables us to effectively follow changes in development stages without the issue of checking plated states. It is basic and simple; be that as it may, different strategies regularly give progressively point by point, quantitative data and are favored when increasingly exact information is important. Turbidimetric strategies can regularly be utilized close by these different systems as a support to patterns in the information gathered. Regardless of whether it is utilized alone or close by different methods, it is a basic and proficient methodology when gathering information for standard development bends.

Experiment 2: pH Meter -Preparation of Buffer

Principle of the Method

Buffers are mixtures of weak acids and their salts with strong bases or mixtures of weak bases and their salts with strong acids. They can keep up roughly a similar pH esteem after the expansion of a little measure of solid corrosive or base. After the expansion of a little sum of solid corrosive to the support, the pH changes pretty much nothing. The support base is a hold which forestalls a more prominent change in pH. Buffers also resist pH changes after dilution.

Materials and Instruments

- Glass Beakers
- A Graduated Pipette
- An Automatic Pipette
- A Pipette Pump
- A Glass Rod

- A pH Tester
- Checker

Chemicals

- Phosphate-Citrate Buffer (Citric Acid/ Na_2HPO_4) - A, B, C
- 0.1 mol/l HCl

Procedure of Measurement by a pH Meter

1. Turn on (“ON”) or turn off (“OFF”) the pH meter by a switch (button) which is on the red cover of the pH meter.
2. Turn on the pH meter and wait until the display shows the measurement mode “pH”.
3. Blend completely the deliberate arrangement before the measurement.
4. Dip the electrode into the test solution and wait until the value on the display stabilizes.
5. Peruse the deliberate pH esteem on the display.
6. The electrode must be dipped in the solution only for necessary period of time.
7. It is necessary to rinse the electrode with distilled water after each measurement.
8. If pH measurements are not performed immediately after each other, it is necessary to keep the electrode between measurements in a test tube with the storage solution.
9. After the measurement wash cautiously the anode with refined water and plunge it back into the storage solution.
10. Turn off the pH meter.

Procedure of pH Measurement of Buffers

1. Measure pH of the undiluted phosphate-citrate buffer in the beaker A, B or C.
2. Dilute the undiluted phosphate-citrate buffer ten times in the following way: Pipet 5 ml of the buffer to an empty beaker. Then pour water after the mark of 50 ml. blend it with a glass rod.
3. Measure pH of the undiluted and the diluted phosphate-citrate buffer in the beakers using the pH meter.
4. Pour 0.5 ml of 0.1 mol/l HCl to both beakers using the automatic pipette, stir and measure pH. 5. Twice repeat the Step Number 4.
6. Write all measured values in the tables.
7. Find out the change of pH value of the phosphate-citrate buffer after dilution. Compare the changes of pH values of both buffers after the addition of HCl.

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Table 1: pH Measurement

Solution	pH		δ (Delta) pH
(a) undiluted phosphate-citrate buffer			
(b) + 0.5 ml 0.1 mol/l HCl		a-b	
(c) + 0.5 ml 0.1 mol/l HCl		a-c	
(d) + 0.5 ml 0.1 mol/l HCl		a-d	
Solution	pH		δ (Delta) pH
(e) 10x diluted phosphate-citrate buffer			
(f) + 0.5 ml 0.1 mol/l HCl		e-f	
(g) + 0.5 ml 0.1 mol/l HCl		e-g	
(h) + 0.5 ml 0.1 mol/l HCl		e-h	
Solution	pH		δ (Delta) pH
(a) undiluted phosphate-citrate buffer			
(e) 10x diluted phosphate-citrate buffer		e-a	

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Experiment 3: Spectrophotometry- Wavelength Scan

Determination of Wavelength of Maximum Absorbance

Colored solutions are colored because they absorb certain wavelengths of light while allowing other wavelengths of light to pass through. As spectators, we see the wavelengths of light that are not absorbed. By estimating the measure of light assimilated, we can discover the grouping of arrangements. Before doing this kind of ghostly investigation, the wavelength at which absorbance is most noteworthy should be resolved. This is the wavelength to be used for the analysis.

The procedure includes recording the absorbance over the scope of 350 nm to 650 nm, typically in interims of 25 nm. The information can be charted to picture the most astounding absorbance or the information sets can be inspected to decide the wavelength. Water with food color added works very well to teach this procedure. Alternatively, solutions of colored ions may be used.

Purpose

To find the wavelength of maximum absorbance of a colored solution using a spectrophotometer.

Equipments / Materials

- Spectrophotometer
- Dropper Bottle Containing Colored Water
- Wash Bottle Containing Distilled Water
- 2 Cuvets

Procedure

1. Select a dropper bottle containing one of the four available colored solutions.
2. Hold cuvet by the harsh sides. Wash the cuvet with a little measure of the hue arrangement and after that fill the cuvet 3/4 full.

3. Fill the second cuvet with distilled water to be used as a blank.
4. Press A/T/C Button on the Spectronic 20 Genesys. Select absorbance.
5. Press the **nm** arrow up or down and select 350 **nm**.
6. Insert the blank into the cell holder and close the door. Position the cell so that the light passes through clear walls.
7. Press **0 ABS/100% T** to set the blank to 0 A.
8. Remove the blank and insert sample into the cell holder. The sample measurement appears on the LCD display. Record the absorbance on the data sheet.
9. Reset the wavelength to 375 nm and repeat steps 6, 7, and 8.
10. Repeat Steps 5, 6, 7 and 8, recording absorbance at every 25 nm using this technique until you reach 650 nm.
11. Locate the 50 nm region in which the absorbance is highest and record the absorbance every 10 nm in this region repeating Steps 5, 6, 7 and 8.
12. Determine the wavelength of maximum absorbance by creating a graph of the data - placing wavelength on the X-axis and absorption on the Y-axis.

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Experiment 4: Chromatography - Paper and Thin Layer Chromatography - Separation of Amino Acid

Objective

The separation of amino acid using thin layer chromatography.

Chromatography

Chromatography is by a wide margin the most valuable general group of techniques accessible for the detachment of closely related compounds in a mixture. Here the separation is effected by differences in the equilibrium distribution of the segments between two immiscible stages, like the stationary and the mobile stages/phase. These distinctions in the equilibrium distribution are a consequence of nature and level of collaboration of the parts with these two stages. The stationary stage is a permeable medium like silica or alumina, through sample mixture percolates under the influence of a moving solvent (the mobile stage/phase). There are various connections between the example and the stationary stage and these have been well exploited to effect the separation of compounds.

Thin Layer Chromatography [TLC]

Thin Layer Chromatographic (TLC) technique readily provides qualitative information and with careful attention to details, it is possible to obtain quantitative data.. Thin layer chromatography is a procedure used to isolate and recognize mixes of intrigue. A TLC plate is comprised of a thin layer of silica clung to glass or aluminum for help. The silica gel goes about as the stationary stage/phase and the dissolvable blend goes about as the mobile stage/phase. In the perfect dissolvable system the compounds of interest are soluble to different degrees. Separation

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results from the partition equilibrium of the components in the mixture. In the most straightforward type of the strategy, a thin zone or spot of the example blend to be isolated is connected almost one end of the TLC plate and permitted to dry. The strip or plate is then placed with this end dipping in to the solvent mixture, taking care that the sample spot/zone is not immersed in the solvent. As the solvent moves towards the opposite end of the strip, the test blend isolates into different segments. This is called as the development of TLC plates. The division relies upon a few factors:

- (a) **Solubility:** The more soluble a compound is in a solvent, the quicker it will move up the plate.
- (b) **Attractions:** The attraction between the compound and the silica, the more the compound interacts with silica, the lesser it moves
- (c) **Size of the Compound/Molecule:** The bigger the compound/molecule the slower it move up the plate.

The plate is evacuated after an development time and dried and the spots/zones are distinguished utilizing a reasonable area reagent. A vital trademark utilized in thin layer chromatography is RF values.

RF Values = (Distance moved by the substance from the origin/ Distance moved by the solvent from the origin)

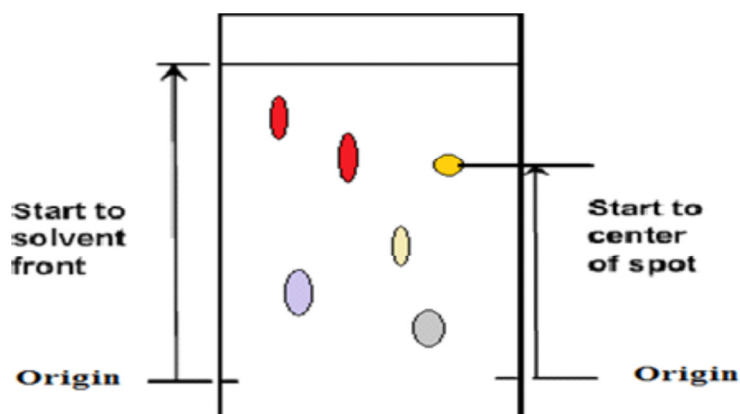


Fig. 1: TLC

Chromatographic Separation of Amino acids

The present experiment utilizes the strategy of thin layer chromatography to isolate the amino acids in a given mixture. Every one of the 20 of the basic amino acids are α -amino acids. They have a carboxyl group and an amino group bonded to the same carbon atom (the α - carbon). They contrast from one another in their side chains, or R groups, which fluctuate in structure, size, and electric charge. The interaction of the amino acids with the stationary phase like silica varies depending on their 'R' groups (Refer Figure 2). The amino acid that interacts strongly with silica will be conveyed by the solvent to a little separation, though the

one with less interaction will be moved further. By running controls close by, it is conceivable to recognize the segments of the mixture.

Since amino acids are colorless molecules/compound, ninhydrin is utilized for identifying them. To distinguish this, after development, the TLC plate is splashed with ninhydrin reagent and dried in an oven, at 105°C for around 5 minutes. Ninhydrin responds (reacts) with α -amino acids that outcomes in purple coloured spots. Rf esteems can be determined and contrasted with the reference value with identify the amino acids.

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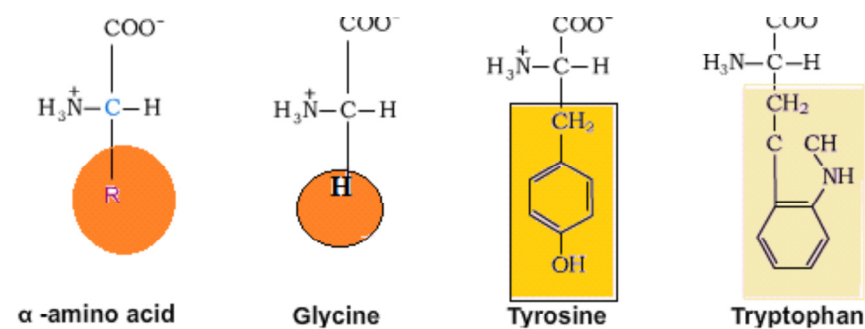


Fig. 2: Amino Acid with their R Groups (With Different Size of R)

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MICROBIAL PHYSIOLOGY

Experiment 1: Carbohydrates: Quantitative Estimation of Glucose, Glycogen from Bacterial and Yeast Cell

Estimation of Protein

Objective

To estimate the amount of Protein present from bacterial yeast cell.

Principle

First take the sample of bacterial yeast cell and make a solution by using any base solution and take the Alkaline CuSO_4 catalyzes the oxidation of aromatic amino acids with subsequent reduction of sodium potassium molybdate tungstate of Folin's reagent giving a purple shading complex the force of the shading is directly proportion to the concentration of the aromatic amino acid in the given sample solution.

Reagents Required

1. Stock Solution: Bovine Serum albumin (serum albumin protein extract from cows. It is often used as a protein concentration standard in lab experiments) of 100mg is weighed accurately and dissolved in 100mL of distilled water in a standard flask and take 10 mL is distilled to 100mL with distilled water in a standard flask (concentration 100 mg/mL).

2. Folin's Phenol Reagent: Folin's Phenol Reagent is mixed with distilled water in the ratio 1:2.

3. Alkaline Copper Reagent:

Solution A: 2% Sodium Carbonate in 0.1 N Sodium Hydroxide.

Solution B: 0.5% Copper Sulphate in 1% Sodium Potassium Tartarate.

Solution C: Alkaline Copper Solution (Mix 50mL of A and 1mL of B) A, B, C is mixed in the proportion of 50:1:0.5.

Unknown Preparation

First take the sample of bacterial yeast cell and make a solution by 100 ml with distilled water.

Procedure

Working standard of 0.2 - 1mL is pipette out into clean test tube and marked as S1-S5. Test arrangement of 0.2ml is taken into test tube and named as T1. The volume is made upto 1ml of refined water. Distill water of 1mL fill in as clear. To all the test tube 4.5mL of basic CuSO_4 reagent is included and brooded at room temperature for 10 minutes. All the test tube 0.5mL of folin's phenol reagent is included. The substance are blended well and the blue shading created is perused at 640 rpm following 15 minutes. From the standard chart shown in Table 1 the measure of protein in the given obscure arrangement is determined.

Table 1: Measure o Protein

S.No	Reagent	B	S ₁	S ₂	S ₃	S ₄	S ₅	T ₁	T ₂
1	Working standard Volume (ml)								
2	Concentration of working standard (mg)								
3	Prepared solution Volume (ml)								
4	Distilled water volume (ml)								
5	Alkaline copper reagent volume (ml)								
6	Folin's phenol reagent volume								
The contents are mixed well and kept at room temperature for 10 minutes. The blue colour developed is read at 640nm									
7	Optical density 640nm								

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Result

Protein amount present (µg of protein).

Estimating the Amount of Glucose/Glycogen

Objective: To estimate the amount of glucose/glycogen present from bacterial yeast cell using benedict's quantitative reagent.

Principle

Benedict's quantitative reagent is an adjustment of subjective. It contains copper sulfate, sodium acetate and sodium carbonate. It likewise contains potassium thio cyanate and little measure of potassium ferricyanide. The consideration of acetate prevents the precipitation of copper carbonate by chelating Cu³⁺ion. The thiocyanate causes the precipitation of white cuprous thio cyanate instead of red cupric oxide. On decrease of Cu³⁺ particles which empowers the end purpose of the titration i.e., the change from blue to white to be promptly recognizable. Methylene blue will be utilized an additional indicator. The little measure of potassium ferricyanide prevents the re-oxidation of copper. A non-stoicheometric response is on which does not pursue a characterized pathway and can't be depicted by a condition either quantitatively or subjectively. The decrease of Cu³⁺ particles by sugar is a non-stoicheometric condition and is just consistent over a little scope of sugar concentration. To acquire precise outcomes the volume of sugar included must be with in 6-12 mL for 10 mL of Benedict's reagent. In the event that the

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fundamental titre value falls outside this range the sugar arrangement must be titrations are rehashed.

Reagents Required

1. Standard Glucose Solution

200 mg of glucose was weighed accurately and made upto 100 mL with distilled water (concentration: 2 mg / mL)

2. Benedict's Quantitative Reagent

100 mL of solution acetate, 37.5 g of sodium carbonate and 62.5 g of potassium thiocyanate were dissolved in 300 ml of distilled water by warming gently and filtered. 9 g of copper sulphate is dissolved in 50 mL of water, added with continuous stirring. 2.5 mL of potassium ferricyanide is added and the volume is made upto 500 mL with water.

3. Anhydrous Sodium Carbonate

Procedure

100 mL of benedict's reagent was pipetted out into a clean conical flask. Around 600 mg of anhydrous sodium carbonate was added to furnish the required allcaling with a couple of porcelain bits and warmed to bubbling over a moderate fire. The standard glucose arrangement is taken in the burette when the Benedict's answer bubbles, glucose arrangement is included drop by drop (one drop for each second) till the last trace of blue shading vanishes. The volume of glucose once-over is noted and the titrations are rehashed for concordant value. The given obscure sugar arrangement was made upto 100 mL in a standard flagon with refined water. Then the burette was filled with sugar extract from the bacterial/yeast solution and the benedict's reagent was titrated as before. The volume of sugar solution rundown was noted and titrations are repeated for concordant values.

Standardisation of Benedict's Reagent

Table 2 gives the detailed readings to estimate the Benedict's reagent.

Table 2: Benedict Reagent vs Standard Solution Glucose

S.No	Benedict's reagents Volume (ml)	Burette Readings		Standard glucose Volume (ml)	Indicator
		Initial (ml)	Final (ml)		

Estimation of Glucose

Standardised Benedict's Reagent vs Glucose (Unknown Amount of Glucose in Solution)

S.No	Benedict's reagents Volume (ml)	Burette Readings		Standard glucose Volume (ml)	Indicator
		Initial (ml)	Final (ml)		
					self

Calculation

Glucose solution (Standard) : 2 mg / mL

Benedict-solution (5 mL) react with..... mL of the glucose solution (Standard).

.....mL of standard glucose solution which contains $\dots \times 2 = \text{mg}$

Benedict's solution (5 mL) reacts with mg of unknown glucose solution

100 mL of unknown glucose contains is $\dots \times 100 \times$

Result

Glucose amountpresent in 100 mL of given solution

Estimating the Various Microbes in a Given Sample

Objective: To isolate and enumerate (and purify) various microbes from the given sample.

Principle

The procedures regularly utilized for isolation of discrete colonies initially require that the quantity of organisms in the inoculum be decreased. The subsequent lessening of the populace estimate guarantees that, following inoculation, individual cells will be adequately far separated on the surface of the agar medium to impact a separation of the diverse species present. The sequential weakening is utilized to achieve this. There are three techniques to do isolation of pure cultures.

Requirement Materials

- Sterile Blanks (9mL)
- 12 Sterile Test Tubes for Slants
- 1 Sterile Blank (10 mL) Samples
- 18-20 Petriplates (20 mL)
- 10 Sterile Pipettes (1 mL)
- Bacterial Growth Medium-Nutrient Agar ($5 \times 100 \text{ mL}$)
- Inoculation Loop
- Wire
- Burner
- Marker Pen
- Sterile Chamber

Procedure

1. Serial Dilution

Precisely 1 ml of the given sample was added to 9 ml of blank,. This arrangement was marked as 10^{-1} . From this test tube, 1 mL of solution was taken and pours in to a 9 mL blank, blended it equitably and named as dilution 10^{-2} for a similar way,

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the method was isolated during sterile pipettes for each exchange, until dilutions up to 10^{-5} are acquired. For the given soil test, dilutions up to 10^{-3} and 10^{-4} were taken for parasitic separation and dilutions 10^{-4} were taken for fungal isolation and dilutions 10^{-4} and 10^{-5} were fungal isolation and dilutions 10^{-4} and 10^{-5} were taken for bacterial detachment.

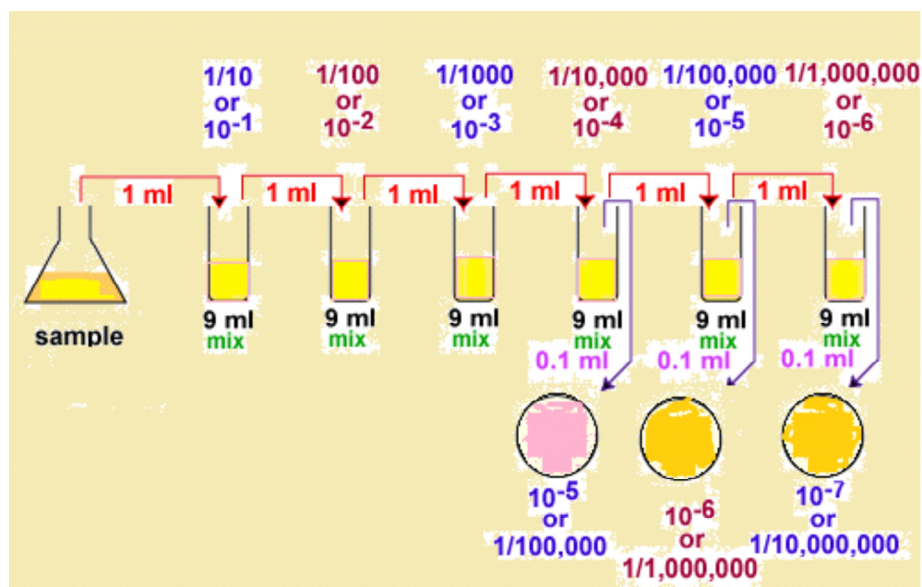


Fig. 1: Serial Dilution

Isolation of Microbes

Pour Plate Method

Pour plating is a technique good for isolation and enumeration of microbes 1 ml of the selected dilutions were pipetted into sterile petriplates in close proximity to the flame. Molten agar was poured over the inoculum and the plates were swirled to evenly distribute the inoculum. Plates of a particular dilution were prepared in duplicates. The agar was allowed to solidify and the plates were incubated at a temperature of 35°C in an inverted fraction for a period of 24-72 hours.

Spread Plate Method

- The **spread-plate** technique requires that a previously diluted mixture of microorganisms be used. During inoculation, the cells are spread over the surface of a solid agar medium with a sterile, L-shaped bent rod. The step-by-step procedure for this technique is as follows:
- Place the bent glass rod into the beaker and add a sufficient amount of 95% ethyl alcohol to cover the lower, bent portion.
- With a sterile loop, place a loopful of culture in the center of the appropriately labeled nutrient agar plate that has been placed on the turntable. Replace the cover.

- Remove the glass rod from the beaker and pass it through the Bunsen burner flame, with the bent portion of the rod pointing downward to prevent the burning alcohol from running down your arm. Allow the alcohol to burn off the rod completely. Cool the rod for 10 to 15 seconds.
- Remove the Petri dish cover and spin the turntable.
- While the turntable is spinning, lightly touch the sterile bent rod to the surface of the agar and move it back and forth. This will spread the culture over the agar surface.
- When the turntable comes to a stop, replace the cover. Immerse the rod in alcohol and reflare.
- Keep the plate for incubation.

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Enumeration of Microbes Observations and Calculations Bacterial Culture

The number of bacteria in a given sample is usually too great to be counted directly. However, if the sample is serially diluted and then plated out on an agar surface the number of colonies can be used as a measure of the number of viable cells in that known dilution. However, keep in mind that if the organism normally forms multiple cell arrangements, such as chains, the colony-forming unit may consist of a chain of bacteria rather than a single bacterium. In addition, some of the bacteria may be clumped together. Therefore, when doing the plate count technique, we generally say we are determining the number of **Colony-Forming Units (CFUs)** in that known dilution. By extrapolation, this number can in turn be used to calculate the number of CFUs in the original sample.

Normally, the bacterial sample is diluted by factors of 10 and plated on agar. After incubation, the number of colonies on a dilution plate showing **between 30 and 300 colonies** is determined. A plate having 30-300 colonies is chosen because this range is considered statistically significant. If there are less than 30 colonies on the plate, small errors in dilution technique or the presence of a few contaminants will have a drastic effect on the final count. Likewise, if there are more than 300 colonies on the plate, there will be poor isolation and colonies will have grown together.

Generally, one wants to determine the number of CFUs **per milliliter (mL) of sample**. To find this, the number of colonies (on a plate having 30-300 colonies) is multiplied by the number of times the original ml of bacteria was diluted (the **dilution factor** of the plate is counted).

Enumeration of Bacteria - Viable Count (Plate Count) and Total Count

The quantity of practical 'germs' in a culture can be found out by deciding the quantity of colony forming units CFU with the colony counting technique. Somewhere in the range of 20 and 200 CFU can be relied on a regular Petri plate. Microbial societies of high thickness must be weakened before they are plated. A known volume [0,1mL] of these weakenings is plated onto appropriate development medium in the Petri dish. After the plates have been brooded up to

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seven days, the normal number of provinces on plates is resolved. The quantity of suitable microorganisms per milliliter (or g) of the underlying society (test) can be determined from the normal CFU's and the realized weakening element. The practical tally is constantly not exactly the aggregate cell check since it quantifies just cells fit for partitioning. The real detriments of the colony plating technique are:

- (i) The brooding time frame is protracted
- (ii) Sterile media , pipets and plates are required
- (iii) Examining and weakening mistakes happen

Two methods of this examinations are differentiated:

- 1) In the **pour–plate method**, an example from a precise weakening of organisms/test is pipetted onto a Petri - dish, at that point agar medium is poured over the fluid and blended.
- 2) In the **spread– plate strategy**, for the most part 0.1 ml of the weakened example is pipetted onto the surface of a hardened agar medium and spread with a disinfected, bowed, glass bar.

The hypothesis behind the technique of CFU builds up that a solitary organism can develop and turn into a colony through division. These provinces are plainly unique in relation to one another, both infinitesimally and visibly. This technique enables the client to know what number of CFU's are available per mL in the example. In this manner, it empowers us to know the microbiological stack and the extent of the contamination in people and creatures, or the level of defilement in tests of water, vegetables, soil or leafy foods mechanical items and theequipment.

Quantifying Bacteria by Spread Plate

The quantity of microscopic organisms in an answer can be promptly measured by utilizing the spread plate technique. In this technique, the example is properly weakened and a little aliquot is exchanged to an agar plate. The microscopic organisms are then circulated uniformly over the surface by a exceptional streaking technique. After states are developed, they are tallied and the quantity of microscopic organisms in the first example is determined. The end purpose of our examination is the quantity of colony forming units per mL (CFU/mL) since we are counting the quantity of provinces as opposed to the genuine number of microorganisms. CFU/ mL is all things considered a more valuable assurance than counting every one of the microorganisms under a magnifying instrument, since in numerous bacterial populaces, a critical number will be dead cells and along these lines of nointerest.

Diluting the Bacteria. Microorganisms usually grow up to densities around 10⁹ CFU/mL, despite the fact that the greatest densities shift immensely relying upon the types of microorganisms and the media they are developing in. therefore, to get promptly countable quantities of microorganisms, we need to make an extensive variety of weakenings and examine every one of them with the objective of having one or two weakenings with countable numbers. We do this by making sequential

10-overlap weakenings of the microbes that cover the whole plausible scope of focuses. At that point we exchange 0.1 mL of every weakening to an agar plate, which essentially makes another 10-overlap weakening, since the last unit is CFU/mL and we just streak 0.1 mL.

Inoculating the Plate. Streaking in this technique is finished utilizing a twisted glass pole. 0.1 mL of bacterial suspension is set in the focal point of the plate utilizing a sterile pipette. The glass pole is disinfected by first dunking it into a 70% liquor arrangement and afterward going it rapidly through the Bunsen burner fire. The consuming liquor cleans the pole at a cooler temperature than holding the pole in the burner fire, in this way decreasing the possibility of you consuming your fingers.

At the point when all the liquor has consumed off and the pole has air-cooled, mark the pole forward and backward over the plate working all over a few times. Not at all like streaking for disconnection, you need to backtrack commonly so as to convey the microbes as equally as could be expected under the circumstances. Turn the plate 90 degrees and rehash the side to side, here and there streaking. Turn the plate 45 degrees furthermore, mark a third time. Try not to sanitize the glass bar between plate turnings. Cover the plate furthermore, hold up a few minutes previously flipping around it for brooding. This will permit the stock to drench into the plate so the microscopic organisms will not dribble onto the plate cover.

Counting bacteria. Colonies are most readily counted using a plate counter. The plate counter has a light source and a magnifying glass making colonies easier to see. If at all possible, you do not want to count plates with more than 300 or less than 30 colonies. In the former case, the colonies, run together, and, in the latter, there are too few to allow statistically accurate counts. Once you count the colonies, multiply by the appropriate dilution factor to determine the number of CFU/ml in the original sample.

Total Counts

Direct Counts

Microscopic determination of the numbers of microorganisms which generally give the highest estimates. Usually applied with staining, in particular fluorescent staining is used with environmental samples.

Acridine Orange: binds to nucleic acids and fluoresce either green or orange.

DAPI: Fluoresce bright blue.

INT: Stains respiring microorganisms (viable).

Counting Chamber: Counting numbers cells in known volumes, such as haemocytometer.

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Preparing Hemocytometer

1. If using a glass hemocytometer and coverslip, clean with alcohol before use. Moisten the coverslip with water and affix to the hemocytometer. The presence of Newton's refraction rings under the coverslip indicates proper adhesion.
2. If using a disposable hemocytometer (for example, INCYTO DHC-N01), simply remove from the packet before use.

Preparing Cell Suspension

1. Gently swirl the flask to ensure the cells are evenly distributed.
2. Before the cells have a chance to settle, take out 0.5 mL of cell suspension using a 5 mL sterile pipette and place in an Eppendorf tube.
3. Take 100 μL of cells into a new Eppendorf tube and add 400 μL 0.4% Trypan Blue (final concentration 0.32%). Mix gently.

Counting

1. Using a pipette, take 100 μL of Trypan Blue-treated cell suspension and apply to the hemocytometer. If using a glass hemocytometer, very gently fill both chambers underneath the coverslip, allowing the cell suspension to be drawn out by capillary action. If using a disposable hemocytometer, pipette the cell suspension into the well of the counting chamber, allowing capillary action to draw it inside.
2. Using a microscope, focus on the grid lines of the hemocytometer with a 10X objective.
3. Using a hand tally counter, count the live, unstained cells (live cells do not take up Trypan Blue) in one set of 16 squares. When counting, employ a system whereby cells are only counted when they are set within a square or on the right-hand or bottom boundary line. Following the same guidelines, dead cells stained with Trypan Blue can also be counted for a viability estimate if required.
4. Move the hemocytometer to the next set of 16 corner squares and carry on counting until all 4 sets of 16 corners are counted.

Viability

To Calculate the Number of Viable Cells/mL

1. Take the average cell count from each of the sets of 16 corner squares.
2. Multiply by 10,000 (10^4).
3. Multiply by 5 to correct for the 1:5 dilution from the Trypan Blue addition.
The final value is the number of viable cells/mL in the original cell suspension.

Experiment 2: Effect of Temperature and pH on Bacterial Growth

Environmental conditions affect microbial growth like - Temperature, Oxygen, pH, Water Activity, Pressure, Radiation, Lack of Nutrients (these are the primary ones) (Refer Figure 1). The physical characteristics of the environment and the adaptations of microbes generally studied.

pH Effect

pH is defined as the negative logarithm of the hydrogen ion concentration of a solution, expressed in molarity. The pH scale ranges from 0 to 14, with 0 representing an extremely acidic solution (1.0 M H^+) and 14 representing an extremely alkaline solution ($1.0 \times 10^{-14} \text{ M H}^+$). Each pH unit represents a tenfold change in hydrogen ion concentration, meaning a solution with a pH of 3 is 10x more acidic than a solution with a pH of 4.

Typically cells would prefer a pH that is similar to their internal environment, with cytoplasm having a pH of 7.2. That means that most microbes are neutrophiles ('neutral lovers'), preferring a pH in the range of 5.5 to 8.0. There are some microbes, however, that have evolved to live in the extreme pH environments.

Acidophiles ('acid lovers'), preferring an environmental pH range of 0 to 5.5, must use a variety of mechanisms to maintain their internal pH in an acceptable range and preserve the stability of their plasma membrane. These organisms transport cations (such as potassium ions) into the cell, thus decreasing H^+ movement into the cell. They also utilize proton pumps that actively pump H^+ out.

Alkaliphiles ("alkaline lovers"), preferring an environmental pH range of 8.0 to 11.5, must pump protons in, in order to maintain the pH of their cytoplasm. They typically employ antiporters, which pump protons in and sodium ions out.

Temperature

Microorganisms have no real way to control their inside temperature so they should develop adjustments for the earth they might want to live in. Changes in temperature have the greatest impact on proteins and their movement, with an ideal temperature that prompts the quickest digestion and coming about development rate. Temperatures underneath ideal will prompt a decline in chemical movement and slower digestion, while higher temperatures can really denature proteins, for example, catalysts and transporter proteins, prompting cell passing. Thus, microorganisms have a development bend in connection to temperature with an ideal temperature at which development rate crests, and additionally least and greatest temperatures where development proceeds yet is not as strong. For a bacterium the development extend is ordinarily around 30 degrees. The psychrophiles are the cool sweethearts, with an ideal of 15°C or lower and a growth scope of -20°C to 20°C . A large portion of these organisms are found in the seas, where the temperature is regularly 5°C or colder. They can likewise be found in the Arctic and the Antarctic, living in ice wherever they can discover

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pockets of fluid water. Adjustment to the cool required advancement of explicit proteins, especially catalysts, that can even now work in low temperatures. Likewise, it additionally expected change to the plasma film to keep it semifluid. Psychrophiles have an expanded measure of unsaturated and shorter-chain unsaturated fats. Ultimately, psychrophiles create cryoprotectants, uncommon proteins or sugars that keep the improvement of ice precious stones that may harm the cell. Psychrotrophs or cool tolerant organisms have a scope of 0-35°C, with an ideal of 16°C or higher.

People are best familiar with the mesophiles, organisms with a development optima of 37°C and a scope of 20-45°C. All of the human microflora fall into this classification, and in addition every single human pathogen. The mesophiles possess similar situations that people do, as far as sustenances that we eat, surfaces that we contact, and water that we drink and swim in.

On the hotter end of the range is the place we discover the thermophiles ('warm sweethearts'), the organisms that like high temperatures. Thermophiles regularly have a scope of 45-80°C, and a development ideal of 60°C. There are additionally the hyperthermophiles, those organisms that like things additional fiery. These organisms have a development optima of 88-106°C, at least 65°C and a most extreme of 120°C. Both the thermophiles and the hyperthermophiles require specific warmth stable catalysts that are impervious to denaturation and unfurling, mostly because of the nearness of defensive proteins known as chaperone proteins. The plasma film of these life forms contains increasingly immersed unsaturated fats, with expanded dissolving focuses.

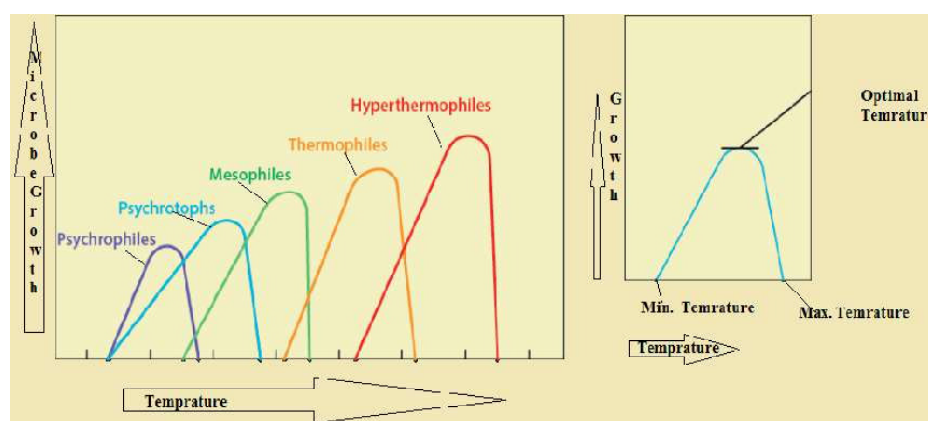


Fig. 1: Bacterial Growth

Experiment 3: Nitrogen Metabolism in Bacteria - Nitrate Reduction Test

Nitrate reduction test is utilized for the separation of members of Enterobacteriaceae on based on their capacity to produce nitrate reductase enzyme that hydrolyze nitrate (NO_3^-) to nitrite (NO_2^-) which may then again be degraded to various nitrogen products like nitrogen oxide, nitrous oxide and ammonia (NH_3) depending on the enzyme system of the life form and the climate in which it is developing.

Principle

In inoculum of test microorganism is incubated in nitrate broth. After 4 hrs incubation, the broth is tested for reduction of nitrate (NO_3^-) to nitrite (NO_2^-) by adding sulfanilic acid reagent and α -naphthylamine.

1. If the microorganism has reduced nitrate to nitrite, the nitrites in the medium will form nitrous acid. When sulfanilic acid is added, it will react with the nitrous acid to produce diazotized sulfanilic acid. This reacts with the α -naphthylamine to form a red-colored compound. Hence, if the medium turns red after the addition of the nitrate reagents, it is considered a positive result for nitrate reduction.
2. If the medium does not turn red after the addition of the reagents, it can mean that the microorganism was unable to reduce the nitrate, or the microorganism was able to denitrify the nitrate or nitrite to produce ammonia or molecular nitrogen. Therefore, another step is needed in the test. Add a small amount of powdered zinc. If the tube turns red after the addition of the zinc, it means that unreduced nitrate was present. Therefore, a red color on the second step is a negative result.

Materials Requirement

1. **Media:** Nitrate Broth with Inverted Durhams Tube
2. **Reagents:** Sulphanilic Acid Reagent, Alpha Naphthylamine Reagent, Zinc Dust
3. **Others:** Inoculating Loop, Burner, Dropper

Procedure

1. Inoculate nitrate broth with a heavy growth of test microorganism using aseptic technique.
2. Incubate at an appropriate temperature for 24 to 48 hours.
3. Add one dropperfull of sulfanilic acid and one dropperfull of α -naphthylamine to each broth.
 - (i) At this point, a color change to RED indicates a POSITIVE nitrate reduction test. If you get a red color, then you can stop at this point.
 - (ii) No color change indicates the absence of nitrite. This can happen either because nitrate was not reduced or because nitrate was reduced to nitrite, then nitrite was further reduced to some other molecule. If you DO NOT get a red color, then you must proceed to the next step.
4. Add a small amount of zinc (a toothpick full) to each broth. Zinc catalyzes the reduction of nitrate to nitrite.
 - (i) At this point, a color change to red indicates a negative nitrate reduction test because this means that nitrate must have been present and must have been reduced to form nitrite.

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- (i) No color change means that no nitrate was present. Thus no color change at this point is a positive result.

Figure 1 illustrates the process.

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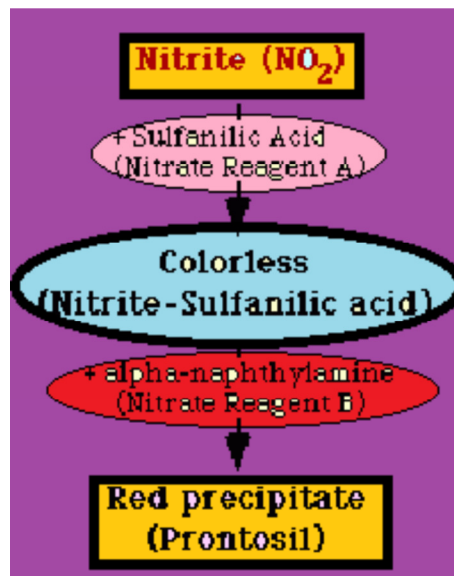


Fig. 1: Process Representation

Result

1. **Nitrate Reduction Positive:** (Red After Sulfanilic Acid + Alpha-Naphthylamine; No Color After Zinc)
2. **Nitrate Reduction Negative:** (No color After Sulfanilic Acid + Alpha-Naphthylamine followed by Red after zinc)

Experiment 4: Microbial Degradation of Azo Dyes (Study)

Unpredictable disposal of industrial effluents into water bodies represents a noteworthy risk to nature. Azo dyes are the biggest and most flexible class of dyes, which share over half of the dyes created every year. Benzidine has for some time been perceived as a human urinary bladder cancer-causing agent and tumorigenic in an assortment of research center/Lab animals. The detoxification and transfer of slop is an issue to the textile dye units. Microbiological treatment of slop is the most ideal route for detoxification. A consecutive anaerobic and high-impact treatment process dependent on blended culture of microscopic organisms separated from textile dye effluent contaminated soil has been used degrade sulfonated azo dyes orange-G, amido black, direct red. Different reactor designs have been proposed in order to obtain an effective continuous anaerobic/aerobic treatment of azo dyes.

Azoreductase in Biodegradation

Enzymatic biodegradation of azo dyes catalyzes reductive cleavage of azo gatherings (-N=N-) under mellow conditions. Azoreductase catalyzes the reductive

cleavage of azo linkages in benzidine based dyes and different mixes containing an azo attach to deliver fragrant amines. Numerous bacterial strains have rather unspecific cytoplasmic compounds, which go about as ‘azoreductases’ and under anaerobic conditions exchange electrons through solvent flavins to azo dyes. Azoreductases have been distinguished in liver cells and a few anaerobic microscopic organisms. The intestinal microbial vegetation of people, monkeys and rodents diminish azo dyes to amines. Azoreductases of these microbes are comparable in their capacities.

Process

The recombinant strains expressing high level of flavin reductase in the presence of oxygen, is assumed an azoreductase activity. It has been found that under aerobic conditions there may be some decrease in the concentration of dye. Almost identical oxidation rates for NADH have been observed under aerobic and anaerobic conditions. There are some reports about the presence of unspecific aerobic azoreductases in bacteria such as *E. coli*. Several bacterial azoreductase activities have been described in cell extracts. When azo dye is incubated in oxygen-free buffer with

NADH as a source of reduction equivalents, a slow decolourization of azo dye has been observed. Addition of cell extract in the presence of flavin adenine dinucleotide (FAD) to the reaction mixture enhances the reaction rate after a few minutes and shortened the lag phase. A possible explanation for this pronounced effect is that FAD is reduced enzymatically by NADH and FADH₂ can then spontaneously reduce the azo dye. Cofactors like FADH₂, FMNH₂, NADH and NADPH, as well as the enzymes reducing these cofactors are located in the cytoplasm. Lysis of cells would release cofactors in the extracellular environment. Hence, it has been reported that cell extracts of starving or lysed cells show higher azo dye reduction rates than intact or resting cells. However, a membrane transport system may be a prerequisite for reduction of azo dyes by these cofactors in intact cells. In addition, FAD and FMN cannot readily cross cell walls. In contrast, riboflavin is able to move across cell membranes

(Sources: - <https://pdfs.semanticscholar.org/232e/08a8c10f7257a182bcb64a2a6b29cb6ff6eb.pdf>)

Result: A FMN dependent NADH- azoreductase of (*E. coli*) *Microorganism* has been purified and N- terminal sequence of the azoreductase.

Experiment 5: Cytochrome Oxidase Assay

Cytochrome c oxidase is the last enzyme in the respiratory electron transport chain of mitochondria. Its main function is to convert molecular oxygen to water and aid in establishing mitochondrial membrane potential. Cytochrome c oxidase locates to the inner membrane which separates the mitochondrial matrix from the intermembrane space. This colorimetric assay is based on observation of the decrease in absorbance at 550 nm of ferrocytochrome c caused by its oxidation to ferricytochrome c by cytochrome c oxidase.

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Mitochondria cytochrome c oxidase activity was measured on rodent liver tissue and human dermal fibroblasts derived mitochondria in serial dilution. The cytochrome c oxidase activity (OD550nm) is proportional to the amount of mitochondria in reaction.

Reagents and Equipments

1. Spectrophotometer
2. 1mL Cuvettes
3. Ultrapure Water
4. Mitochondria Isolation Kit

Sample and Buffer Preparation

1. **Mitochondria Isolation:** Isolate mitochondria from cultured cells or tissue by using mitochondria isolation kit.
2. **Cytochrome c Working Solution (1X):** Thaw and dilute cytochrome c stock solution in ultra-pure water (1:4).
3. **Substrate:** Add 10 μ L DTT solution per mL cytochrome c working solution (1X), mix and leave it in room temperature for 15~ 20 minutes.
4. **Checking the Efficiency of Reducing Cytochrome c:** Mix 50 μ L substrate with 950 μ L of assay buffer read its OD at 550nm and 560nm. The optimal ratio (550nm/560nm) should be between 10 and 20.

Procedure for Cytochrome C Oxidase Activity Assay (1mL Cuvette)

1. Set the spectrophotometer at 550 nm on a kinetic program:
Duration: 30 seconds
Interval: 5 seconds
2. Warm the assay solutions to room temperature before starting the reaction.
Mix well.
3. Prepare sample reactions according to the reaction scheme (Refer Table 1)

Table 1: Reactions

Assay Buffer	940-X μ L
n-Dodecyl β -D-Maltoside solution	10 μ L
Mitochondrial protein (0.5~2 μ g)	X μ L

4. Mix solution in cuvette. Note: If several samples are required for measuring, n-Dodecyl β -D-Maltoside solution is recommended to be added just before blanking.
5. Blank spectrophotometer with reaction mixture.
6. Add 50 μ L substrate solution and mix (covered by parafilm and shake).
7. Immediately read and record decrease in OD for 30 seconds.

8. Calculate $\Delta A/\text{min}$ by using of the maximum linear rate. The oxidation of cytochrome c by cytochrome c oxidase is biphasic reaction with a fast initial burst of activity followed by a slower reaction rate. ΔA = change in OD reading.

9. Calculate cytochrome c oxidase activity of the sample.

$$\text{Unit/mg Mitochondria} = (\Delta A/\text{min}) / (\epsilon \times \text{mg mitochondria})$$

$$\Delta A/\text{min} = (\text{Change in OD Reading})/\text{Time}$$

$\epsilon = 19.6 \text{ mM}^{-1} \text{ cm}^{-1}$ and ϵ is extinction coefficient of reduced cytochrome c solution at 550 nm.

Unit Definition: One unit would oxidize 1.0 μmole reduced cytochrome c per minute at pH 7.2 at 25 °C.

(Source:- <https://www.sciencellonline.com/PS/8278.pdf>)

Experiment 6: Estimation of Alkaline Phosphatase Activity (Enzyme Based)

Principle

Alkaline Phosphatase (ALP) is an enzyme naturally present in all raw milks, which is utilized as an indicator of proper milk pasteurization. Complete pasteurization will inactivate the enzyme to below levels which are detectable by conventional methods. Because the warmth dependability of ALP is more prominent than that of pathogens which might be available in milk, the enzyme serves as an indicator of product safety. Pasteurisation is an essential process in the production of milk which is safe and free from pathogens. Alkaline Phosphatase test is used to indicate whether milk has been adequately pasteurised or whether it has been contaminated with raw milk after pasteurisation. This test is based on the principle that the alkaline phosphatase enzyme in raw milk liberates phenol from a disodium para-nitro phenyl phosphate and forms a yellow coloured complex at alkaline pH. The intensity of yellow colour produced is proportional to the activity of the enzyme. The test is not applicable to sour milk and milk preserved with chemical preservatives.

Apparatus Required

1. Water-Bath-Maintained at $37 \pm 1^\circ\text{C}$, Thermostatically Controlled.
2. Comparator - With Special Discs of Standard Colour Glasses Calibrated in μg p-Nitrophenol Per mL Milk, and 2×25 mm Cells.
3. Test Tubes - of Size 16×1.50 mm and Rubber Stoppers to fit.
4. Pipettes - 1, 5, and 10 mL.
5. Filter Paper
6. Litmus Paper

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Reagents

1. Sodium Carbonate-Bicarbonate Buffer - Dissolve 3.5 g of anhydrous sodium carbonate and 1.5 g of sodium bicarbonate in one litre of distilled water.
2. Buffer Substrate - Dissolve 1.5 g of disodium p-nitrophenyl phosphate in one litre of sodium carbonate-bicarbonate buffer. This solution is stable if stored in a refrigerator at 4°C or less for one month but a colour control test should be carried out on such stored solutions

Procedure

1. Pipette 5 mL of buffer substrate into a clean, dry test tube followed by 1 mL of the milk to be tested. Stopper the tube, mix by inversion and place in the water-bath.
2. At the same time place in the water-bath a control tube containing 5 mL of the buffer substrate and 1 mL of boiled milk of the same kind as that under test that is pasteurized homogenized, low fat.
3. After 2 hours, remove the tubes from the bath, invert each and read the colour developed using the comparator and special disc, the tube containing the boiled milk control being placed on the left of the stand and the tube containing the sample under test on the right. Record readings which lie between two standard colour discs by adding a plus (+) or minus (-) sign to the figure of the nearest standard.

Result

The colour intensity is measured by direct comparison with standard colour discs in a Lovibond comparator.

APPENDIX - I

*LAB I – General
Microbiology, Microbial
Physiology and
Biochemistry*

Morphology of Bacteria

Microbiology is the study of microscopic organisms that are invisible to the naked eye and can be observed only under a microscope. They can be:

- (i) Unicellular (single-celled)
- (ii) Multicellular (having numerous cells), or
- (iii) Acellular (lacking cells)

Such organisms include bacteria, fungi, protozoa and viruses. Microbiology deals with the study of the growth, characteristics, morphology and various other related aspects of such an organism. This acquired knowledge can be beneficially used to study their relationship with human beings and also the treatment and prevention of the diseases caused by these organisms.

Some Important Definitions

Microbiology has simplified the process of understanding the biology of microorganisms. However, with this simplification, it has also brought some challenges, which have influenced the society in both positive and negative ways. The most difficult challenges for the basic understanding of microbial cells are the spread of diseases like Acquired Immunodeficiency Syndrome (AIDS) that appear without showing any signs of their presence. The functioning style of cells at the elementary level can be now easily understood by the help of new discoveries in the field. How the microorganisms play a crucial role in the environment can be understood through the microbial ecology.

A biofilm is considered as a powerful form of organization of microbial communities. A huge number of unculturable microbes can be analysed and their characteristic features can be studied with the help of genomic tools.

Microorganisms play a more positive role than simply causing infectious diseases. They can provide humanity with an even better and more healthful existence.

- **Bacteriology (study of bacteria):** The smallest, simplest, single-celled prokaryotic microorganisms and archaea–prokaryotic microorganisms that form an ancient group intermediate between the bacteria and eukaryotes.
- **Mycology (study of fungi):** Microscopic eukaryotic forms (molds and yeasts), higher forms (mushrooms, toadstools and puffballs), and slime molds.
- **Virology (study of viruses):** Infectious agents containing either DNA or RNA that require living cells for their replication/ or reproduction) and viral diseases

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- **Parasitology:** Study of parasitism and parasites that include pathogenic protozoa, helminth worms and some insects
- **Microbial ecology:** Study of interrelationships between microbes and the environment.
- **Microbial morphology:** Study of detailed structures of microorganisms.
- **Microbial systematics:** Classification, naming and identification of microorganisms and constructions of the phylogenetic tree of life.
- **Microbial physiology:** Study of metabolism of microbes at the cellular and molecular levels.
- **Microbial biochemistry:** Study of the discovery of microbial enzymes and the chemical reactions carried out by them.
- **Molecular microbiology:** Study of genome (i.e., genomics) of microorganisms and construction of phylogenetic tree based on rRNA.
- **Microbial genetics:** Study of heredity and variation in varieties.
- **Molecular biology:** Advanced study of the genetic material (DNA, RNA) and protein synthesis.
- **Immunology:** The immune system that protects against infections and attempts to understand many phenomena responsible for both acquired and innate immunity, in addition to the study of antibody–antigen reactions in the laboratory.
- **Agricultural microbiology:** Study of relationships of microbes and crops with an emphasis on control of plant diseases and improvement of yields.
- **Food Microbiology:** Interaction of microorganisms and food in relation to food bioprocess.
- **Dairy Microbiology:** Study of the production and maintenance of quality dairy products.
- **Industrial Microbiology:** Industrial uses of microbes in the production of alcoholic beverages, vitamins, amino acids, enzymes, antibiotics and other drugs.
- **Marine Microbiology:** Study of microorganisms and their activity concerning human and animal health in fresh, estuarine and marine waters.
- **Air Microbiology:** Role of aerospora in contamination and spoilage of food and dissemination of plant and animal diseases through air.
- **Exomicrobiology:** Exploration for microbial life in outer space.
- **Diagnostic Microbiology:** Explores the fundamental principles and techniques involved in the study of pathogenic organisms as well as their application in the diagnosis of infectious diseases.

- **Epidemiology and Public:** Monitoring, control and spread of diseases in communities.
- **Biotechnology:** The scientific manipulation of living organisms, especially at the molecular and genetic level to produce useful products.

To observe and study the microorganisms the concept of microscopy comes into the picture. It is the technical field of using microscopes to view the disease-causing organisms which cannot be seen with the unaided eye. So microscopy is considered an inherent part of Microbiology.

Micrometry

The method of measuring a microscopic organism under a microscope with the help of a calibrated scale is known as micrometry. Devices like the eyepiece micrometer and stage graticule are used in the measurement and calibration processes. This fundamental methodology is applied to the measurement of any specimens noticed in the microscope and for performing measurements at high magnifications in compound optical microscopy must be brought into practice.

The most common of these is the application of eyepiece reticles in combination with stage micrometers where measurements made with compound microscopes fall into the size range of 0.2 micrometers to 25 millimeter.

A lot of approaches have been employed for measuring linear, area, and volume specimen dimensions with the microscope called micrometry or morphometrics.

Classification of Bacteria

As has been mentioned earlier bacteria are microorganisms which cause a wide range of diseases in humans. So, to study the diseases caused by this group of organisms it is very essential that you first classify them accordingly.

Bacteria can be classified into various types. At first, you will be introduced to the phenotypic classification of bacteria.

Phenotypic Classification

- (i) The prokaryotes consist of genetically distinct unicellular organisms.
- (ii) They lack structural diversity.
- (iii) They show a wide range of genetic and physiological diversity.

The phenotypic characteristics include:

- (a) Gram stain
- (b) Morphology (rods, cocci, etc.)
- (c) Motility
- (d) Presence of spores, filaments, sheaths, appendages

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(e) Physiological features like photosynthesis, anaerobiosis (capability of the microorganisms to grow in the absence of oxygen), methanogenesis (Methanogenesis or biomethanation is the formation of methane by microbes known as methanogens), lithotrophy (A lithotroph is an organism that uses an inorganic substrate (usually of mineral origin) to obtain reducing equivalents for use in biosynthesis (e.g., carbon dioxide fixation) or energy conservation via aerobic or anaerobic respiration)

Figure 1 broadly shows the different bacteria present categorized on the basis of their phenotypes (i.e. external characters or characters which are visible).

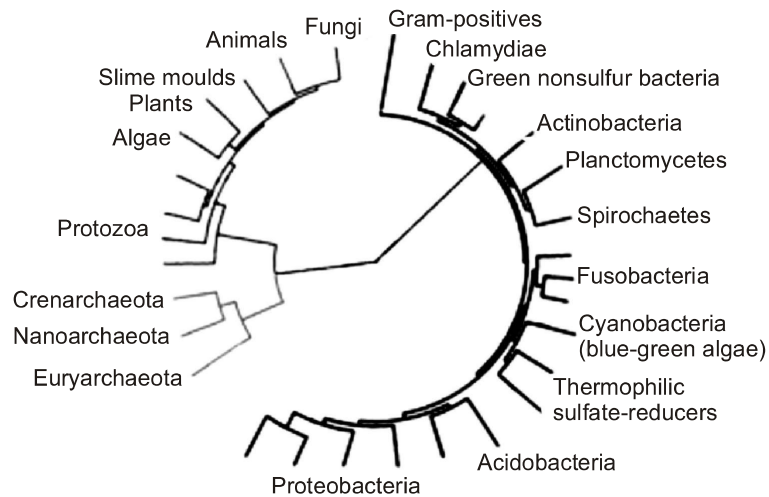


Fig. 1: Phenotypic Classification of Bacteria

Source: <http://www.google.co.in/search?q=phenotypic+classification+of+ bacteria>—accessed 15/02/13

Figure 2 shows the phylogenetic tree of life based on comparative *ssrRNA* sequencing.

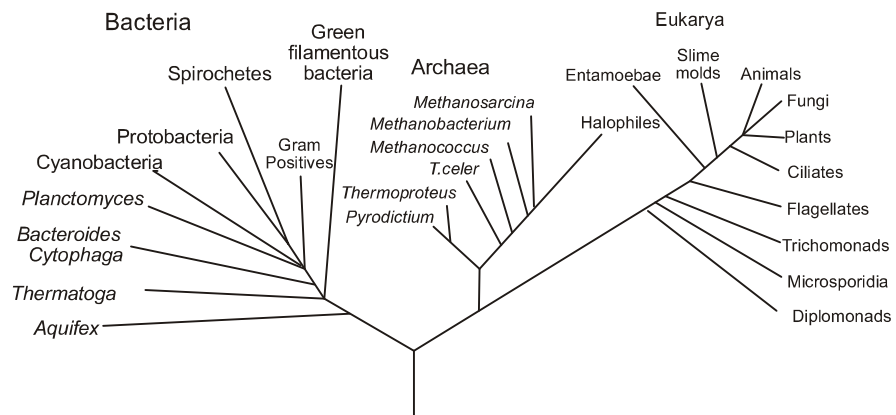


Fig. 2: The Phylogenetic Tree of Life based on Comparative *ssrRNA* Sequencing

Source: <http://textbookofbacteriology.net/themicrobialworld/procaryotes.html>—accessed 15/02/13

It is important to study multiple isolates within a given species. This is done to determine whether they represent a single strain or multiple strains. A strain is a species of bacteria isolated and cultivated in the laboratory; a single isolate with distinctive characteristics represents a strain.

Members in the species having small differences between them can be distinguished by additional methods, and subdivided into subspecies, subgroups, biotypes, serotypes and variant. This process of differentiating strains based on their phenotypic and genotypic differences is called 'typing'.

Typing Methods

Clinical microbiology laboratories determine biological profiles (biotypes), antimicrobial susceptibility patterns of bacteria commonly isolated from specimens. Laboratories also determine serological profiles by establishing facilities for serologic typing (serotypes), bacteriophage susceptibility patterns (phage typing), bacteriocin production patterns, bacteriocin susceptibility patterns, plasmid analysis and chromosomal DNA analysis for a variety of bacteria, mycobacteria, viruses and fungi causing nosocomial infections.

- (i) **Resistotyping:** This is a typing technique that encompasses comparison of several isolates to a given set of antibiotics. Once these isolates differ in their susceptibilities, they are considered to be different strains. This is useful in identifying an outbreak, that is, the identification of definite patterns of antibiotic resistance in these isolates that are cultured from multiple patients. This is an indication of an outbreak. This technique is easy to perform and interpret with a fair amount of reproducibility. The antibiotic sensitivity pattern of isolates recorded in a period of time, which represents the same strain, could differ for one or more antibiotics because of the acquisition of antibiotic resistance.
- (ii) **Serological Typing:** Serological methods are based on the concept that the microorganisms show variations in antigenic constitution between distinctly related, unrelated and within groups of closely related organisms. The identification of specific antigens of microorganisms by using specific antibodies permits very fine subdivision of microbial taxa, which permits surveillance and control of spread of pathogenic microorganisms.

The antigens used for serotyping are:

- (a) Cell wall
- (b) Capsule
- (c) Slime
- (d) Flagellae
- (e) Pili

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(f) Endotoxins and exotoxins

(g) Extracellular enzymes

Any of these listed antigens can be used for typing monoclonal antisera against these antigens or polyclonal antisera containing separate antibodies to various antigenic determinants to permit specific identification. Serotyping is based on specificity but cross-identification between protein antigens and carbohydrate antigens is commonly seen. However, protein antigens are less cross-reactive and are better used for serotyping. The antigens used in serotyping are determined by genetic make-up which may be due to structural genes or extraneous genetic elements, such as bacteriophages, plasmids and episomes. Some gram-negative cocco-baccili may show variation. The antigens with single known determinants make antibodies of only one specificity. For example, Salmonella 'O' antigen with determinant 11 will form specific antibodies anti 11.

Serotyping involves precipitation reaction between an antigen in a colloidal solution and an antibody to form a lattice and give visible precipitation because of antigens bound to latex or bentonite or tanned red blood cells.

Examples are

- Cytolysis used for *Vibrio cholerae* and gram-negative rods
- Neutralization tests for typing toxins of *Clostridium botulinum*

(iii) **Bacteriophage Typing:** Plaque means a zone of clearing on a lawn of bacteria caused by the lysis of cells by infecting bacteriophages (Figure 3). The size, shape and nature of plaques differ from phage to phage. The specificity of phage–bacterium interaction is made use of in the identification of typing of bacteria. Phages exhibit different degrees of host specificity. Some phages possess wide host ranges, covering many bacterial genera, while others have a narrow range limited to certain bacteria only. With some phages, a serial passage in a strain of bacterium makes them specific for that strain and related strains (adaptation of host range). Phages lyse the following:

- All members of a bacterial genus (for example, genus-specific bacteriophage for *Salmonella*)
- All members of a species (for example, specific bacteriophage for *B. anthracis*)
- All members of a biotype or subspecies.

The most important application of phage typing is in intra-species typing of bacteria, such as in *S. typhi* and staphylococci. The highest dilution of the phage preparation that just produces confluent lysis is known as Routine Test Done (RTD).

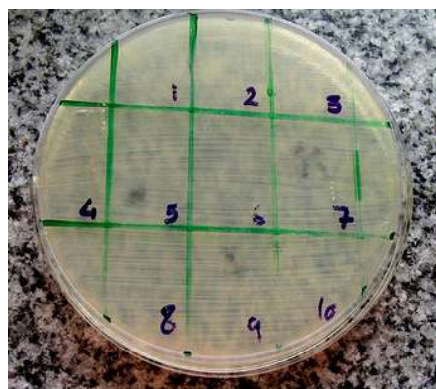


Fig. 3: Bacteriophage Typing of *Staphylococcus aureus*

Source: medadteam.org

(iv) **Biotyping:** Biotyping is a typing method that uses the inherent pattern of metabolic activities being expressed by a particular isolate or its colonial morphology and are hence called ‘biotypes’. Biotyping is performed manually or by using automated systems as follows:

- (a) Reactions in sugar fermentation
- (b) Amino acid decarboxylation/deamination; enzymatic tests like citrate, urease.
- (c) Tolerance to pH, chemicals and dyes
- (d) Haemagglutination and/or haemolysis

Disadvantages: They have poor discriminatory power. Variation in gene expression is the most common reason for isolates that represent single strain to differ in one or more biochemical reactions.

Genotypic Classification

Till the late 19th century, bacteria were classified on the basis of phenotypic classification. At present, the genotypic methods are more widely used for a more satisfactory classification of bacteria.

Genotypic methods include the various nucleic acid-based typing systems, which are discussed as follows:

- (i) **Plasmid Analysis:** This is a convenient and beneficial technique for epidemiologic typing of different types of organisms. In outbreaks, plasmid profiling is a rapid and suitable method to follow the spread of the epidemic strain. It is more specific than other typing methods. Plasmid analysis is the earliest technique applied to the diagnosis of infectious diseases. Plasmids are found in many types of bacteria. These are small self-replicating circular double stranded DNA.
- (ii) **Restriction Fragment Length Polymorphism (RFLP) Analysis:** In molecular biology, RFLP refers to the difference between two or

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more samples of homologous DNA molecules arising from differing locations of restriction sites, and to a related laboratory technique by which these segments can be distinguished. In RFLP analysis, the DNA sample is broken into pieces (digested) by restriction enzymes and the resulting *restriction fragments* are separated according to their lengths by gel electrophoresis. Today, this analysis is largely obsolete.

- (iii) **Pulsed Field Gel Electrophoresis (PFGE):** PFGE, a standard gel electrophoresis technique for separation of DNA molecules, uses a specialized electrophoresis device to separate chromosomal fragments produced by enzymatic digestion of intact bacterial chromosomal DNA. Bacterial suspensions are first embedded in agarose plugs, where they are lysed to release intact chromosomal DNA; the DNA is then digested using restriction endonuclease enzymes. As large-sized DNA fragments are produced, the resolution of bonding patterns requires the use of pulsed electrical fields across the agarose gel.
- (iv) **Ribotyping:** The analysis of restriction fragments with probes that detect rRNA is known as ribotyping. It involves enzymatic digestion of chromosomal DNA followed by southern hybridization using probes for genes that encode ribosomal RNA, i.e., 16s or 23s.
- (v) **Random Amplification of Polymorphic DNA (RAPD):** RAPD is a type of polymerase chain reaction (PCR), but the segments of DNA that are amplified randomly. RAPD markers are DNA fragments from PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequence.
- (vi) **DNA Microarray:** DNA microarray is a multiplex technology used in molecular biology and in medicine. It consists of an arrayed series of thousands of microscopic spots of DNA oligonucleotides, called features, each containing picomoles (10–12 moles) of a specific DNA sequence, known as ‘probes’ (or *reporters*). This technology promises to monitor the whole genome on a single chip so that researchers can have a better picture of the interactions among thousands of genes simultaneously. Gene chip is used as a general terminology that refers to a micro array technology. Hybridization is the central event between an array of ‘targets’ positioned at designated spots on a solid support (the chip) and the mixture of labelled ‘probes’. Hybridization at each spot is scanned and data produced is managed and analysed by appropriate software systems to produce colourful data arrays.

Microbial Cells: Prokaryotes and Eukaryotes

After studying the phenotypic and genotypic classification the next important step will be to know about the basic structure of a bacterial cell.

There are two types of cells: prokaryotic and eukaryotic. Both cells commonly contain the following structures:

- Cell membrane
- Cytoplasm
- Genetic material
- Energy currency
- Enzymes and co-enzymes

Differentiating Properties of Bacteria and Viruses

Bacteria and viruses are different. How will you differentiate between the two? Here are certain characteristics that will help you distinguish one from the other.

- Viruses are the smallest and simplest life form known. They are 10–100 times smaller than bacteria.
- The biggest difference between viruses and bacteria is that viruses must have a living host, like a plant or animal to multiply, whereas most bacteria can grow on non-living surfaces.
- Bacteria contain both DNA and RNA while viruses contain either DNA or RNA.
- All viruses are harmful but not all bacteria are harmful. There are some useful bacteria as well.
- Antibiotics can kill bacteria but not viruses.
- An example of a disease caused by bacteria is streptococcal throat infection and an example of an affliction caused by a virus is the flu.

Size and Major Groups of Bacteria

Medically important bacteria may be divided into groups depending on their shape:

- **Cocci** are spherical, or round.
- **Bacilli** are relatively straight, rod-shaped or cylindrical cells; when the length of the cells is equal to width, these bacillary forms are called as coccobacilli.
- **Vibrios** are comma-shaped rods.
- **Spirilla** are rigid spiral or helical forms.
- **Spirochaetes** are coiled.
- **Mycoplasma** refers to the genus of cell wall-deficient bacteria that occur as round or oval bodies and interlacing filaments.

Figure 4 demonstrates the different shapes of bacteria such as coccus, bacillus, spirilla and so on.

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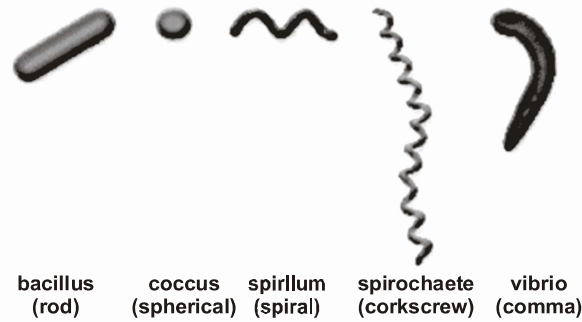


Fig. 4: Different Shapes of Bacteria

Source: <http://www.microbiologyonline.org.uk/about-microbiology/introducing-microbes/bacteria>—accessed 08/03/2013)

The pathogenic bacteria show characteristic of cellular arrangement or grouping that is determined by the plane through which binary fission takes place and these are grouped as:

- **Diplococci:** Cocci arranged in pairs
- **Streptococcus, enterococcus, and lactococcus:** Cocci in chains are noted.
- Grape-like clusters (staphylococci)
- Cubical packets of eight cocci
- Bacilli as flat, rounded, cigar-shaped, or bifurcated arranged in chains (streptobacilli)
- Bacilli in various angles resembling the letters V or Chinese letter arrangement is characteristic of *Corynebacterium diphtheriae*

Measurement of Bacterial Size

The size of bacteria can be measured through the following stepwise procedure:

- First, the diameter of the microscope's field of view is measured. The low-power objective is used to look through the microscope, and the ruler is placed under the field of view. Then the diameter is measured in millimeters. For instance, you may find that the diameter of the field of view to be 5 mm.
- The measurement of the diameter is then converted from millimeters to micrometers as bacteria are usually measured in smaller units such as micrometers. There are 1,000 μm in 1 mm. If the diameter of the field of view is 5 mm (as considered above), then multiply 5 by 1,000; so the field of view will appear as 5,000 μm in diameter.
- Then the bacteria are observed under the microscope under low power. Place the bacteria slide on the stage of the microscope, and then bring it into focus using the fine-course adjustment knobs.

- An individual bacterium is located among several bacteria which the slide will contain. Then estimate how many times it will fit across the field of view. For example, you may find that a single rod-shaped bacterium will fit across the field of view about three times.
- Divide the diameter of the field of view by the number of times that the bacterium fits across the field of view. For example, if the diameter is 5,000 μm , and the bacterium under consideration fits across the field of view three times, then you divide 5,000 by 3. In this case, the bacterium is approximately 1.667 μm .

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Structure of a Bacterial Cell

The structure of bacterial cell includes capsule, cell wall, appendages, cytoplasm, intra-cytoplasmic inclusions, bacterial spores; capsule, pili, fimbriae and L-forms.

Structurally, there are three architectural regions: appendages (attachments to the cell surface) in the form of flagella and pili (or fimbriae); a cell envelope consisting of a capsule, cell wall and plasma membrane; and a cytoplasmic region that contains the cell chromosome (DNA) and ribosomes and various sorts of inclusions. These structural features have been shown in Figure 5.

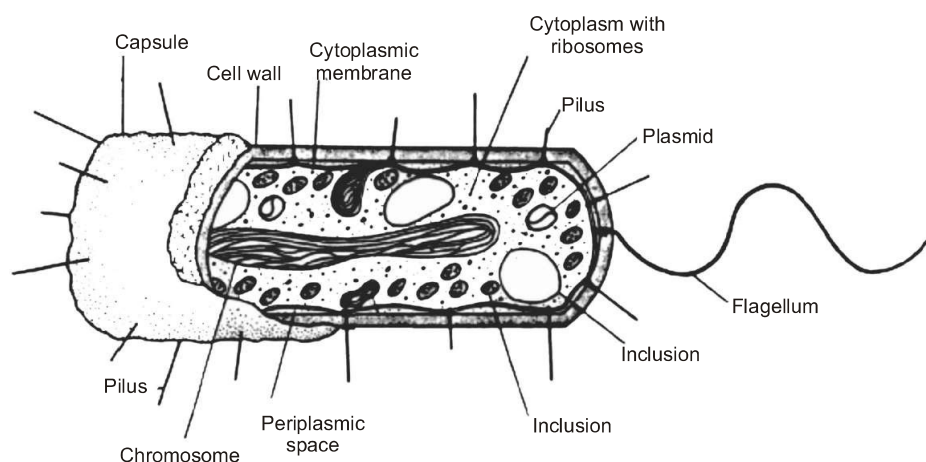


Fig. 5: Cutaway Diagram of a Typical Bacterial Cell

Source: <http://textbookofbacteriology.net/structure.html>—accessed 15/02/13

- Cell envelope or capsule:** Prokaryotic cells, particularly bacterial cells, have a chemically complex cell envelope in which three basic layers can be identified as the outermost *gluco-oligosaccharides* glycocalyx, followed by a cell wall and a cell membrane or plasma membrane. Altogether, each layer of cell envelope performs a distinct function but together they act as a single protective unit: glycocalyx is the outermost layer made up of macromolecules like proteins, polysaccharide, starch, etc. It protects the cell and helps in bacterial adhesion to the host cell. It is a virulence factor in many

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pathogenic bacteria. Some bacteria show a loose sheath called the slime layer which protects the cell from loss of water and nutrients. Others may have a thick and tough covering known as capsule and these are made up of polysaccharides, proteins.

(ii) **Cell wall:** The cell wall is 10–25 nm thick and relatively rigid, with some elasticity. It is openly porous and freely permeable to solute molecules smaller than 10 kDa in mass and 1 nm in diameter. The cell wall is made of peptidoglycan formed by N-acetyl glucosamine and N-acetyl muramic acid molecules. Antibiotics interfere with the construction of the cell wall, peptidoglycan. In gram-positive bacteria, the cell wall consists mainly of peptidoglycan and teichoic acids, whereas in gram-negative bacteria the cell wall is more complex in both the anatomical and chemical sense and includes the thinner peptidoglycan and outer membrane. The cell wall is the second layer of the cell envelope lying below the glycocalyx. It provides strong structural support to prevent a bacterium from collapsing in a hypotonic solution, meaning a solution having low salt concentration. This layer is rigid because of macromolecules called peptidoglycan. Gram-positive organisms contain more teichoic acid in the cell wall while gram-negative organism contains them in lesser quantity.

(iii) **Flagellum:** The bacterial flagellum is composed of the following three parts:

- (a) Filament
- (b) Hook
- (c) Basal body

The filament is the longest and the most obvious portion of the flagellum. It extends from the cell surface. It is made up of protein called flagellin. The filament is about 20 m in diameter and 1–70 nm in length. The hook is different from the filament and is made up of different protein sub-units.

The basal body is the most complex part of the flagellum. In Gram-negative bacteria, it is composed of four rings connected to the central rod. The Gram-positive cells have two basal body rings. The inner ring is connected to the plasma membrane while the outer ring is connected to a peptidoglycan layer. The flagella remain rotated and push the bacterium in various directions.

(iv) **Pilus and fimbriae:** Pili and fimbriae are elongated, tubular structures made up of special protein called pilin, that protrude from the bacterial cell surface. They are basically involved in the mating process between F-positive and F-negative bacterial forms, especially Gram-negative bacilli. It is through them that genetic material is exchanged between bacteria.

(v) **The bacterial cytoplasmic membrane:** The bacterial cytoplasmic membrane is made up of a phospholipid bilayer. It functions to act as a permeability barrier for most molecules and serving as the location for the

transport of molecules into the cell and also in energy conservation as the location about which a proton motive force is generated.

- (vi) **The bacterial chromosome and plasmids:** The bacterial chromosome is located in the bacterial cytoplasm and the transfer of cellular information through the processes of translation, transcription and DNA replication occurs within the same compartment and can interact with other cytoplasmic structures, most notably ribosomes. The bacterial chromosome exists as a highly compact supercoiled structure. Along with chromosomal DNA, most bacteria also contain small independent pieces of DNA called plasmids. These encode for traits that are advantageous but not essential to their bacterial host. Plasmids can be easily gained or lost by a bacterium.
- (vii) **Bacterial inclusion bodies and storage structures:** Bacteria store excess carbon in the form of polyhydroxyalkanoates or glycogen and soluble nutrients such as nitrate in vacuoles. Sulphur gets stored as elemental (S^0) granules that are deposited either intracellularly or extracellularly in bacteria that use hydrogen sulphide as an electron source.
- Gas vesicles are spindle-shaped structures that are found in some planktonic bacteria. This gives buoyancy to cells by decreasing their overall cell density, required to keep the cells in the upper to perform photosynthesis.
 - **Carboxysome:** These are intracellular structures found in many autotrophic bacteria like Cyanobacteria, Knallgasbacteria. They are proteinic structures that resemble phage heads in morphology. They harbour enzymes for carbon dioxide fixation.
 - **Endospore:** These are bacterial survival structures which are highly resistant to chemical and environmental stresses. Endospore formation permits the survival of some bacteria for hundreds of millions of years (e.g. in salt crystals) and is limited to several genera of Gram-positive bacteria such as *Bacillus* and *Clostridium*.
- (viii) **L-forms:** L-forms or spheroplasts are synthesized from complete bacteria by the action of penicillin on them. They retain a residual cell wall. They are osmotically less sensitive than the protoplasts and can grow on agar-based culture media. L-forms are unstable spheroplasts discovered by Kleiueberger-Nobel upon studying *Streptobacillus moniliformis*. They form colonies with a ‘fried egg’ appearance and are non-pathogenic in laboratory animals in animal experiments, as has been shown in Figure 6.

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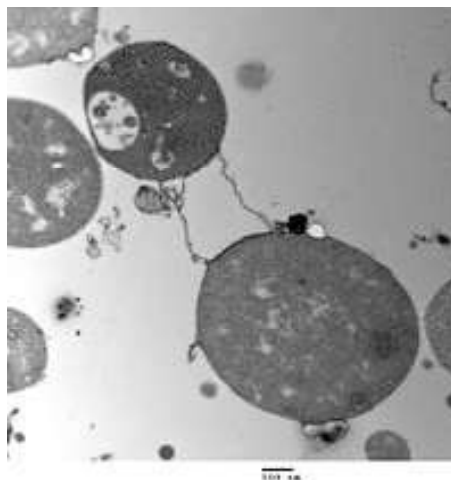


Fig. 6: L-form Bacterium

Source: [Google mages en.wikipedia.org](https://www.google.com/search?q=Google+mages+en.wikipedia.org)

Staining Techniques

In the previous section you got to know about the morphology, i.e. the basic form and structure of a bacterium. As these organisms are microscopic, i.e. not visible by the naked eye, so something requires to be done so that they become visible under the microscope. This is where the process of staining comes into picture.

Staining techniques are used to observe components that are otherwise not easily visible under an ordinary light microscope. This could be due to the lack of colour contrast between the object being examined and the background or because of the limited resolving power of the light microscope.

The following are a list of staining techniques commonly used:

- (i) **Simple Staining:** Simple staining methods are used to demonstrate the presence and the morphology of bacteria and cells. They employ simple stains.

Methylene blue stains: Neutral methylene blue, Loeffler's alkaline methylene blue and polychrome methylene blue solutions are useful for the identification of anthrax bacilli by Mcfadyean's reaction.

- (ii) **Differential Staining:** It generally refers to a number of specific processes. It is used to describe staining processes which use more than one chemical stain. Using multiple stains can better differentiate between different microorganisms or structures/cellular components of a single organism.

It also describes medical processes used to detect abnormalities in the proportion of different white blood cells in the blood. The process or results are called a WBC differential. This test is useful because many diseases alter the proportion of certain WBCs. By analysing

these differences in combination with a clinical exam and other lab tests, medical professionals can diagnose a disease.

One commonly recognizable use of differential staining is the Gram stain. Gram staining uses two dyes: Crystal violet and Carbol Fuchsin (which is the counterstain) to differentiate between Gram-positive (large peptidoglycan layer on outer surface of cell) and Gram-negative bacteria.

- (iii) **Negative Staining:** It is an established method, often used in diagnostic microscopy, for contrasting a thin specimen with an optically opaque fluid. In this technique, the background is stained, leaving the actual specimen untouched, and thus visible. This contrasts with 'positive staining', in which the actual specimen is stained.

For bright field microscopy, negative staining is typically performed using a black ink fluid such as nigrosin. The specimen, such as a wet bacterial culture spread on a glass slide, is mixed with the negative stain and allowed to dry. When viewed under the microscope the bacterial cells, and perhaps their spores, appear light against the dark surrounding background.

- (iv) **Special Staining Techniques:** Some of the specialized techniques used for staining have been discussed as follows:
- (a) **The spore staining** technique helps observe the spores of spore-forming bacteria. The structures are protected by a spore coat which helps them to show resistance against various chemical and physical agents. This technique uses rather vigorous heat treatment to force a primary stain into the spores. This is the reason that the presence of spores can be observed and differentiated from vegetative cells very easily.
 - (b) **Flagella stain:** The purpose of the protocol is to stain bacterial flagella, thus to reveal the presence or absence of flagella as well as their arrangement on the perimeter of the cell. These traits can be used to characterize bacteria phenotypically, as not all bacteria are flagellated and those that are will possess these structures in various locations extending from the cell membrane. Solid or liquid media cultures can be used for flagella staining. Cultures should be incubated between 16 and 20 hours before staining, as older cultures tend to lose flagella. For example, newer cultures are particularly important for *Bacillus* spp. that undergo spore formation and lose flagella during this developmental process.
 - (c) **Capsule stain:** Capsules are the structures positioned outside the cell wall of an organism and that is why these are in direct contact with the environment. Many bacteria produce capsules

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under the right conditions. Bacterial capsules are most often composed of long polymers of sugar or sugar derivatives, which are known as polysaccharides. Some of the capsules are composed of polyalcohols or amino acid polymers.

- (d) **Spirochaetes:** Spirochaetes fall into the category of a phylum of distinctive double-membrane bacteria, most of which have long spiral-shaped cells. These spirochaetes are differentiated from other bacterial phyla by their position of their flagella. These flagella are sometimes called axial filaments. They run lengthwise between the bacterial inner membrane and outer membrane in periplasmic space. These cause a twisting motion which allows the spirochaete to move about. A spirochaete will undergo asexual transverse binary fission during reproduction.

APPENDIX - II

Growth and Multiplication of Bacteria

Classification of Bacteria on the Basis of Nutrition: Phototrophs, Autotrophs and Heterotrophs

All types of bacteria need sources of energy and conducive environments, in nature or in the laboratory, for their growth. A bacterium cannot grow in the absence of a source of carbon and other necessary nutrients, apart from a permissive range of physical conditions like oxygen concentration, temperature and pH.

Bacteria are often referred to as individuals or groups based on their growth pattern under different chemical, nutritional and physical conditions. You can understand this with the help of the following example: to grow, organisms like phototrophs use light as an energy source. Similarly, anaerobes, another type of organism, cannot grow in the presence of oxygen. The third types are thermophiles which require high temperatures for their growth.

The above mentioned examples show that all living organisms need a source of energy for proper growth. Organisms which require radiant energy (light) to grow are known as **phototrophs**. Organisms that need an organic form of carbon for their growth are put into the category of **heterotrophs** or (chemo)heterotrophs. The organisms that oxidize inorganic compounds are categorized as **lithotrophs**.

The requirement of carbon is fulfilled by organic carbon which is a chemical compound with a carbon–hydrogen bond, or by carbon dioxide. The organisms that fall into the category of **heterotrophs** use organic carbon. Similarly, the organisms that require carbon dioxide as the only source of carbon for growth are termed **autotrophs**.

The various means of obtaining energy by various bacteria are depicted in Figure 1.

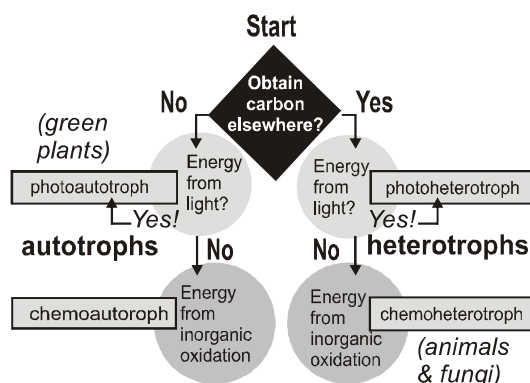


Fig. 1: Different Methods through Which Bacteria Obtain Energy

Source: geozoo.org-

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Bacterial Growth

An infection spreads only when the causal organism multiplies itself and increases in number, i.e. when growth and development occurs. So from the point of view of causing a disease it is very important for you to know the pattern of growth of the pathogen, in this case, the bacteria.

Growth may be defined as the orderly increase of all the chemical constituents of the cell. Bacterial growth involves both an increase in the size of cells and an increase in the number of cells. Whatever the balance between these two processes, the net effect is an increase in the total mass (biomass).

Bacterial Cell Division

Bacteria divide to yield two progenies of approximately equal size. Nuclear division precedes cell division and, therefore, in a growing population, many cells carrying two nuclear bodies can be seen. The cell division occurs by a constrictive or pinching process, or by the in-growth of a transverse septum across the cell. The daughter cells may remain partially attached after division in some species. Figure 2 shows the division of a bacterial cell.

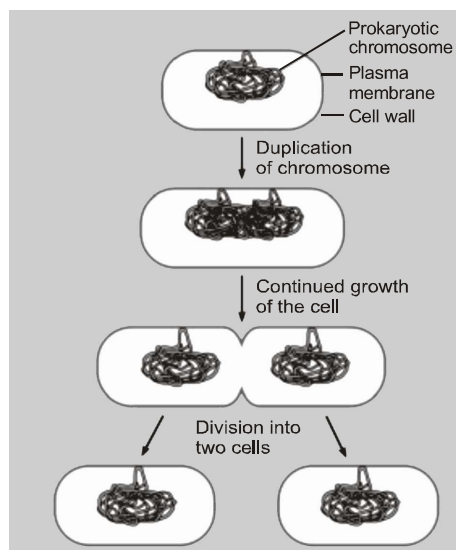


Fig. 2: Multiplications of Bacteria

Source: biology.about.com

Generation Time

Time required for a single bacterium to give rise to two daughter cells under optimum conditions is referred to as the generation time or doubling time. Generation time for different bacteria is given below:

- In *Escherichia coli*, it is about 20 minutes
- In *Mycobacterium tuberculosis*, it is about 20 hours
- In *M. leprae*, it is about 20 days

Bacterial Count

How do you think bacteria reproduce? Through geometric progression. A single bacterial cell can give rise to 10^{21} progenies in 24 hours. In actual practice, though, the exponential growth cannot be sustained indefinitely in a batch culture with limited available nutrients. When bacteria grow on solid media they form colonies which represent a clone of cells that are derived from a single parent cell.

Each bacterial colony is made up of a clone of cells derived from a single parent cell. In culture media or in a clinical specimen, bacterial counting is done as follows:

- (i) **Viable count:** Here, only viable (living) cells which are capable of growing and producing a colony on media are counted. Viable count is determined by the following methods:
 - (a) **Plating method:** Appropriate dilutions are inoculated on solid media by the pour plate method or spread plate method where serial dilutions are placed on the surface of plates and colony counts are made.
 - (b) **Dilution method:** A suspension is diluted till quantities do not yield growth when inoculated into the liquid media. Varying dilutions with several tubes are inoculated. The viable counts are calculated statistically from the number of tubes showing growth. The method is used in the presumptive coliform count in drinking water.
- (ii) **Total bacterial count:** Here, the total number of bacteria, irrespective of whether they are living or dead, are counted under the microscope using the counting chamber and by comparing the growth with standard opacity tubes.

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Bacterial Growth Curve

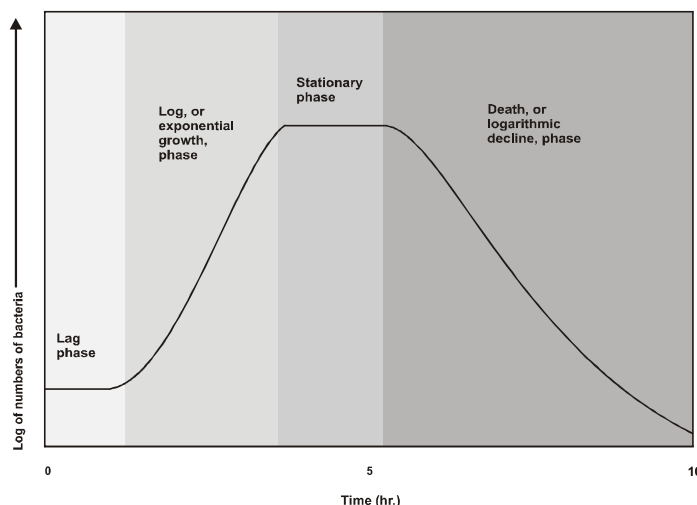


Fig. 3: The Four Phases of Bacterial Growth Beginning From the Lag to the Decline Phase of Growth

Source: amyuasheisme.wordpress.com

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You can carry out the determination of a bacterial growth curve in a liquid medium that is inoculated with bacterium and incubated under appropriate conditions as shown in Figure 3. You can take small samples at regular intervals after inoculation, take their counts and plot them in relation to time. The changes of slope on such a graph indicate the transition from one phase of development to another.

You can divide the bacterial growth curve into four major phases:

- (i) Lag phase
 - (ii) Exponential or log phase
 - (iii) Stationary phase
 - (iv) Decline phase
- (i) **Lag phase:** Microorganisms in fresh culture medium, after inoculation, start dividing. There is an increase in cell size at a time when little or no cell division is occurring. This period is the time required for adaptation to the new environment. Here, the necessary enzymes and metabolic intermediates are built. The lag phase varies considerably in length with the species, nature of the medium, size of inoculum and environmental factors, such as temperature and nutrients present in the new medium.
- (ii) **Log (logarithmic) or exponential phase:** Following the lag phase, the cells start dividing, their numbers increase exponentially, in geometric progression, and when viable count is plotted against time, a straight line is found. The exponential phase cultures are usually used in biochemical and physiological studies. The log phase is the time when cells are most active metabolically and is preferred for industrial purposes; microorganisms are particularly sensitive to adverse conditions.
- (iii) **Stationary phase:** The cell division stops due to depletion of nutrients and accumulation of toxic products. Growth slows down, and the bacterial cell number reaches a maximum. The growth curve becomes horizontal and the viable count is stationary.
- (iv) **Decline or death phase:** Finally the rate of death exceeds the rate of reproduction. The number of viable cells declines. Death is exponential and cells die due to autolysis besides nutrient deprivation and build-up of toxic wastes. All the cells die and culture becomes sterile.

Measurement of Bacterial Growth

You can carry out the measurement of bacterial growth in the following ways:

- By measuring direct cell counts
- By calculating viable cell counts
- By measuring cell products
- By measuring biomass

Cell counting is done by Coulter counters and flow cytometers to take count of total cells in solutions or flow cytometers to count microorganisms that are linked to dyes. Microscopic count is done by the direct microscopic count method where a measured amount of bacterial suspension is placed on a microscope slide called a *Petroff-Hausser cell counter*. Viable cell counts are done by the *viable count* of number of living cells capable of multiplication. Measuring of biomass is done through turbidity, dry weights or rapid calorimetric assays for measurement of the amount of cell protein, acids and gases produced by microbial fermentation, and consumption or utilization of ATP.

Growth factors are the following:

- (i) **Purines and Pyrimidines:** These are required for synthesis of nucleic acids (DNA and RNA).
- (ii) **Amino acids:** These are required for the synthesis of proteins.
- (iii) **Vitamins:** These are needed as coenzymes and functional groups of certain enzymes. The function(s) of these vitamins in essential enzymatic reactions gives a clue as to why, the cell must be provided exogenously if it cannot make the vitamin, for growth to occur.

Bacterial Nutrition and Metabolism

It is but natural that for any kind of growth to occur, certain nutrients are required by your body to complete the metabolic processes. So is the case with bacteria.

The elements essential for nutrition of a bacterium include C, H, O, N, S, P, K, Mg, Fe, Ca, Mn, and traces of Zn, Co, Cu, and Mo.

These elements are found in water, inorganic ions, small molecules and macromolecules, which serve either a structural or a functional role in the cells.

Environmental Factors Affecting Bacterial Growth

Just as you, as a human being, require air and nutritious food to grow well, bacteria also require certain conditions for their growth.

- (i) **Oxygen:** Based on their O₂ requirements, aerobic bacteria require oxygen for growth. Obligate aerobes have an absolute or obligate requirement for oxygen. The vibrio cholerae or facultative anaerobes are aerobic. They can also grow in the absence of oxygen, e.g. *Staphylococcus* spp., *Escherichia coli*, etc. Most bacteria of medical importance are facultative anaerobes or microaerophilic organisms that grow most efficiently at 5%, for example, *Campylobacter* spp. Anaerobic bacteria grow in the absence of oxygen and are also called obligate anaerobes; they die on exposure to oxygen, e.g. *Clostridium tetani*.

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- (ii) **Carbon dioxide:** *Brucella abortus* requires much higher levels of carbon dioxide (5–10%) for growth (capnophilic). *Pneumococci* and *Gonococci* are other capnophilic bacteria which grow better in air supplemented with 5–10% CO₂.
- (iii) **Moisture and drying:** Water is an essential ingredient of bacterial protoplasm. Therefore, drying is lethal to cells.
- (iv) **pH:** Bacteria multiply within pH 5 (acidic) to pH 8 (basic) and have a neutral pH 7. Pathogenic bacteria grow most efficiently at a neutral pH; acidophilic bacteria like *Lactobacilli* grow under acidic conditions; *Vibrio cholerae* grows above pH 8.
- (v) **Light:** Bacteria are sensitive to ultraviolet light. Mercury lamp is bactericidal. Bacteria get killed by ionizing radiations. On the other hand, photochromogenic mycobacteria form pigments only on exposure to light.
- (vi) **Osmotic effect:** Besides mycoplasma and cell wall-defective organisms, the majority of the bacteria are osmotically tolerant. However, if placed in a hypertonic environment, water leaves, membrane shrinks away from the wall, and a process called plasmolysis is observed in Gram-negative bacteria.
- (vii) **Mechanical and sonic stresses:** Bacteria may be ruptured by mechanical stress or vigorous agitation employing glass beads or by exposure to ultrasonic vibration.

Bacterial Metabolism

The prokaryotes, as a group, conduct all the same types of basic metabolism and its diversity is expressed by their great variation in modes of energy generation and metabolism. This allows prokaryotes to flourish in all habitats suitable for life. *Escherichia coli* can produce energy for growth by fermentation or respiration, aerobically using O₂ as a final electron acceptor or respire anaerobically using NO₃ as a terminal electron acceptor.

E. coli can use glucose or lactose as a sole carbon source for growth while *Rhodospirillum rubrum* has the heterotrophic capabilities as *E. coli* and the ability to grow by photoautotrophic, photoheterotrophic or lithotrophic means.

Metabolism is defined as the series of changes in carbohydrate, protein or fat within a bacterial cell. It may be **aerobic** or **anaerobic**. It results in chemical reactions that release and require energy. The energy production is carried out by aerobic respiration, anaerobic respiration and fermentation.

APPENDIX - III

LAB I – General
Microbiology, Microbial
Physiology and
Biochemistry

Sterilization and Disinfection

Let us begin by defining the two key words used in this unit, i.e. ‘sterilization’ and ‘disinfection’, properly so that you can derive the actual meaning of these terms from their definitions. This will help you understand the concepts better.

The processes of sterilization and disinfection are defined in various ways. Some of the important definitions of these processes are as follows:

Sterilization means complete destruction or elimination of all viable organisms in or on a substance being sterilized. Sterilization procedures involve the use of heat, radiation or chemicals, or physical removal of cells.

Sterilization is defined as the process by which an article, surface or medium is freed of all living microorganisms either in the vegetative or spore state. When sterilization is done by a chemical agent, the chemical is called **sterilant**.

Sterilization is absolute and it means that all of the microorganisms have either been removed or killed.

Disinfection: It is the killing, inhibition or removal of microorganisms that may cause disease. Disinfection simply means that you reduce the microbial load on an object.

Since this is usually done to render the object less likely to be involved in the transmission of infection, a good disinfection procedure is aimed at specifically reducing the numbers of potentially pathogenic organisms in the context of the use of the object being disinfected.

Classification of Sterilization Methods

The sterilization process is performed by physical and chemical methods. As classification of these methods is a very broad topic so it will be taken up in the next section.

Physical Methods of Sterilization

You must have some kind of general knowledge about the physical methods sterilizing equipment in a laboratory. The most reliable and authentic physical methods of sterilization are as follows:

- (i) **Sunlight:** It has bactericidal activity. It spontaneously sterilizes under natural conditions primarily due to its content of ultraviolet rays.
- (ii) **Heat:** It is the most reliable and universally applicable method of sterilization. Dry or moist heat may be applied and the materials that may be damaged by heat can be sterilized at lower temperature, for longer periods or by repeated cycles.

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(iii) **Filtration:** Sterilizing a solution by filtering is one of the most popular methods of sterilization. The solution can be sterilized through this method without heating it. A filter works by passing the solution through a filter with a pore diameter which is very small and so microbes cannot pass through it.

Figure 1 shows the various components which make up a Millipore filtration unit. The filter is made from 0.3 micron pleated glass fibre with a carbon core.



Fig. 1: Millipore Filtration Unit

Source: textbookofbacteriology.net

The various types of filters include:

- Earthware filters
- Asbestos filters
- Sintered glass filters
- Membrane filters
- Syringe filters
- Vacuum and 'in-line' filters
- Pressure filtration
- Air filters

(iv) **Radiation:** Another physical method using which you can sterilize is radiation. This is performed by using electron beams, X-rays, gamma rays, or subatomic particles.

The process of non-ionizing radiation sterilization uses ultraviolet light. The process of irradiation is useful only for sterilization of surfaces and some transparent objects. Ultraviolet irradiation is regularly done to sterilize the interiors of biological safety boxes. The other useful process of sterilization is gamma radiation which involves beam radiation. These are different types of ionizing radiation mostly used in the healthcare industry. The gamma rays are very useful in getting rid of microorganisms.

Very similar to gamma radiation is e-beam irradiation. These e-beams are concentration of various chemical and molecular bonds and are highly charged streams of electrons. Acceleration and conversion of electricity generate these e-beams. These e-beams and gamma radiation sterilize a wide range of devices, including syringes and cardiothoracic devices.

- (v) **Moist Heat:** You need temperature below 100°C to kill vegetative bacteria but not spores, like pasteurization of milk employed for disinfection at 63°C for 30 minutes or 72°C for 15–20 seconds followed by rapid cooling to 13°C. All non-sporing pathogens such as mycobacteria, brucellae and salmonellae are destroyed by these processes. Vaccines are inactivated in a water bath at 60°C for 1 hour; serum containing congealable proteins can be sterilized by heating for 1 hour at 56°C. Lowenstein–Jensen and Loeffler’s serum are remade sterile by heating at 80–85°C for half an hour on three successive days.

Steam under pressure provides greater lethal action of moist heat; is quicker in heating up articles to be sterilized and can penetrate easily porous material such as cotton wool stopper.

The various types of steam sterilizers include displacement laboratory autoclave, bench top autoclave, multipurpose laboratory autoclave, high security autoclave, porous load sterilizer and low-temperature steam. An autoclave for use in a laboratory or hospital setting is a large pressure cooker; it operates by using steam under pressure as the sterilizing agent. Figure 2 gives a diagrammatic explanation of the working of an autoclave. High pressures enable steam to reach high temperatures, thus increasing its heat content and killing power. Heating power of steam comes from its latent heat of vapourization. This amount of heat content is too large. It takes 80 calories to make 1 liter of boiled water, but 540 calories to convert that boiling water to steam. Thus steam at 100°C has almost seven times more heat than boiling water.

Uses: This is used for sterilizing culture media, laboratory supplies, aqueous solutions, rubber material, dressing materials, gowns, dressing, linen, gloves, instruments and pharmaceutical products.

Sterilization controls include a biological control made of an envelope containing a filter paper strip impregnated with 10⁶ spores of *Bacillus*

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stearotherophilus. After sterilization the strip is inoculated into broth and incubated at 56°C for 5 days. No growth of *B. stearotherophilus* indicates proper sterilization; a Browne’s tube containing red solution changes to green when exposed to temperature of 121°C for 15 minutes in autoclave. It indicates proper sterilization.

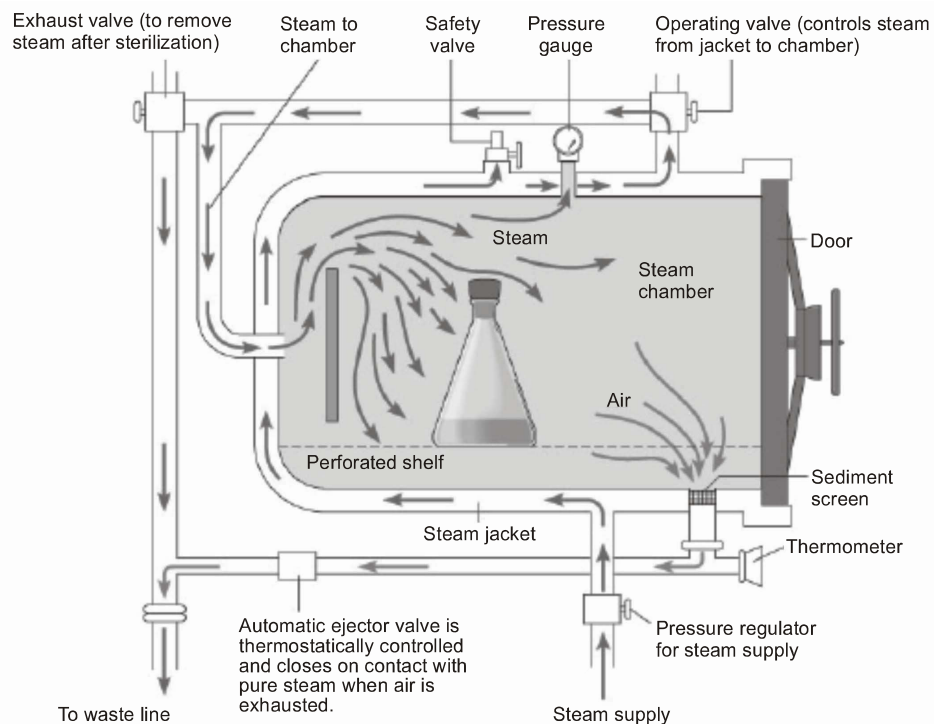


Fig. 2: Working of an Autoclave

Source: uah.wikispaces.com

Table 1 shows the permissible limit of the use of heat to control bacterial growth.

Table 1: Use of Heat to Control Bacterial Growth (permissible limit)

Treatment	Temperature	Effectiveness
Incineration	>500°	Vapourizes organic material on non-flammable surfaces; however, it may spoil valuable elements in the process
Boiling	100°	Half an hour of boiling destroys microbial pathogens and vegetative forms of bacteria; however, it may fail to kill bacterial endospores
Periodic boiling	100°	Three half an hour of intervals during the process of boiling, followed by periods of cooling destroys bacterial endospores

Autoclave and pressure cooker (steam under pressure)	121°/15 minutes at 15# pressure	Kills all forms of life including bacterial endospores. The substance being sterilized must be maintained at the effective temperature for the full time
Dry heat (hot air oven)	160°/2 hours	For materials that must remain dry and which are not destroyed at temperature between 121° and 170°. Good for glassware, metal, but not for plastic or rubber items
Dry heat (hot air oven)	170°/1 hour	Same as above. Note increasing temperature by 10 degrees shortens the sterilizing time by 50%
Pasteurization (batch method)	63°/30 minutes	Kills most vegetative bacterial cells including pathogens such as streptococci, staphylococci and <i>Mycobacterium tuberculosis</i>
Pasteurization (flash method)	72°/15 seconds	Effect on bacterial cells similar to batch method; for milk, this method is more conducive to industry and has fewer undesirable effects on quality or taste
Ultra pasteurization (direct method)	140°/2 seconds	Effect on most bacterial cells is lethal. For milk, this method creates a product with relatively long shelf life at refrigeration temperatures.

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(vi) **Dry Heat Sterilization:** The lethal effect of dry heat is due to protein denaturation, oxidative damage and toxic effects of elevated levels of electrolytes. Moist heat actually kills microorganisms by coagulation and denaturation of their enzymes and various structural proteins. This is the most important and widely used method. The type of heat, and most importantly, the time of application and temperature are important to ensure destruction of all microorganisms.

Dry heat sterilization is used for needles, inoculating wires, glassware, etc. and objects not destroyed in the incineration process. The rules of relating time and temperature apply, but dry heat is not as effective as moist heat (i.e., higher temperatures are needed for longer periods of time). 160°/2 hours or 170°/1 hour is necessary for sterilization. Hot air sterilizer is the most widely used method of sterilization by dry heat, and it is used for processing materials which can withstand high temperatures for length of time needed for sterilization.

Hot air oven is electrically heated, with heating and it should be fitted with a fan to provide forced air circulation throughout the oven chamber, a temperature indicator, a control thermostat and timer, open mesh shelving and adequate wall insulation. The sterilization hold time is set to 160°C for 2 hour. Oils, glycerol and dusting powder for 1 hour are kept at 150°C. For oils, glycerol, cooling may take up to several hours. Glassware is liable to crack if cold air is admitted suddenly while it is still very hot.

The sterilization is performed by using the physical and chemical methods. Table 2 shows the physical agents which are used in the sterilization.

Table 2: Physical Agents Used in Sterilization

Method	Mechanism of action	Use
i. Heat		
(a) Dry heat		
(b) Flaming	Burning contaminants to ashes	Very effective method of sterilization. Scalpel blades, glass slides, mouth of culture tubes and bottles
(c) Incineration	Burning to ashes	Very effective method of sterilization for paper cups, contaminated dressings, animal carcasses, bags and wipes
(d) Hot air sterilization	Oxidation	Needles, and glass syringes, sealed materials like oils, grease, dry powder, etc.
ii. Boiling		
	Protein denaturation	Kills vegetative bacterial and fungal pathogens and almost all viruses within 10 min Less effective on endospores
iii. Pasteurization		
	Protein denaturation	Heat treatment for milk (72°C for about 15 seconds) that kills all pathogens and most non-pathogens. Milk, cream, and certain alcoholic beverages (beer and wine)
iv. Autoclaving		
	Protein denaturation	Very effective method of sterilization; at 15 psi of pressure (121°C). All vegetative cells and their endospores are killed in about 15 min. Microbiological media, solutions, linens, utensils, dressings, equipment, and other items that can withstand temperature and pressure
v. Filtration		
	Separation of bacteria from suspending liquid	Useful for sterilizing liquids (enzymes, vaccines) that are destroyed by heat
vi. Radiation		
(a) Ionizing	Destruction of DNA	Used for sterilizing pharmaceuticals and medical and dental supplies
(b) Non-ionizing	Damage to DNA	Control of closed environment with UV (germicidal) lamp

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Chemical Methods of Sterilization

Apart from the physical methods, in some cases, you need to also use chemicals for sterilizing and disinfecting. In this section you will get to know about the chemical methods.

Chemicals used for sterilization include gases ethylene oxide and formaldehyde, and liquids such as glutaraldehyde, ozone and hydrogen peroxide.

Several groups of chemical substances such as alcohols, aldehydes, phenols, halogens and so on are used for chemical sterilization.

(i) Types of Antimicrobial Agents

The following are some of the microbial agents which are used frequently.

- (a) **Disinfectants:** These are the agents that kill microorganisms, but not necessarily their spores, and so are not safe for application to living tissues. They are used on inanimate objects such as tables, floors, utensils, etc. Examples include, hypochlorite, chlorine compounds, lye, copper sulphate, quaternary ammonium compounds, formaldehyde and phenolic compounds.
- (b) **Antiseptics:** These are applied to the skin and mucous membrane as they are absolutely harmless microbicidal agents. However, their internal use is prohibited. These antiseptics and disinfectants are frequently used in hospitals and other treatment centres for a number of topical and hard-surface applications. The antiseptics are used as deterrents for the growth of microorganisms. Table 3 shows the list of antiseptics and disinfectants used on regular basis.

Table 3: Common Antiseptics and Disinfectants and Their Uses

Chemical	Action	Uses
Ethanol (50–70%)	Denatures proteins and solubilizes lipids	Antiseptic used on skin
Isopropanol (50–70%)	Denatures proteins and solubilizes lipids	Antiseptic used on skin
Formaldehyde (8%)	Reacts with NH ₂ , SH and COOH groups	Disinfectant and kills endospores
Tincture of iodine (2% I ₂ in 70% alcohol)	Inactivates proteins	Antiseptic used on skin; disinfection of drinking water
Chlorine (Cl ₂) gas	Forms hypochlorous acid (HClO), a strong oxidizing agent	Disinfect drinking water; general disinfectant
Silver nitrate (AgNO ₃)	Precipitates proteins	General antiseptic and used in the eyes of newborns
Mercuric chloride	Inactivates proteins by reacting with sulphide groups	Disinfectant, although occasionally used as an antiseptic on skin
Detergents (e.g. quaternary ammonium compounds)	Disrupts cell membranes	Skin antiseptics and disinfectants
Phenolic compounds (e.g. carbolic acid, lysol, hexylresorcinol, hexachlorophene)	Denature proteins and disrupt cell membranes	Antiseptics at low concentrations; disinfectants at high concentrations
Ethylene oxide gas	Alkylating agent	Disinfectant used to sterilize heat-sensitive objects such as rubber and plastics
Ozone	Generates lethal oxygen radicals	Purification of water, sewage

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Table 4 shows the various chemical agents used in sterilization and disinfection processes.

Table 4: Chemical Agents Used in Sterilization and Disinfection Processes

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Chemical agent	Mechanism of action	Use	Comments
Surface-active agents			
Soaps and acid anionic detergents	Mechanical removal of microbes through scrubbing	Skin de-germing and removal of debris	Much antibacterial soap contains antimicrobials.
Acid-anionic detergents	Not certain; may involve enzyme inactivation or disruption	Sanitizers in dairy and food-processing industries	Wide spectrum of activity; nontoxic, no corrosive, fast-acting
Cationic detergents (quaternary ammonium compounds)	Enzyme inhibition, protein denaturation, and disruption of plasma membranes	Antiseptic for skin, instruments, utensils, rubber goods	Bactericidal, bacteriostatic, fungicidal, and virucidal against enveloped viruses
Phenol and phenolics			
Phenol	Disruption of plasma membrane, denaturation of enzymes	Rarely used, except as a standard of comparison	Seldom used as a disinfectant 'or antiseptic because of its irritating qualities and disagreeable odour
Phenolics	Disruption of plasma membrane, denaturation of enzymes	Environmental surfaces, instruments, skin surfaces, and mucous membranes	Derivatives of phenol that are reactive even in the presence of organic material; O-phenylphenol is an example
Bisphenols	Probably disruption of plasma membrane	Disinfectant hand soaps and skin lotions	Triclosan is an especially common example of a bisphenol Broad spectrum, but most effective against gram-positives
Alcohols	Protein denaturation and lipid dissolution	Thermometers and other instruments; in swabbing the skin with alcohol before an injection, most of the disinfecting action probably comes from a simple wiping away (de-germing) of dirt and some microbes	Bactericidal and fungicidal, but not effective against endospores or non-enveloped viruses; commonly used alcohols are ethanol and isopropanol

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Organic acids	Metabolic inhibition, mostly affecting molds; action not related to their acidity	Sorbic acid and benzoic acid effective at low pH; parabens much used in cosmetics, shampoos; calcium propionate used in bread	Widely used to control molds and some bacteria in foods and cosmetics
Heavy metals and their compounds	Denaturation of enzymes and other essential proteins	Silver nitrate may be used to prevent gonorrheal ophthalmia neonatorum; mercurochrome disinfects skin and mucous membranes; copper sulphate is an algicide	Heavy metals such as silver and mercury are biocidal
Halogens	Iodine inhibits protein function and is a strong oxidizing agent; chlorine forms the strong oxidizing agent hypochlorous acid, which alters cellular components	Iodine is an effective antiseptic available as a tincture and an iodophor; chlorine gas is used to disinfect water; chlorine compounds are used to disinfect dairy equipment, eating utensils, household items, and glassware	Iodine and chlorine may act alone or as components of inorganic and organic compounds
Peroxygens (oxidizing agents)	Oxidation	Contaminated surfaces; some deep wounds, in which they are very effective against oxygen sensitive anaerobes	Ozone is widely used as a supplement for chlorination; hydrogen peroxide is a poor antiseptic but a good disinfectant. Peracetic acid is especially effective
Aldehydes	Protein denaturation	Glutaraldehyde (Cidex) is less irritating than formaldehyde and is used for disinfection of medical equipment	Very effective antimicrobials
Gaseous sterilants	Protein denaturation	Excellent sterilizing agent, especially for objects that would be damaged by heat	Ethylene oxide is the most commonly used

Laboratory Evaluation of Disinfectants

Before a disinfectant can be used in a laboratory, it is very essential that you test its potency and effectiveness. In order to achieve this you should perform certain tests under laboratory conditions. Some of these tests have been detailed out as follows:

Rideal Walker Test and Chick-Martin Test

In this test, the potency of a new disinfectant is compared with that of phenol. Serial dilutions of phenol and the new disinfectants are inoculated with the test bacterium, be it gram-positive or gram-negative. The suspensions containing equal

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numbers of bacteria are subjected to the action of varying concentrations of phenol and of the new disinfectant. The dilution of new disinfectant which sterilizes the suspension in a given time, divided by the corresponding dilution of phenol, is defined as phenol coefficient.

- A. **Objective:** This test is done to determine the effectiveness of a new disinfectant relative to phenol in killing bacteria.
- B. **Test method:** The standard test procedure is described in the official methods of analysis of the Association of Official Analytical Chemistry International (16th Ed., Vol. 1) and Locator 6.1.02, AOAC Official Method 955. It determines the phenol coefficient of the disinfectant against *Staphylococcus aureus* (ATCC No. 6538).
- C. **Interpretation of assay:** The phenol coefficient is the value obtained by dividing the highest dilution of the test solution by the highest dilution of phenol that sterilizes the given culture of bacteria under standard conditions of time and temperature.

Probable test results that are achieved: A phenol coefficient of 5.26 was obtained.

D. Observations:

Phenol coefficient against *S. aureus* (ATCC No. 6538) is given in the following table.

Disinfectant (dilution)	Bacterial activity		
	5 minutes	10 minutes	15 minutes
1 to 10	No growth	No growth	No growth
1 to 50	No growth	No growth	No growth
1 to 100	No growth	No growth	No growth
1 to 500	Growth	No growth	No growth
1 to 640	Growth	Growth	No growth

Phenol (dilution)	Bacterial activity		
	5 minutes	10 minutes	15 minutes
1 to 90	No Growth	No Growth	No Growth
1 to 95	Growth	No Growth	No Growth
1 to 100	Growth	Growth	No Growth

Chick–Martin test is a modification of Rideal Walker test where the disinfectant acts in the presence of organic matter (dried yeast or feces) to simulate natural situations.

Kelsey-Sykes Test (Capacity Test)

In this test, inoculums, organisms are put in consideration with or without organic matter. These give a measure of the capacity of the new disinfectant to act on bacterial cultures. It is similar to the natural conditions under which the disinfectants are used in the hospitals.

S. aureus and *Pseudomonas aeruginosa* are added to the disinfectant and after leaving them in 0, 10 and 20 minutes in clean and dirty conditions. Samples are transferred at 8, 18 and 28 minutes to a broth culture medium. The disinfectant is validated by its ability to kill bacteria as noted by its growth or no growth in recovery media.

In-Use Test

The ability of a disinfectant to inactivate a known number of a standard strain of a pathogenic *Staphylococcus* on a given surface within a certain time determines its efficiency and this is the underlying principle of this test. Generally, the results of such tests are more useful than those of phenol coefficient test and its modifications. For general purposes, use 0.5% 'Tween 80'; the bottle of dilute disinfectant is transported to the laboratory within an hour of the addition of the disinfectant.

The presence of bacterial colonies on one or both of a pair of plates provides an evidence of the survival of bacteria in the particular jar, from which the sample was taken. One or two colonies on the plate may be ignored. A disinfectant is not a sterilant. The presence of a few live bacteria is to be expected. The growth of five or more colonies should arouse suspicion and may be indicative of poor bactericidal activity.

NOTES

APPENDIX - IV

NOTES

Staining Methods

Staining methods are used to make bacterial cells and the internal structures of these cells conspicuous under the microscope. The purpose of staining of microorganisms is to improve visibility, emphasize certain morphological features and preserve the specimen for future use. A stain is a substance that adheres to the cell and gives the characteristic colour.

Single-celled prokaryotic microorganisms are referred to as bacteria. Their length comes around to a few micrometers along with an extensive range of shapes, encompassing spheres to rods and spirals. The empirical method involving differentiation of bacterial species in two large groups (gram-negative and gram-positive) on the basis of the physical and chemical properties of their cell walls is known as Gram staining or Gram's method. Gram-positive bacteria can be distinctly identified through their violet or dark blue stains in the staining protocol, while gram-negative bacteria are unable to withhold their crystal violet dye. Gram-positive bacteria, due to the high content of peptidoglycan in their cell wall, are able to keep their crystal violet stain. The gram-negative bacteria consist of an outer membrane which is not present in the gram-positive bacteria. After colouring all gram-negative bacteria with pink or red colour, a counterstain (or safranin) is added to perform a Gram stain test. This test is useful in order to categorize two distinct bacterial types on the basis of structural differences of their cell walls.

The different methods used are as follows:

- (i) **Gram Stain:** It divides the bacteria into gram-positive and gram-negative groups.
- (ii) **Ziehl–Neelsen Stain:** It differentiates between acid fast and nonacid fast bacteria used for the diagnosis of *Mycobacterium tuberculosis*.
- (iii) **Albert's Stain:** It identifies *Corynebacterium diphtheria*.

The colony characteristics identify the major group of bacteria, to be supported by staining and/or a hanging drop preparation for motility. However, it has been observed that these methods of bacteria identification are not explicit in their specification. The difference between Gram-positive and Gram-negative organisms can be determined by Gram staining. Similarly, the difference between coccus- and bacillus can also be done by Gram staining. One of the drawbacks of this method is that a specific identification is not possible.

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I - Semester



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