Practical-A

1. Microbiology Laboratory Practices and Biosafety.
2. To study the principle and applications of important instruments (biological safety cabinets, autoclave, incubator, BOD incubator, hot air oven, light microscope, pH meter).
3. Preparation and sterilization of culture media for bacterial cultivation.
4. Study of different shapes of bacteria, fungi, algae, protozoa using permanent slides/pictographs.
5. Staining of bacteria using Gram stain.
6. Isolation of pure cultures of bacteria by streaking method.
7. Estimation of CFU count.

REFERENCES

Atlas, R.M., (1997). Principles of Microbiology. 2nd edition. W M.T.Brown Publishers.

Pelczar, M.J, Chan, E.C.S., and Krieg, N.R., (1993). Microbiology. 5th edition. McGraw Hill Book Company .

Kannan, N., (2003). Laboratory Manual in Microbiology, Panima Publishing Corporation, Bangalore.

Experiments

S.No	Experiment Duration Hour	Topics to be Covered	Support Material/Page Nos
1	4	Microbiology Laboratory Practices and Biosafety.	W1, P3-P4
2	8	To study the principle and applications of important instruments (biological safety cabinets, autoclave, incubator, BOD incubator, hot air oven, light microscope, pH meter).	R2
3	8	Preparation and sterilization of culture media for bacterial cultivation.	R1, R2
4	8	Study of different shapes of bacteria, fungi, algae, protozoa using permanent slides/pictographs.	R3
5	8	Staining of bacteria using Gram stain	R1
6	8	Isolation of pure cultures of bacteria by streaking method.	R3
7	4	Estimation of CFU count.	R2
	Total No Of Hours 48		

REFERENCES

R1. Atlas, R.M., (1997). Principles of Microbiology. 2nd edition. W M.T.Brown Publishers.

R2. Pelczar, M.J, Chan, E.C.S., and Krieg, N.R., (1993). Microbiology. 5th edition. McGraw Hill Book Company .

R3. Kannan, N., (2003). Laboratory Manual in Microbiology, Pa https://www.researchgate.net/publication/306018042_Microbiology_Laboratory_Manualnima Publishing Corporation, Bangalore.

W1; https://www.researchgate.net/publication/306018042_Microbiology_Laboratory_Manual

LECTURE PLAN

DEPARTMENT OF MICROBIOLOGY

Microbiology Lab Practices and BioSafety

Wash your hands with disinfectants when you arrive at the lab and again before you leave.
Wear laboratory coats in the lab. Students with long hair must put up the hair.
At the start and end of each laboratory session, students should clean their assigned bench-top area with a disinfectant solution provided. That space should then be kept neat, clean, and uncluttered throughout each laboratory period.
Eating or drinking in the laboratory is not permitted. No mouth pipetting.
Label everything clearly. Sterilize equipment and materials.
Avoid loose fitting items of clothing. Wear appropriate shoes in the laboratory.
Report any breakage of equipment to the instructor.
Report any personal accidents such as cuts to the instructor at once.
Turn off Bunsen burner when not in use.
Discard all cultures and used glassware into the container labeled CONTAMINATED. (This container will later be sterilized.) Plastic or other disposable items should be discarded separately from glassware in containers to be sterilized.
Never place contaminated pipettes on the bench top.
When you flame sterilize with alcohol, be sure that you do not have any papers under you.
Before beginning your laboratory exercise, wash off the bench top with the disinfectant provided. When exercises are completed, wash off the bench top again. Always wash your hands with soap and water before leaving the laboratory.
Before leaving the laboratory, see that all the equipments are in the proper location and gas and water turned off.
Purchase a fine point, waterproof marker and small roll of masking tape. Use them to clearly label your cultures.
Disinfect work areas before and after use with 70% alcohol or fresh 10% bleach. Laboratory equipment and work surfaces should be decontaminated with an appropriate disinfectant on a routine basis and especially after spills, splashes or other contamination.
Replace caps on reagents, solution bottles and bacterial cultures. Do not open petri dishes in the lab unless absolutely necessary.
Cultures are not to be removed from the laboratory unless the instructor gives permission.
Always place culture tubes (broth and slants) in the upright position in a rack or basket for incubation or disposal.
Dispose off all solid waste materials in a biohazard bag and autoclave it before discarding in the regular trash.

Treat all cultures as potentially pathogenic, *i.e.*, flood areas with disinfectant if cultures are spilled, wash hands after contact and notify your instructor at once.

Read the instructions carefully before beginning an exercise. Also, make sure you have all the materials needed for the exercise at hand before you commence the experiment. Ask the instructor for clarification of any points about which you are in doubt.

Flame the inoculating loop or needle immediately before and after use. If viscous material is present on the loop or needle, dry it at the side of the flame before placing it directly in the flame.

Laboratory note books must be kept up-to-date. Illustrations should be done when requested.

Make sure you consult the instructor to dispose of the cultures that are not needed any longer. Remove all labels and markings from the tubes before disposing of them; do not discard anything into the sinks.

Please inform your instructor if you have any medical condition that could potentially affect your safety in the laboratory (eg: diabetes, epilepsy, immunosuppression etc.). This information will help the instructor to deal with any emergency that would arise. The information will be treated confidentially and it will not affect their ability to participate in the laboratory activities.

Be systematic and logical. Keep a faithful record of all the experiments and observations. Update it regularly and submit it for evaluation at the end of each exercise.

Work either using laminar air flow chamber or light the burner at least five minutes prior to making any inoculations and work near the burner.

TO STUDY THE PRINCIPLE AND APPLICATIONS OF IMPORTANT INSTRUMENTS (BIOLOGICAL SAFETY CABINETS, AUTOCLAVE, INCUBATOR, BOD INCUBATOR, HOT AIR OVEN, LIGHT MICROSCOPE, PH METER).

3. MEDIA PREPARATION AND CULTIVATION OF BACTERIA

INTRODUCTION:

To establish the role of a microbial agent to a disease process, it is essential to demonstrate the organism or its component in the diseased tissue. To accomplish this, the organism must be cultivated from the tissue. Though both in vitro and in vivo cultivation methods can be used to isolate the organisms, the easiest way is to grow them in vitro on an artificial culture medium.

ARTIFICIAL CULTURE MEDIA:

A medium is an environment which supplies the ingredients necessary for the growth of an organism. Various kinds of media have been prepared in the laboratory to isolate, grow and identify an organism. Depending on the need to isolate and identify an organism from a particular sample or environment, different kinds of media are formulated.

KINDS OF MEDIA:

1) Basal or supportive media:

Basal medium is one that contains nutrients that allow the growth of most non-fastidious organisms without affording growth advantage to any organism over others.

Example: nutrient agar, trypticase soy agar, brain heart infusion agar.

2) Enriched medium:

Enriched medium is one that contains extra nutrients in addition to that present in basal medium which enables it to support the growth of fastidious organisms.

Example: blood agar, chocolate agar.

3) Differential medium:

The differential medium is one, which enables one to differentiate two types of organisms by their characteristic growth.

Example: Blood agar : Haemolytic and non-heamolytic colonies can be differentiated.

Mac Conkey agar : Lactose fermenters and non-lactose fermenters can be differentiated.

Many different kinds of media can act as differential medium too.

4) Selective Medium:

Selective medium is one that contains ingredients that afford growth advantage to particular organism over others by allowing the growth of the particular organism and inhibiting the others.

Eample : Salmonella shigella agar : Allows the growth of Salmonella and shigella sp. And inhibits E.coli

AIM :

To distinguish the growth characteristics of microorganisms in various differential, selective and enriched media.

Principle:

Much of the study of microorganisms depends on its ability to grow in the laboratory, and this is possible only if suitable culture media are available for the growth of microorganism. A culture medium is defined as a solid or liquid preparation used for the growth, transport, and storage of microorganisms. The effective culture medium must contain all the nutrients required for the growth of the microorganism.

Specialized media are widely employed for the isolation and identification of microorganisms, testing the antibiotic sensitivities, analysis of water and food, industrial microbiology, and other activities. Although all microorganisms need sources of energy, nitrogen, carbon, phosphorus, sulfur, and various minerals, the exact composition of a satisfactory medium will rely on the species one is trying to identify and cultivate because nutritional requirements vary so greatly among the microorganisms.

Knowledge of microorganism's normal habitat is often useful in selecting a suitable culture medium because its nutrient requirements reflect its natural surroundings. A medium is used to select and growing specific microorganisms or to help identifying a particular species. In these cases, the function of the medium also depends on its composition. In addition to nutrients necessary for the growth of all bacteria, special-purpose media contain one or more chemical compounds that are essential for their functional specificity.

These include: **Selective, Differential and Enriched Media.**

Selective media:

Selective media allows the growth of certain type of organisms, while inhibiting the growth of other organisms. This selectivity is achieved in several ways. For example, organisms that have the ability to utilize a given sugar are screened easily by making that particular sugar the only carbon source in the medium for the growth of the microorganism. Like-wise, the selective inhibition of some types of microorganisms can be studied by adding certain dyes, antibiotics, salts or specific inhibitors that will affect the metabolism or enzymatic systems of the organisms. For example, media containing potassium tellurite, sodium azide or thallium acetate at different concentrations of 0.1 - 0.5 g/l will inhibit the growth of all Gram-negative bacteria. Media supplemented with the antibiotic penicillin concentration 5-50 units/ml or crystal violet 2 mg/l inhibit the growth of Gram-positive bacteria. Tellurite agar, is used to select for Gram-positive organisms, and nutrient agar supplemented with the antibiotic penicillin can be used to select for the growth of Gram negative organisms.

Eg., Mannitol salt agar, Hektoen enteric agar (HE), Phenylethyl alcohol agar

Differential media:

Differential media are widely used for differentiating closely related organisms or groups of organisms. Because of the presence of certain dyes or chemicals in the media, the organisms will produce certain characteristic changes or growth patterns that are used for identification or differentiation of microorganism.

Eg., Mac Conkey (MCK) agar, Eosin Methylene Blue (EMB) agar

Enriched media:

Enriched media are media that have been supplemented with highly nutritious materials such as blood, serum or yeast extract for the purpose of cultivating fastidious organisms.

Eg., Blood agar, Chocolate agar

Some of the special-purpose media are as follows:

1. Mannitol Salt Agar (MSA):

Mannitol salt agar is both a selective and differential media used for the isolation of pathogenic *Staphylococci* from mixed cultures.

Components:

- **7.5% NaCl** – selects for species of S*taphylococcus*. This concentration of salt is too high for most other bacteria to withstand and , therefore, inhibits their growth.

- **Mannitol** – alcohol of the carbohydrate mannose. Mannitol fermentation produces acid end products which turn the medium yellow. Yellow indicates mannitol positive and no color change indicates mannitol negative.

- **Phenol red pH indicator** – yellow in acid pH (The same indicator that is used in phenol red carbohydrate fermentation broths).

Figure1 : Mannitol Salt Agar

On MSA, only pathogenic *Staphylococcus aureus* produces small colonies surrounded by yellow zones. The reason for this color change is that *S. aureus* have the ability to ferment the mannitol, producing an acid, which, in turn, changes the indicator color from red to yellow. The growth of other types of bacteria is usually inhibited. This growth differentiates *S.aureus* from *S.epidermidis*, which forms colonies with red zones or both zones.

Formula:

Ingredients per liter of deionized water

Beef extract	1.0 g
Peptone	10.0 g
Sodium chloride	75.0 g
D-mannitol	10.0 g
Agar	15.0 g
Phenol red	0.025 g

2. MacConkey's Agar (MAC):

MacConkey's Agar is both a selective and differential media; it is selective for Gram negative bacteria and can differentiate those bacteria that have the ability to ferment lactose.

Components:

- **Bile salts** - Inhibits most Gram-positive bacteria, except *Enterococcus* and some species of *Staphylococcus i.e. Staphylococcus aureus.*

- **Crystal violet dye**- Inhibits certain Gram-positive bacteria thus selecting for Gram negatives.

- **Lactose**- Some bacteria can ferment lactose acid-end products, others cannot.

- **Neutral pH red indicator** - Stains microbes fermenting lactose

  * hot pink in acid pH
  * rose in neutral pH
  * tan in alkaline pH

- **Peptone** - a source of proteins, amino acids for microbial growth.

Figure2 : MacConkey's Agar

By utilizing the available lactose in the medium, Lac+ (Lactose positive) bacteria such as *Escherichia coli, Enterobacter* and *Klebsiella* will produce acid in the medium, which lowers the pH of the agar below 6.8 and results in the appearance of red or pink colonies. The bile salts in the medium precipitate

in the immediate neighborhood of the colony, causing the medium surrounding the colony to become hazy appearance. Non-lactose fermenting bacteria such as, *Proteus species*,*Salmonella*, *Pseudomonas aeruginosa* and *Shigella* cannot utilize lactose in the medium, and will use peptone instead. This results in the formation of ammonia, which raises the pH of the agar, and leads to the formation of white or colorless colonies in the plate. But, in some cases, they can also look golden to brown with dark centers. They are usually circular colonies and arranged randomly.

Formula:

Ingredients per liter of deionized water

3. Eosin Methylene Blue (EMB) Agar (Levine):

Eosin methylene blue agar (EMB) is both a selective and differential medium used for the detection and isolation of Gram-negative intestinal pathogens.

Components:

- **Lactose** – a disaccharide which can be fermented by some bacterial enzymes to produce acidic end products.

- **Eosin and Methylene Blue** – these are dyes which inhibit the growth of most Gram positive bacteria. They also react with any acidic products resulted from lactose fermentation to color the colonies.

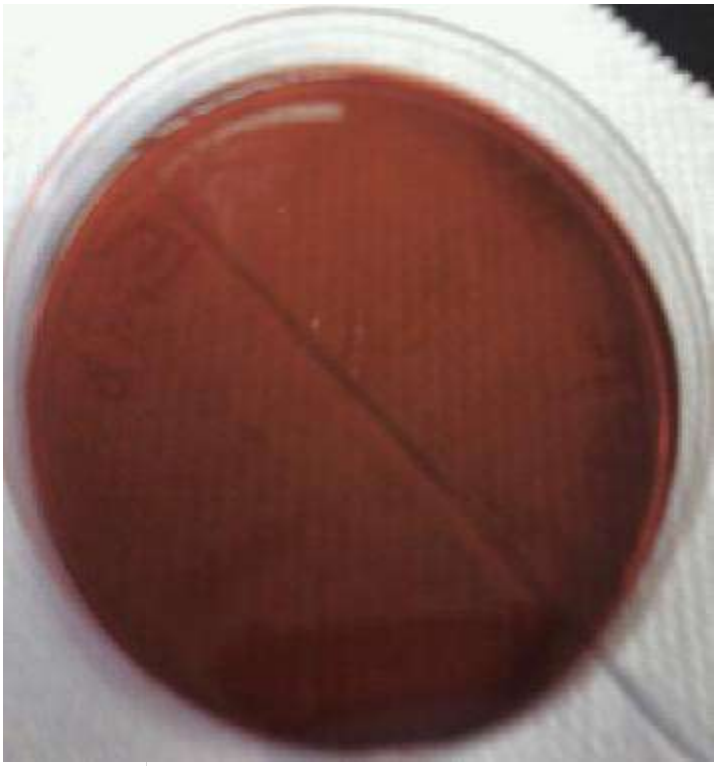


Figure 3: Uninoculated EMB agar plate

Acid production from lactose fermentation causes precipitation of the dyes on the surface of the colony resulting in different colors.

- Large amounts of acid → green metallic sheen
- Small amounts of acid → pink
- No fermentation → colorless

Enterobacter aerogenes produces large colonies which are pink-to-buff around dark centers. *Escherichia coli* produce small, dark colonies with a green metallic sheen. *Pseudomonas, Proteus, Salmonella* and *Shigella sp* produces colorless colonies because it does not ferment lactose.

PREPARATION OF NUTRIENT AGAR

Bacteriological media come an a wide range of types. Nutrient Agar is a **complex** medium because it contains ingredients with contain unknown amounts or types of nutrients.Nutrient Agar contains Beef Extract (0.3%), Peptone (0.5%) and Agar (1.5%) in water. Beef extract is the commercially prepared dehydrated form of autolysed beef and is supplied in the form of a paste. Peptone is casein (milk protein) that has been digested with the enzyme pepsin.Peptone is dehydrated and supplied as a powder. Peptone and Beef Extract contain a mixture of amino acids and peptides. Beef Extract also contains water soluble digest products of all other macromolecules (nucleic acids, fats, polysaccharides) as well as vitamins and trace minerals. Although we know and can define Beef Extract in these terms, each bach can not be chemically

defined. There are many media ingredients which are complex: yeast extract, tryptone, and others. The advantage of complex media is that they support the growth of a wide range of microbes.

Agar is purified from red algae in which it is an accessory polysaccharide (polygalacturonic acid) of their cell walls. Agar is added to microbiological media only as a **solidification agent**. Agar for most purposes has no nutrient value. Agar is an excellent solidification agent because it dissolves at near boiling but solidifies at 45oC. Thus, one can prepare molten (liquid) agar at 45oC, mix cells with it, then allow it to solidify thereby trapping living cells. Below 45oC agar is a solid and remains so as the temperature is raised melting only when >95oC is obtained. In this experiment each student will prepare 200 ml of Nutrient Agar to be used in

MATERIALS

1. Electronic or beam balances.
2. Weigh boats, tongue depressors.
3. Tripods, asbestos wire-gauze, asbestos gloves.
4. 10 ml nonsterile pipettes.
5. pH paper or pH meter with standard buffers.
6. 4 13x100 mm screw capped culture tubes.
7. Graduated Cylinder, 250 ml.
8. 2 500ml Erlenmeyer Flasks
9. Beef Extract, Peptone, Agar.

10. 3 N HCl, 3 N KOH.

11. 16 x 150 mm screw cap culture tubes.

12. Nonabsorbent cotton and gauze to make cotton stoppers.

Nutrient Agar

Beef Extract: 0.3%

Peptone: 0.5%

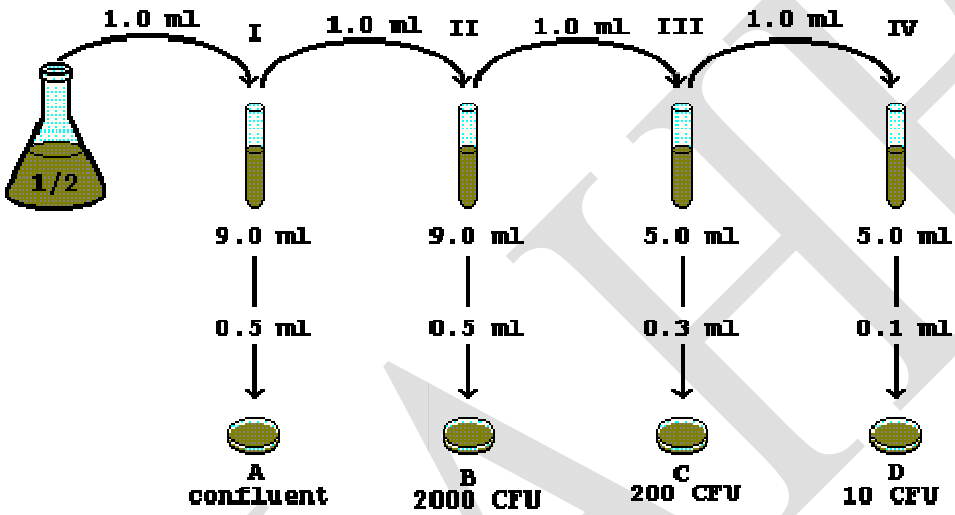
Agar: 1.5%

PROCEDURE

1. To make 200 ml of Nutrient Agar.
2. Tare a weigh boat and weigh out enough Peptone and add that to the flask.
3. Add 200 ml of distilled water and swirl to dissolve the peptone and beef extract. Check the pH, it should be 7.0.
4. Tare a weigh boat and weigh out enough Agar and add that to the flask.
5. With a bunsen burner, tripod, asbestos wire-gauze, heat the medium to boiling to dissolve the agar. CAREFUL: 1) keep the rotating the flasks to prevent the agar from cooking onto the bottom of the flask and 2) watch out: boiling agar can froth and boil out all over the lab bench. As soon as it begins to boil take it off the heat and put it on to the bench. Allow it to cool a few minutes.
6. While the agar is still warm, but not hot, pipette 3 ml each into 4 13x100 mm screw cap culture tubes.
7. Label the flask and your tubes with your name.
8. After preparation of your medium, the instructor will take you to the autoclave.
9. Place your media in the autoclave with those of the rest of the class.
10. After discussion of the parts of the autoclave, autoclave the medium for 20 minutes.
11. The media will be saved and used in other Experiment.

4. SERIAL DILUTION TECHNIQUE

Serial dilutions are used to calculate the concentration of microorganisms. As it would usually be impossible to actually count the number of microorganisms in a sample, the sample is diluted and plated to get a reasonable number of colonies to count. Since each colony on an agar plate theoretically grew from a single microorganism, the number of colonies or Colony Forming Units is representative of the number of viable microorganisms. Since the dilution factor is known, the number of microorganisms per ml in the original sample can be calculated.



A dilution problem such as the one shown above is relatively easy to solve if taken step by step. Follow the steps below.

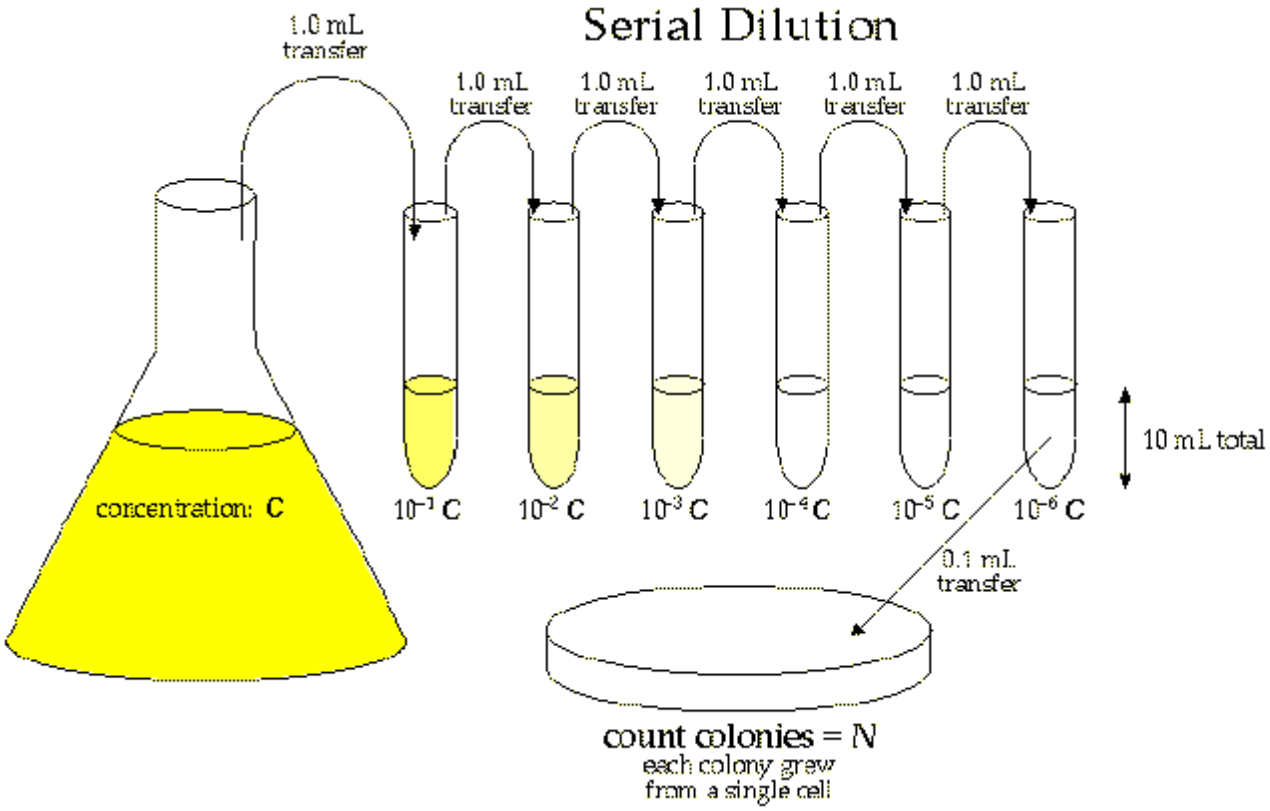
1. First determine which is the **countable plate**.
    - Count the number of colonies on each plate. If there are too many colonies on the plate, the colonies can run together and become indistinguishable as individual colonies. In this case the plate is called confluent or Too Numerous To Count (TNTC). The countable plate has between 30 and 300 colonies. More than 300 colonies would be difficult to count, and less than 30 colonies is too small a sample size to present an accurate representation of the original sample. As stated above, the number of colonies is the number of Colony Forming Units which represents the number of microorganisms per ml.
2. **Sample Dilution Factor (SDF)**

- A sample is often diluted prior to doing the serial dilutions. If it is, the sample dilution factor will be shown in the diagram as above (the 1/2 in the erlenmeyer flask is the sample dilution factor). If the sample remains undiluted, use 1/1 as the Sample Dilution Factor.

3. **Individual Tube Dilution Factor (ITDF)**
   - The individual tube dilution factors are a calculation of how much the sample was diluted in each individual tube. This is just the amount of sample added to the tube divided by the total volume in the tube after adding the sample. In tube I above, 1 ml of sample was added to 9 ml of water, so the ITDF for tube I is: 1ml/1ml + 9 ml = 1/10
4. **Total Series Dilution Factor (TSDF)**
   - The total series dilution factor is a calculation of how much the sample was diluted in all of the tubes combined. This is accomplished by multiplying each of the appropriate ISDF. This series does not include any dilutions after the countable plate. In the example above, since the countable plate was plate C, tube IV is not included in the TSDF. The TSDF for the example above is 1/10 (ITDF for tube I) x 1/10 (ITDF for tube II) x 1/6 (ITDF for tube III) = 1/600.
5. **Plating Dilution Factor (PDF)**
   - When the sample is plated, a dilution factor must also be calculated for this transfer. Since the object of these calculations is to determine CFU/ml, the amount plated for the countable plate is divided by 1 ml to get the PDF. In the example above, 0.3 ml from tube III was plated onto plate C, so the PDF is 0.3ml/1.0 ml = 0.3ml/1.0ml x 10/10 = 3/10.
6. **Final Dilution Factor (FDF)**
   - The FDF takes into account all of the above dilution factors, giving you the total dilution from the original sample to the countable plate. The FDF = SDF x TSDF x PDF, so in this example, the FDF = 1/2 x 1/600 x 3/10 = 3/12000 = 1/4000. This means that the original sample was 4000 times as concentrated as the plated sample from tube III. In other words, it would take 4 L of the sample in tube III to contain the same number of bacteria as 1 ml of the original sample.
7. **Colony Forming Units/ml (CFU/ml)** in original sample
   - To find out the number of CFU/ ml in the original sample, the number of colony forming units on the countable plate is multiplied by 1/FDF. This takes into account all of the

dilution of the original sample. For the example above, the countable plate had 200 colonies, so there were 200 CFU, and the FDF was 1/4000.

- 200 CFU x 1/1/4000 = 200 CFU x 4000 = 800000 CFU/ml = 8 x 10
- CFU/ml in the original sample



5. STRATEGIES FOR OBTAINING PURE ISOLATES

Bergey's Manual correctly stresses the importance of obtaining a pure isolate before attempting to characterize a species. The presence of just one contaminant in an otherwise homogeneous culture can lead to a misleading assay result, if the contaminant grows at a significant rate and responds differently to

assay conditions. A few bacteria are morphologically unique and can be identified without isolation, but nearly all require isolation and characterization by various assay methods. Here is a description of the approach used in our teaching lab to the isolation of pure cultures from a mixture of species.

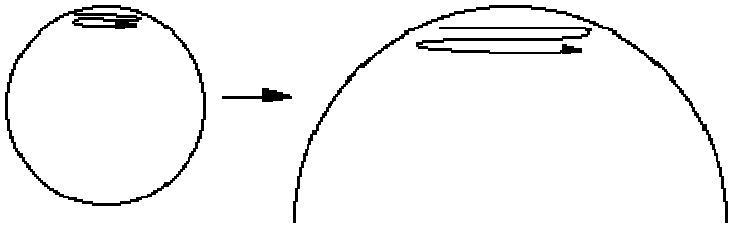
DILUTION STREAKING AND DIFFERENTIAL INCUBATIONS

Sterile equipment must be used when sampling, of course. A specimen should be plated out as soon as possible after sampling. If a specimen is removed from a specialized environment such as bodily fluids, it should be cultured immediately. A sterile solution of 1% peptone can be used as a vehicle for samples to be spread on agar plates. Any liquid sample must be thoroughly vortexed prior to preparation of plates. Non-motile bacteria may settle to the bottom of a sample, particularly if they are associated with particulate matter. Bacteria do not segregate homogeneously, that is, replicate samples from the same mixture may contain vastly different quantities of bacteria. For example, in water analysis several dilute samples from a source may contain zero viable bacteria, while another may contain enough to declare the source contaminated.

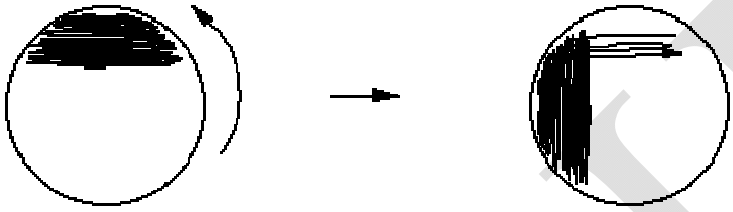
Each viable cell or cluster of viable cells is called a colony forming unit (CFU). The object of thorough vortexing and subsequent dilution streaking is to spread out individual CFUs so as to obtain discrete colonies that may be subcultured. Please keep in mind that a single colony may have been derived from a CFU containing more than one viable species.

To prepare a three way dilution streak plate, a loopful of thoroughly vortexed sample is obtained aseptically and applied to one edge of the agar surface. With back and forth movements about 1/4 of the surface should be streaked while drawing the loop toward the middle of the plate. Streaking should not break the surface of the agar, and there should be many (20 or more) streak lines produced. To dilute the sample the loop must be flamed to destroy all viable material, touched to a clean part of the agar surface to cool it, then streaks made perpendicular to the original inoculum, overlapping that part of the plate once or twice. The second section should cover 1/2 of the remaining sterile surface. This spreads out a small part of the original inoculum, possibly diluting it sufficiently to result in the appearance of individual colonies after incubation. A third section is then streaked perpendicular to the second section,,

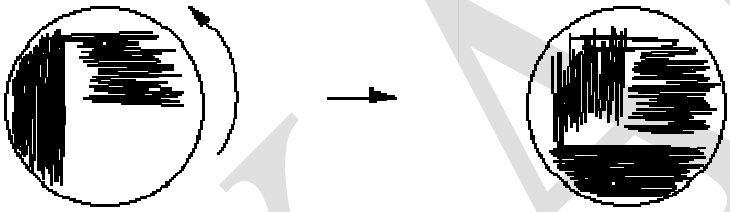
flaming and cooling the loop and overlapping the previous section as before, to further dilute the inoculum.



Start at one edge of the plate, moving the loop back and forth while pulling it back to streak about 1/4 of the plate. Sterilize the loop, and touch a clean part of the surface to cool it.



Rotate the dish 90° and streak one-half of the remaining surface, starting with two streaks that overlap the first area. Sterilize and cool the loop again.



Repeat for the third section. Remember NOT to overlap either previously inoculated section after the first two.

To prepare isolates from a mixed culture we typically prepare replicate streak plates and incubate them under different conditions in an inverted position, to maximize opportunities to differentiate colony types. We vary both temperature of incubation (typically 25, 30, and 37C) and incubate under both aerobic and anaerobic conditions. This approach increases the chances of separating individual species, since different species often have different optimum temperature ranges for growth, and different requirements for oxygen. Aerobically incubated plates should be checked after one day of incubation, since some species may grow very fast and crowd out the others. Any plate should be removed from the incubator after two days and examined for the presence of distinctive colonies. Leaving plates in an incubator only dries them out and clutters up the place.

Some species, such as those of genus Micrococcus, are slow to develop recognizable colonies and may require longer incubation times. You may wish to keep one or more plates for several more days to watch for the appearance of novel colonies. Keep such dishes sealed with Parafilm, and be mindful that contaminants can also show up in older plates (especially near the edges).

How to screw up a streak plate

- Start with a wet plate with liquid on the surface - the liquid will pick up material as the plate is handled, and re-inoculate the plate, making a mess.
- If you start with a liquid culture or sample, dip the loop to take an inoculum without vortexing to make the sample homogeneous.
- Forget to cool the loop before taking an inoculum - you¹ll kill the culture, and nothing will grow.
- Forget to sterilize the loop with a flame before streaking the second and/or third time. You¹ll get luxuriant growth all over the plate, and no individual colonies.
- Make only a few passes with the loop each time you streak. You can write your name with the inoculum, in fact, but you won't get individual colonies.
- Contaminate the plate by taking off the lid completely and/or sneezing on it.
- Contaminate the plate by sticking your face close to it to see the streaked area.

Approaches to identifying and separating colony types

It is important to check the cultures in a timely manner in case one or more species grows so rapidly that it will overwhelm the rest. We typically use a dissecting microscope with transilluminator to distinguish individual colonies. Plates should remain inverted during examination. Colonies should be distinguishable by size, shape, opacity, and texture of colonies. You can indicate colonies to be sampled by putting a small mark next to them on the bottom of the plate. You will have to turn the plate lid up to collect colony material. ***Open lids only sufficiently to introduce a sterile loop or needle, and only for the time it takes to obtain an inoculum.***

For color,surface characteristics, and profile (raised, flat, etc.), you will need to examine colonies with incident light, through the transparent lid. Lids should be left on, otherwise it is a near certainty that the plates will become contaminated. Before turning a plate over make sure that liquid condensate will not

land on the agar surface. If necessary, "tap off" excess water by keeping the plate inverted, quickly removing and inverting the lid, and tapping it on a bench top. Alternatively, use a lid from a new plate to replace the old lid for viewing. Never leave a lid off with agar surface exposed, unless you want to add contaminating species from dust particles in the laboratory to your collection of isolates.

If two colonies overlap and still can be distinguished, then at least two species are present. Colonies usually have a fairly simple, uniform texture. If an area resembles a mosaic, you probably have at least two species. Each unique type of colony should be sampled by taking a needle inoculum and performing a three way dilution streak on a fresh plate. Care must be taken to sample only from the colony of interest. Not much material will be required. Incubate each new streak plate under aerobic conditions at the temperature at which the original plate was incubated.

Keep a record of the source of the isolate, the original incubation temperature of the plate from which the colony was sampled, and whether or not the plate was incubated anaerobically. Species will exhibit temperature optima, indicated by faster growth and/or larger colonies at a temperature closest to ideal. Any colony that is sampled from an anaerobically incubated plate will likely be a facultative anaerobe.

Purifying the isolates

Selection of a single colony from a plate does not ensure that it is pure, since non-growing or slow-growing contaminants may be present within the colony. Thus the second generation plates must be carefully screened for contaminants. Generally, all colonies on a plate containing a pure isolate will be identical in morphology, and cells will be morphologically similar in stains or wet mounts. However, there are several pitfalls of which a student must be aware.

- Bacteria with spreading growth may carry contaminants that are difficult to separate in one generation
- Spores may be misinterpreted as contaminating cell types
- The morphology of older colonies may change, leading one to believe that there are two strains
- Older Gram positive cells may lose the gram positive property
- A single species may have variants that appear morphologically different under the same culture conditions

- Contaminants from outside the plate may form viable colonies. Usually these are distinguisable because they are found only on the edge of a plate and are often not on the track of the inoculating loop.

Narrow down the collection

You are almost certain to collect duplicates of the same species and strain from different streak plates. Many different species and different strains of the same species produce very similar colony types. To narrow the number of isolates to unique species/strains, culture suspected dupicate isolates on the same plate. On two thirds of the surface conduct two "mini-dilutions" to obtain individual colonies for each culture, and on the remaining 1/3 mix them. Incubate, and if you can distinguish the two isolates grown on the same plate and/or the mixed inoculum gives you two distinguishable colony types, then you have two unique isolates. If not, then you have less work to do!

Initial characterization of colonies and preservation of isolates

A common descriptive terminology should be applied to avoid confusion when isolating and characterizing pure isolates. Since many different authors contributed to the Bergey's Manuals the student should be prepared to encounter inconsistencies among descriptive characteristics. For example, what is described as a yellow colony may actually look brown to the student. "White" usually means bone-white, but not always. In fact, many of the descriptive characters are subject to interpretation. Most characteristics, and especially color, should be determined using incident, not transmitted, light. The descriptive terms in this course will be kept relatively simple, although some of the literature makes finer distinctions among forms.

Once an isolate is obtained and purity established by both colony examination and microscopic examination, an agar slant tube should be inoculated, labeled, and incubated at an appropriate temperature with the cap loose to allow gas exchange. After luxurient growth appears, the culture should be described, the cap tightened, and the tube kept at room temperature as a source of pure culture for assays.

In addition to the gross descriptive characterization, a young (<18 h) culture should be Gram stained and results recorded including cell shape and size, sheaths or capsules if evident, and any evidence of spores or similar structures. Relationship to oxygen is the logical next step with which to narrow possible categories. After that, it is the particular combination of Gram stain result, cell type, and relationship to oxygen that determines the next series of steps toward characterizing your isolate.

Descriptive terms for colonies on agar plates

If colonies are present, describe the amount of growth as slight, moderate, or abundant. Note if colonies fail to grow under certain conditions (e.g., certain temperatures). Describe the color and whether or not the colony is opaque, transparent, or translucent. Note whether or not pigmentation has diffused into the medium itself. Describe the general colony appearance, margin, and elevation of the colony.

Growth on agar slants

When a loopful of material is used to inoculate an agar slant, the loop is touched to the surface near the bottom and drawn up toward the top to make a single broad streak. The shape of the growth that results should be described in specific terms.

Growth in broth cultures

Describe the amount of growth as for agar plates. Describe the broth beneath the surface as turbid if it is cloudy, flocculent if large flaky masses are floating in the medium. If there is sediment, note that fact, agitate the tube to bring it into suspension, and describe the sediment by size of particle and whether or not it is viscous (use a sterile loop to determine whether or not it is "gooey."

The surface may be characterized by a coating consisting of a thin membrane or a thick pellicle. There may also be a ring of material (like a pellicle or membrane, but only at the edges), or flocculent material at the top. Note that conditions in an undisturbed broth tube become anaerobic below the top 0.5 cm or so of liquid. Presence/absence of growth in deeper layers may confirm the relationship to oxygen

6 a . SIMPLE STANING

AIM:

To perform simple staining to study and compare the morphology and arrangement of organisms.

MATERIALS REQUIRED:

Glass slides , bacterial culture(24 hour old culture) , crystal violet , methylene blue stain , microscope , tissue paper , loop.

PRINCIPLE:

In simple staining , the bacterial smear is stained with single reagent. Basic stains with the positively charged chromogen(coloring agent) can be preferred because bacterial nucleic acids and

certain cell wall components carry a negative charge that srongly attracts and binds to the cationic chromogen. The purpose of simple staining is to elucidate the morphology and arrangement of bacterial cells. The most commonly used basic stains are methylene blue , crystal violet and carbol fuschin.

PROCEDURE:

1) The smear was prepared on a clean slide using a sterile loop and heat fixed.
2) The smear was flooded with crystal violet stain for 30 to 35 seconds.
3) The smear was washed and observed under oil immersion.

RESULT:

The organism observed under the microscope appeared violet in color and found to be rod shaped(if bacteria is bacillus).

6 b. FUNGAL STANING - LACTO PHENOL COTTON BLUE MOUNT

INTRODUCTION:

Fungi are a group of eukaryotic organisms, it comprises moulds and yeasts. Moulds are filamentous and multicellular organisms. Yeats are unicellular organisms. Fungi are spore bearing protests that lack chlorophyll. They reproduce both sexually and asexually.

AIM:

To observe the morphological characteristics of mould, fungi and yeast.

MATERIALS REQUIRED:

Fungal culture, glass slides, scotch tape, lacto phenol cotton blue stain and microscope.

PROCEDURE:

1) A clean grease free slide was taken to which a drop of lacto phenol cotton blue stain was added.
2) A thin strip of scotch tape was taken and slightly pressed over a colony of fungus.
3) A strip was then placed on the glass slide and observed under microscope.

RESULT:

The fungal morphology was identified.

6 c. HANGING DROP METHOD

INTRODUCTION:

Hanging drop technique is used for the microscopic observation of bacteria without staining and also to observe their motility due to flagella.

AIM:

To observe the motility and the presence of flagella.

MATERIALS REQUIRED

24 hour old culture, cavity slide, cover slip, petroleum jelly and microscope.

PRINCIPLE:

Heat fixing and staining are rather severe treatment for a bacterial cell and they cause considerable changes in morphology. To avoid these changes, bacteria can be observed in their living state by means of wet mount technique. In this method there is usually little difficulty with high motile organisms at feebly motile organisms may require prolonged observation of individual cells. Bacterial movement must be distinguished from 'Brownian movement'. Brownian movement is vibratory movement caused by invisible molecules striking the bacteria displacing them for short distance. True motility of bacteria may lead to the bacteria to move from one place to other relatively longer distance. Therefore it is best to use hanging drop preparation for this purpose. For observing motility of pathogenic organisms it is safer method to inoculate semi solid medium and observed growth and motility.

PROCEDURE:

1) A loop full of bacterial culture was placed on the center of a clean cover slip containing petroleum jelly on the corner.
2) The depression on the slide was placed over the cover slip so that the depression covered the drop of culture.
3) The cavity slide was inverted carefully, so that the drop continued to adhere to the inner surface of the cover slip.
4) The slid was then observed under 45 X objective.

RESULT:

The given bacterial culture contained rod shaped(if bacillus) bacteria showing motility.

7. BIOCHEMICAL ANALYSIS TEST - IMViC

Gram negative enteric bacilli play an important role in the contamination of food. Hence they are the main causative agents of intestinal infection. Gram negative family includes Shigella, Salmonella, Proteus, Klebsiella, Escherichia, Enterobacter etc. Usually four tests are used for differentiation of the various members of Enterobactericeae. They are Indole test,Methyl red test, Voges proskauer test and Citrate test; collectively known as IMViC series of reactions.

INDOLE TEST

Indole tests looks for the presence or absence of tryptophanase enzyme production of the bacteria. If the enzyme is present, it will degrade the aminoacid tryptophan in the media and will produce Indole, ammonia and pyruvic acid. Indole will react with Kovac's reagent to produce a cherry red complex, which indicates a positive indole test. The absence of red color is indicative of tryptophan hydrolysis due to the lack of tryptophanse enzyme(Fig 3).

MATERIALS REQUIRED:-

1. Culture:24-48 hour bacterial culture.
2. Media: Peptone water

PROCEDURE:

1. Sterilize the loop vertically in the blue flame of the Bunsen burner till red hot.
2. Take the test tube containing the nutrient broth culture of organism that has been kept for 24 - 48 hours.
3. Remove the cap of the nutrient agar slant.
4. Using aseptic technique organism from the nutrient agar slant.
5. Take the test tube labeled indole.
6. Inoculate the broth with the inoculation loop containing the inoculum from the nutrient agar slant.
7. Incubate the tube for 24 to 48 hours at 37°C.
8. After incubation add 5 drops of Kovac's reagent .
9. Shake gently for several minutes.
10. Red ring formation within 15 to 20 minutes is a positive result. No red ring formation after 15 to 20 minutes is a negative result.

RESULT

Fig 1: Indole test

Interpretation: **After incubation**: The broth must be turbid. A clear broth indicates that your organism did not grow and cannot be tested. Add a few drops of Indole reagent to the broth culture (tryptone broth). DO NOT SHAKE THE TUBE. A positive result has a red layer at the top. A negative result has a yellow or brown layer.

METHYL RED TEST

This test detects the ability of microorganism to ferment glucose and to produce acidic end products. Enteric organism produces pyruvic acid from glucose metabolism. Some enteric will then use the mixed acid pathway to metabolize pyruvic acid to other acidic products such as lactic acid, acetic acid and formic acids. This will reduce the pH of the media. Methyl red is a pH indicator which is red at the acidic pH (below 4.4) and yellow at alkaline pH (above 7). The formation of red color after the addition of Methyl red reagent indicates the accumulation of acidic end products in the medium and is an indicative of positive test .

MATERIAL REQUIRED:

Medium: MR-VP Broth

PROCEDURE:

1. Sterilize the loop vertically in the blue flame of the Bunsen burner till red hot. From the rack,
2. Take the test tube containing the nutrient broth culture of organism that has been kept for 24 - 48 hours.
3. Remove the cap of the nutrient agar slant.
4. Using aseptic technique organism from the nutrient agar slant.
5. Take the test tube labeled MR
6. Inoculate the broth with the inoculation loop containing the inoculum from the nutrient agar slant.
7. Incubate the tube for 24 to 48 hours at 37°C.
8. After incubation add5 drops of Methyl red reagent.
9. Shake gently for several minutes.
10. Red color formation within 15 to 20 minutes is a positive result. No red color formation after 15 to 20 minutes is a negative result.

Fig 2: Methyl red test

Interpretation: **After incubation**: The broth must be turbid. A clear broth indicates that your organism did not grow and cannot be tested. Remove 1 ml of broth and place into a sterile tube before performing the methyl red test if you are going to use the same broth for the VP test. Add 3-4 drops of methyl red to the original broth. DO NOT SHAKE THE TUBE. A positive result has a distinct red layer at the top of the broth. A negative result has a yellow layer.

VOGES PROSKAUER TEST

This test determines the ability of microorganism to ferment glucose. The end products of glucose metabolism,pyruvic acid, is further metabolized by using Butylene glucol pathway to produce neutral end such as acetoin and 2,3 butanediol. When Barrit's reagent A (40% KOH) and Barrit's reagent B (5% solution of alpha naphthol) is added it will detect the presence of acetoin, the precursor in the 2,3-butanediol synthesis. Acetoin in the presence of Oxygen and Barrit's reagent is oxidized to diacetyl, where alpha naphthol act as a catalyst. Diacetyl then reacts with guanidine components of peptone to produce a cherry red colour.

Materials Required:

Culture:

24-48 hour tryptic soy broth culture.

Media:

MR-VP medium

Media preparation:

Weigh 5 g of glucose, 5 g of peptone and 5 g of dipotassium hydrogen phosphate separately. Suspend all the ingredients in distilled water. Make up to 1000 ml. pH should be 6.9. Dispense 3 ml of the media into each test tube, which are plugged and sterilized at 121°C

Reagents:

Barritt's reagents A and B.

Preparation of Barritt's reagent:

It consists of two solutions;

1. Solution A is prepared by dissolving 6 grams of a-naphtholin in 100 ml of 95% ethyl alcohol.
2. Solution B is prepared by dissolving 16 grams of potassium hydroxide in 100 ml of water.

Equipments

Bunsenburner.
Inoculating loop.

Procedure:

Using sterile technique, inoculate each experimental organism to the appropriately labeled tube of medium by means of loop inoculation. Incubate the cultures for 24-48 hours at 37°C. The experiment should be conducted in the LAF. Arrange the materials required for the experiment in the LAF.

1. Sterilize the loop vertically in the blue flame of the Bunsen burner till red hot. Heat from the base of the wire first and slowly move towards the loop (tip). Heat the wire until it is red-hot.
2. From the rack, take the test tube containing the Tryptic Soy Broth(TSB) cultures that has been kept for 24 - 48 hours.
3. Remove the cap from the TSB tube and flame the neck of the tube.
4. Using aseptic technique take a loop full of the organism from the TSB (tryptic soy broth).
5. Again flame the neck of the tube and replace the cap and place the tube in the test tube rack.
6. Take two sterile MR-VP broth tubes, one named Test and the other Control.
7. Remove the cap of the MR-VP broth tube named 'Test' and flame the neck of the tube.
8. Inoculate the MR-VP broth with the inoculation loop containing the inoculum from the TSB.
9. Again flame the neck of the MR-VP tube and place it in the test tube rack. Inoculate only the broth in the tube named 'Test' using aseptic technique. Leave the broth in the tube named 'Control' uninoculated.
10. Incubate both the tubes (Test and Control) for 24 to 48 hours at 37°C.
11. Remove the broths from the incubator.
12. Remove the cap and add 10 drops of Barritt's A reagent and 10 drops of Barritt's B reagent to each broth.
13. Shake gently for several minutes.
14. Red color formation within 15 to 20 minutes is a positive result. No red color formation after 15 to 20 minutes is a negative result.

RESULT

Positive Result:

Glucose ------Glucose Metabolism-------> Pyruvic Acid.

Pyruvic acid ----------> Acetoin.

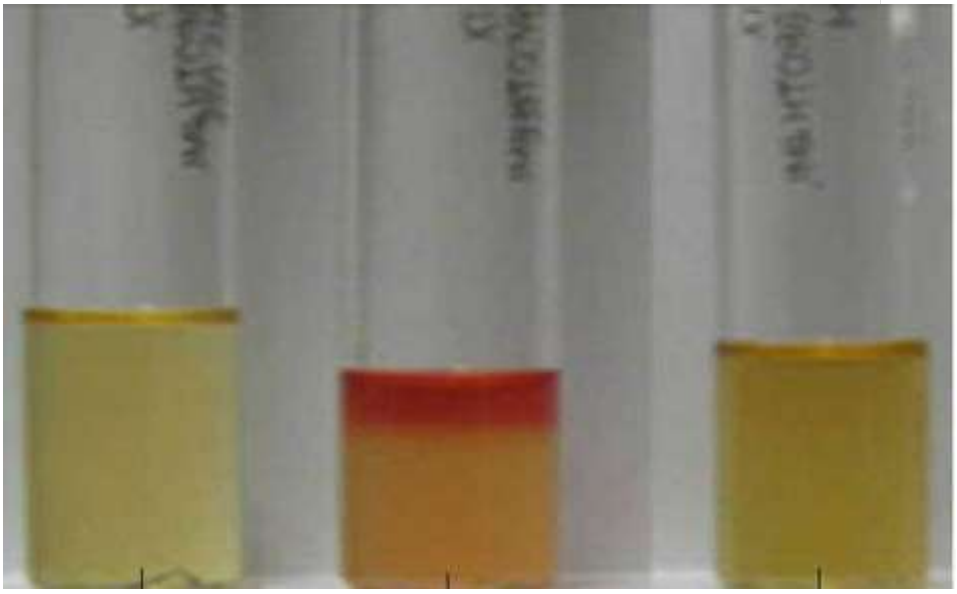
Acetoin + added alpha-naphthol + added KOH = red color.

Negative Result:

Glucose ------Glucose Metabolism-------> Pyruvic Acid.

Pyruvic acid ----------> No Acetoin.

No acetoin + added alpha-naphthol + added KOH = copper color.



Uninoculated **Positive** **Negative**

Fig 3: **Voges proskauer test**

Interpretation: **After incubation**: Read the VP test when you have good turbidity. A clear broth indicates that your organism did not grow and cannot be tested. Barritt's reagent A (VP A) contains naphthol and

Barritt's B (VP B) contains KOH. Test 1 ml of your culture from the MRVP broth. If you have already conducted the methyl red test, you should have already placed 1 ml of untested broth in a sterile tube. If you haven't done this, do so now. Add the entire contents of the VP A reagent (15 drops) and 5 drops of the VP B reagent to the 1 ml of your broth culture. SHAKE WELL. This reaction will take a few minutes before you will see a color change. SHAKE the tube every few minutes for best results. With a positive reaction the medium will change to pink or red indicating that acetoin is present. With a negative reaction the broth will not change color or will be copper colored. Wait at least 15 minutes for color to develop before calling the test negative.

CITRATE UTILIZATION TEST

This test determines the ability of microorganism to utilize Citrate. Some bacteria have the capability to convert the salts of organic acids, for example, Sodium citrate to alkaline carbonates. Sodium citrate is one of the important metabolite of Kreb's cycle. Certain bacteria use citrate as the sole carbon source. Citrate utilization requires a specific membrane transporter and citrate lyase activity. Citrate is converted to Oxalo acetic acid by citrate lyase and oxaloacetate decarboxylase activity will convert oxaloacetate to pyruvate with the release of carbondioxide. The other products of the reaction are acetate, Lactic acid, formic acid etc. The carbondioxide reacts with sodium and water to form sodium carbonate

Medium: Citrate medium

PROCEDURE:

1. Sterilize the loop in the blue flame of the Bunsen burner till red hot and then allowed to cool.
2. Take out an isolated colony of the organism from the nutrient agar tube with the cooled loop aseptically.
3. Again flame the neck of the tube and replace the tube in the test tube rack.
4. Take a sterile citrate slant tube remove the cap and flame the neck of the tube.
5. Inoculate the entire surface of the citrate slant (slope) with the provided growth from the nutrient gar slant culture using the inoculating loop (do not stab the butt). The slant of the medium is inoculated by streaking the surface of the agar in a zigzag manner.
6. Again flame the neck of the citrate tube and place it in the test tube rack
7. Tighten the cap and incubate at 37°C for 24-48 hours.
8. Obtain the tubes from the incubator and observe the colour change.
9. Blue colour formation indicates a positive test and green color indicates the negative test.

Fig 4: Citrate test

Interpretation: **After incubation**: A positive reaction is indicated by a slant with a Prussian blue color. A negative slant will have no growth of bacteria and will remain green.

The significance of these tests is that when testing drinking water for the presence of the sewage indicator *E. coli*, one must be able to rule out *Enterobacter aerogenes*. *E. aerogenes* is not always associated with sewage, and its presence in water would not necessarily indicate sewage contamination.

CONTROLS	Indole	Methyl Red	VP	Citrate
Escherica. coli	+	+	-	-
Enterobacter. aerogenes	-	-	+	+

8. MEASUREMENT OF BACTERIAL GROWTH RATE OF BACTERIA

AIM

1. To study the different phases of bacterial growth.
2. To plot standard growth curve of *Staphylococcus aureus.*
3. To determine the generation time of given bacteria.

PRINCIPLE:

The increase in the cell size and cell mass during the development of an organism is termed as growth. It is the unique characteristics of all organisms. The organism must require certain basic parameters for their energy generation and cellular biosynthesis. The growth of the organism is affected by both physical and Nutritional factors. The physical factors include the pH, temperature, Osmotic pressure, Hydrostatic pressure, and Moisture content of the medium in which the organism is growing. The nutritional factors include the amount of Carbon, nitrogen, Sulphur, phosphorous, and other trace elements provided in the growth medium. Bacteria are unicellular (single cell) organisms. When the bacteria reach a certain size, they divide by binary fission, in which the one cell divides into two, two into four and continue the process in a geometric fashion. The bacterium is then known to be in an actively growing phase. To study the bacterial growth population, the viable cells of the bacterium should be inoculated on to the sterile broth and incubated under optimal growth conditions. The bacterium starts utilising the components of the media and it will increase in its size and cellular mass. The dynamics of the bacterial growth can be studied by plotting the cell growth (absorbance) versus the incubation time or log of cell number versus time. The curve thus obtained is a sigmoid curve and is known as a standard growth curve. The increase in the cell mass of the organism is measured by using the Spectrophotometer. The Spectrophotometer measures the turbidity or Optical density which is the measure of the amount of light absorbed by a bacterial suspension. The degree of turbidity in the broth culture is directly related to the number of microorganism present, either viable or dead cells, and is a convenient and rapid method of measuring cell growth rate of an organism. Thus the increasing the turbidity of the broth medium indicates increase of the microbial cell mass (Fig 1) .The amount of transmitted light through turbid broth decreases with subsequent increase in the absorbance value.

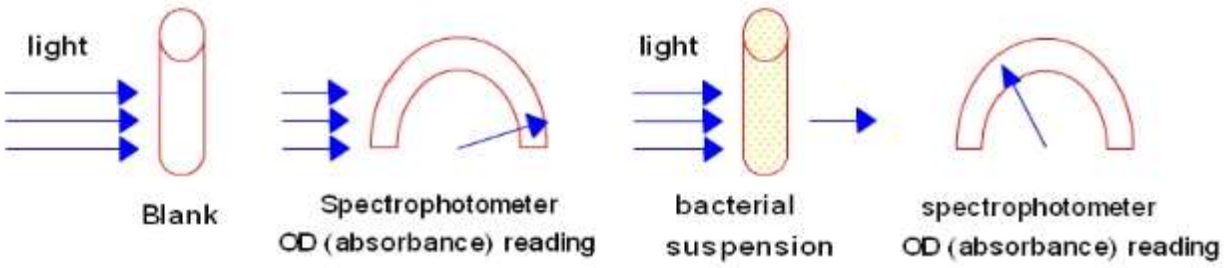


Fig 1: Absorbance reading of bacterial suspension

The growth curve has four distinct phases (Fig 2)

1. Lag phase

When a microorganism is introduced into the fresh medium, it takes some time to adjust with the new environment. This phase is termed as Lag phase, in which cellular metabolism is accelerated, cells are increasing in size, but the bacteria are not able to replicate and therefore no increase in cell mass. The length of the lag phase depends directly on the previous growth condition of the organism. When the microorganism growing in a rich medium is inoculated into nutritionally poor medium, the organism will take more time to adapt with the new environment. The organism will start synthesising the necessary proteins, co-enzymes and vitamins needed for their growth and hence there will be a subsequent increase in the lag phase. Similarly when an organism from a nutritionally poor medium is added to a nutritionally rich medium, the organism can easily adapt to the environment, it can start the cell division without any delay, and therefore will have less lag phase it may be absent.

2. Exponential or Logarithmic (log) phase

During this phase, the microorganisms are in a rapidly growing and dividing state. Their metabolic activity increases and the organism begin the DNA replication by binary fission at a constant rate. The growth medium is exploited at the maximal rate, the culture reaches the maximum growth rate and the number of bacteria increases logarithmically (exponentially) and finally the single cell divide into two, which replicate into four, eight, sixteen, thirty two and so on (That is 2^0, 2^1, 2^2, 2^3.....2^n, n is the number of generations) This will result in a balanced growth. The time taken by the bacteria to double in number during a specified time period is known as the generation time. The generation time tends to vary with different organisms. *E.coli* divides in every 20 minutes, hence its generation time is 20 minutes, and for *Staphylococcus aureus* it is 30 minutes.

3. Stationary phase

As the bacterial population continues to grow, all the nutrients in the growth medium are used up by the microorganism for their rapid multiplication. This result in the accumulation of waste materials, toxic metabolites and inhibitory compounds such as antibiotics in the medium. This shifts the conditions of the medium such as pH and temperature, thereby creating an unfavourable environment for the bacterial growth. The reproduction rate will slow down, the cells undergoing division is equal to the number of cell death, and finally bacterium stops its division completely. The cell number is not increased and thus the growth rate is stabilised. If a cell taken from the stationary phase is introduced into a fresh medium, the cell can easily move on the exponential phase and is able to perform its metabolic activities as usual.

4. Decline or Death phase

The depletion of nutrients and the subsequent accumulation of metabolic waste products and other toxic materials in the media will facilitates the bacterium to move on to the Death phase. During this, the bacterium completely loses its ability to reproduce. Individual bacteria begin to die due to the unfavourable conditions and the death is rapid and at uniform rate. The number of dead cells exceeds the number of live cells. Some organisms which can resist this condition can survive in the environment by producing endospores.

MATERIALS REQUIRED

1. Nutrient broth
2. Sterile petriplates
3. Micropipettes
4. Cuvette
5. Conical flask
6. Sterile tips
7. Culture – Overnight culture of *Staphylococcus aureus*
8. Colorimeter

PROCEDURE

- An isolated colony of the organism (*Staphylococcus aureus*) was inoculated into 15 ml nutrient broth and kept for overnight incubation
- Following day, the OD of this culture was measured and confirmed.
- In order to adjust the OD of the inoculum to the standard value (0.05) the following dilution formula was used

OD1V1 = OD2V2 Where,

OD1 = OD of the broth culture, inoculated the previous day.

V1 = volume of this broth culture to be added to the inoculums

OD2 = OD of the inoculum (as a standard, this value was adjusted to 0.05)

V2 = volume of the inoculums (in this experiment, 50 ml)

- Substitute the values in the equation and V1 was calculated.
- That much amount (V1) of the inoculums was pipetted out before adding an equivalent amount of the broth to it, so that the net volume remains constant.
- The OD was checked at every 30 minutes interval and recorded.
- Using this OD value, a standardized growth curve of the organism was plotted. (Absorbance verses time).
- Generation time was calculated.

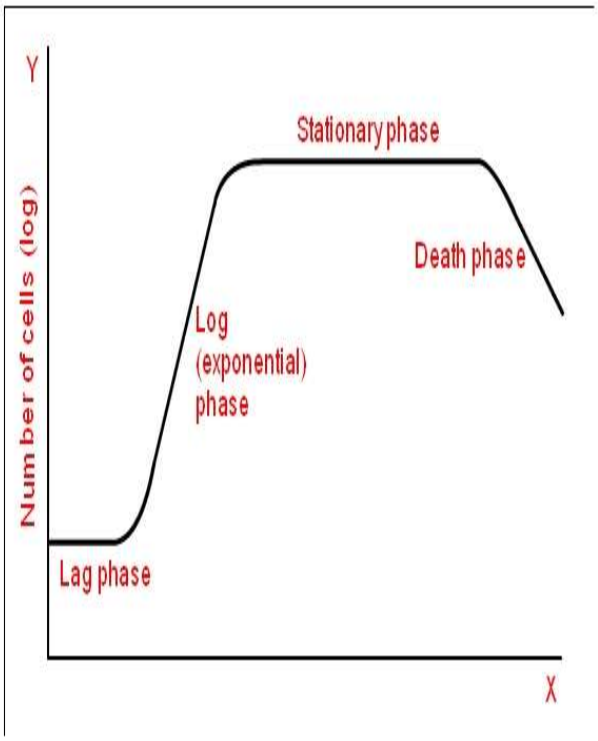


Fig 2: Different phases of growth of a bacteria

RESULT and CALCULATION:

The generation time can be calculated from the growth curve(Fig 3).

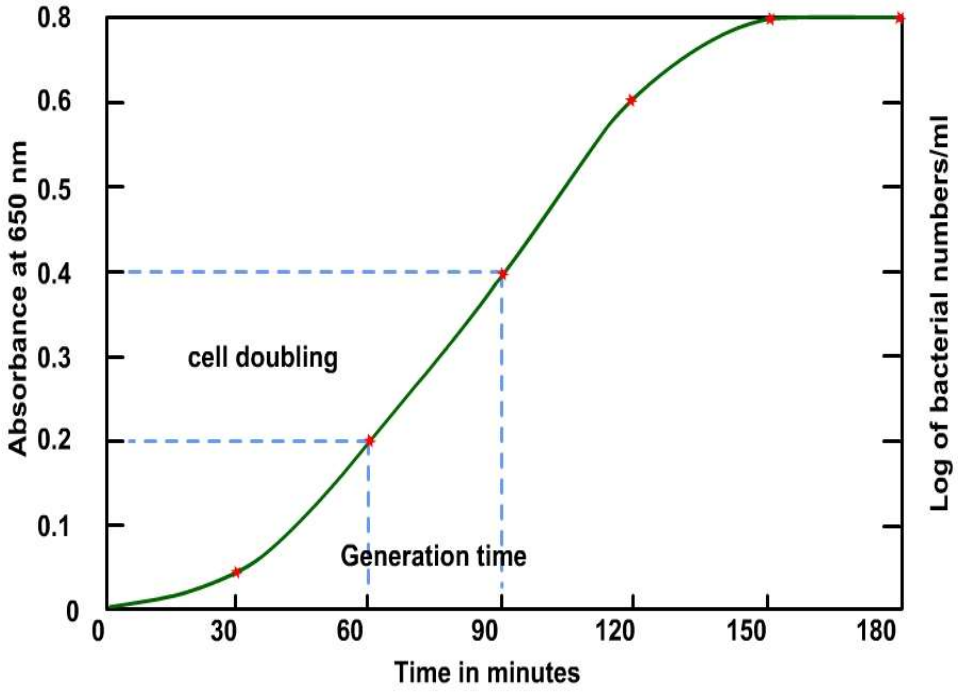


Fig 3: Calculation of generation time

The exactly doubled points from the absorbance readings were taken and, the points were extrapolated to meet the respective time axis.

Generation Time = (Time in minutes to obtain the absorbance 0.4) – (Time in minutes to obtain the absorbance 0.2)

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