SRI VENKATESWARA INTERNSHIP PROGRAM FOR RESEARCH IN ACADEMICS (SRI-VIPRA)

Project Report of 2022: SVP-2246

"Microbial Culture Techniques"

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SRIVIPRA-2022

(Sri Venkateswara College Internship Program in Research and Academics)

This is to certify that this project on **Microbial Culture Techniques**

was registered under SRIVIPRA and completed under the mentorship of Prof . Om Prakash and Dr. Ajaib Singh during the period from 21st June to 7th October 2022.

Sharda Pasricha and S. Krishnakumar

Coordinators

Prof. C Sheela Reddy

Principal

SRIVIPRA PROJECT 2022

Title : Microbial Culture Techniques

Name of Mentor: Prof. Om Prakash and Dr. Ajaib Singh

Name of Department: Zoology

Designation : Professor and Assistant Professor

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Signature of Mentors

Certificate

This is to certify that the aforementioned students from Sri Venkateswara College have participated in the summer project SVP- 2246 entitled "**Microbial Culture Techniques"**. The participants have carried out the research project work under my guidance and supervision from 21st June 2022 to 7th October 2022. The work carried out is original and carried out in an offline mode.

Signature of Mentors

Acknowledgements

We would like to express our sincere gratitude to Dr. Ajaib Singh and Prof. Om Prakash of Department of Zoology, Sri Venkateswara College, who provided sincere guidance, insights and hands - on training during this internship which helped us to get well versed with microbial culture techniques. During the internship we learned various techniques of raising bacterial cultures such as autoclaving, serial dilution, spread plating, streaking and preservation to name a few. We are thankful to Dr. Sharda Pasricha and Mr. Krishnakumar S , the Convenors of Sri Vipra - 2022 summer internship program for successfully organizing this activity and providing support throughout. This Summer Internship was supported by our respected Principal, Professor. C. Sheela Reddy. We thank her for giving us this opportunity and providing the needful support throughout the internship.

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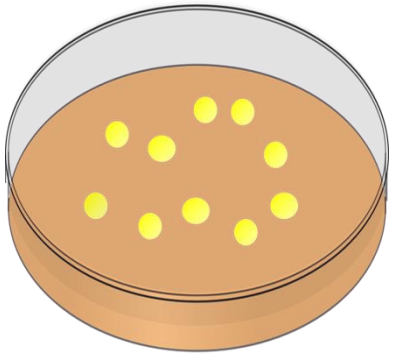
I. INTRODUCTION: MICROBIAL CELL CULTURE

A microbial culture is a method of multiplying microbial organisms by letting them reproduce predetermined culture media under controlled laboratory conditions. It is a method used extensively as a research tool in molecular biology.



COMMON INGREDIENTS OF CULTURE MEDIA

- **Peptone-** source of carbon and nitrogen
- **Beef extract**- source of amino acid, vitamins and minerals
- **Yeast extract-** source of vitamin, carbon and nitrogen
- **Distilled water**
- **Agar-** solidifying agent.

PURPOSE OF CULTURING

- Isolation

- Properties of bacteria
- To create antigens for laboratory use
- Typing with Bacteriophages and Bacteriocins susceptibility
- To test for Antibiotic sensitivity
- Estimate viable counts
- Maintains stock cultures.

How To Prepare culture media?

- Weigh the amount of ingredients powder on weighing machine.
- Dissolve the ingredients in distilled water.
- Adjust PH of the media if needed.
- Add agar and boiled it to dissolve.
- Pour the media into flask.
- Autoclave the media when ingredients fully dissolve.
- Sterilization is done in autoclave to prevent from contamination, at 121 degree Celsius for 15 minute at 15lbs.
- After autoclave place the media flask in laminar air flow.
- Sterilize the laminar flow with 70% alcohol.
- A bit cools down the media and pours into sterile Petri-plates for solidification.
- Then sample is ready to spread/ streak.
- Inoculation loop on the medium for identification or isolation of microbes.
- Sealed the Petri plates with paraffin, label them.
- Keep them inverted in incubator at 37 degree Celsius for 24hrs.
- Observe the result next day colonies formation is visible on the media.

TYPES OF CELL CULTURE METHOD

❖ Streak culture- Streak culture is a method of culture which has developed by drawing an inoculated needle in a straight line over the surface of a medium.

- **Stab or Stick culture-** These cultures are prepared in solidified and liquid media by inserting straight an inoculation needle for some distance. The main points to note in these cultures are grown of microorganisms along the line of puncture and the changes occurring in the medium.
- **Stock culture-** These are the pure cultures of known species of microorganisms which are maintained in viable conditions in laboratory for a longer duration of time. Actually, these cultures are the stocks of particular microbial species or their strain, and one can take sample from them for study. There are many stock established throughout the world to help microbiologists obtaining cultures various studies.
- **Starter culture-** Many microorganisms have been identified which act upon specific end-products. Many commercial laboratories prepare cultures, either of known species and supply to the manufacturers to use to start fermentative processes. These cultures are called starter culture. For instance, several commercial laboratories specialized in preparing starter culture for the dairy industry; the principal microorganisms involved are the species of *Lactobacillus*, *Streptococcus*, *Leuconostoc* which are responsible for the desirable changes in manufacturing fermented milk-products.
- **Enrichment culture-** It is the culture in which the growth of a particular microorganism is favoured as against a mixed population by adjusting the nutritional requirements and environment factors.
- **Batch culture-** Growth of microorganism in a limited volume of culture medium is referred to as a batch culture. A flask containing a constant volume of culture medium inoculated with a bacterium (e.g.; *E. coli*) is an example of a batch culture.
- **Continuous culture-** Unlike batch culture, a continuous culture is that in which fresh nutrients are supplied and the end-products continuously removed through a siphon-flow. A continuous culture, therefore, helps maintaining

exponential growth of bacterial population at a constant rate for over a long period of time.

- Synchronous culture- A synchronous culture is that in which all microbial cells growing are physiologically identical and are in the same stage of division-cycle. A synchronous culture can be obtained either by manipulating environmental conditions or by physical separation of cells.

II. MEDIA PREPARATION AND AUTOCLAVING

Culture media preparation is one of the routine tasks common to many microbiology laboratories. Medium (media pl.) is the substance that provides nutrients for the growth of microorganisms. The nutrients on which microorganisms are cultivated is called culture medium (pl. culture media). Bacteria use the nutrients from the medium as food for its growth and reproduction. Culture media are employed in the isolation and maintenance of pure colonies of bacteria. It can also be used to identify the bacteria depending upon the morphological characteristic of the colony formed by the given species. It makes the study of biochemical and physiological properties, easier and better. It is also used to store r transport microorganisms.

A culture medium (Pl. media) can be a solid or liquid preparation containing all the nutrients required by microorganisms for growth and multiplication. The liquid culture medium is called broth. It can be solidified by adding solidifying agent agar-agar in the ratio of 1.5 – 2.0% for complete solid agar and less than 1% for semi-solid medium. Agar-agar is a sulfonated mucopolysaccharide containing mainly D-galactose, D-glucuronic acid and 3,6 anhydro L galactose. It is derived from red seaweed e.g., *Gelidium* and *Gracilaria.*

Based on chemical composition, media can be classified into:

1. Natural medium: Culture media of which, the exact chemical composition is not known is called natural or empirical culture media. Examples- Milk, urine, diluted blood, vegetable juices, meat extracts, beef and tomato juice, blood etc
2. Semi-synthetic: Culture media, the chemical components of which are partially known and partially obscure are termed as semisynthetic culture media. Examples- Potato dextrose agar (PDA), Czapek-Dox agar, oat meal agar (OMA), corn meal agar (CMA), beef peptone agar and nutrient agar.
3. Synthetic medium: Such media are composed of substances that are chemically known. These media are very useful in studying the physiology, metabolic nature and nutritional requirements of microbes. Both autotrophs and heterotrophs can be grown in these media. Examples- Mineral glucose medium, Richard's solution, Raulins medium etc.

Based on consistency the media are of three types as:

1. Liquid medium: Agar is not added or used while preparing the medium. After inoculation and later incubation, the growth of cells becomes visible in the form of a small mass on the top of the broth. It is used for inoculation preparation, growth of pure bacterial culture and for the isolation of pathogens from a mixture. eg. LB broth.
2. Solid medium: If agar is added to a nutrient broth, it becomes the solid medium. It is used for isolating microbes and determining the characteristics of colonies. It remains solid on incubation and is not destroyed by proteolytic bacteria. 2% agar is added for solidification. Colony morphology, pigmentation, and haemolysis can be studied using solid media. Eg. LB agar, nutrient agar, blood agar etc.
3. Semi-solid medium: It contains 0.5% agar. It is used to check the motility of the bacteria.
No single medium can support the growth of the majority of microbes. Different composition of culture media is used for different microbes; determined by the species to be cultivated. But the one we used for our experimentation is LB media. It is also the most widely used bacterial culture, mainly because it is easy to prepare and provides a broad base of nutrients. Its creator, Giuseppe Bertani, intended LB to stand for lysogenic broth, but LB has also come to be referred as Luria Broth, Lennon Broth or Luria-Bertani medium.
There are several formulations of LB. Although they are different, they generally share a somewhat similar composition of ingredients used to promote growth, including the following:

- Peptides and casein peptones
- Vitamins
- Trace elements (eg. Nitrogen, Sulphur, Magnesium)
- Minerals

Our preparation of LB broth per Litre contains:

- 10g Tryptone (a mixture of peptides formed by the digestion of casein with the pancreatic enzyme trypsin)
- 5g Yeast extract (an autolysate of yeast cells)
- 5g NaCl

NaCl provides the sodium ions for the transport of osmotic balance. Tryptone is used to provide essential amino acids while yeast extract is used to provide organic compounds helpful for bacterial growth; including vitamins and trace elements.

For broth preparation 500 mL distilled water was taken in a conical flask. Tryptone (10g), yeast extract (5g) and NaCl (5g) were measured separately on high accuracy weighing balance and were added to the distilled water. The mixture was then set on a magnetic stirrer to dissolve all the ingredients. Once everything dissolved, the pH of the mixture was checked and adjusted accordingly; to make the pH 7.5 by adding a few sodium hydroxide palettes as required. After adjusting the pH at 7.5 the mixture was poured into a large cylindrical measuring flask and the volume was made up to 1L by adding distilled water to the mixture. The mixture was then transferred to autoclave bottles. 800mL of media was transferred to a 1L autoclave bottle and the rest 200 mL of media was transferred to a 500mL autoclave bottle. The caps were placed but not tightened completely. Autoclave tapes were placed onto the neck of the flask and the media name, individual's name and date were written on it. The bottles were then placed in the autoclave at 121ºC for 20 minutes. After the bottles are taken out of the autoclave the caps are tightened completely. And left to cool down to room temperature. This procedure gives a transparent yellow LB broth.

An autoclave is a machine that uses steam under pressure to kill unwanted microbes. The items are heated to an appropriate sterilization temperature for a given amount of time. The moisture in the steam efficiently transfers heat to the items to destroy the protein structure of the bacteria and spores. Autoclaving the media is a necessary process so as to kill the microbes within the broth and the bottle and make it sterile. So, the growth of other microbes is prohibited in the nutrition-rich broth. Sterilized environment for bacterial growth is essential before starting with the experiments.

Figure 1. An Autoclave

For solid media another 1L of media was prepared following the same procedure as stated above. An additional step of adding 20g, 2% agar to the mixture after the pH had been adjusted, is done. So as to make solid media.

Once the bottles are taken out from the autoclave are cooled. Translucent yellow solidified media is produced. The bottles are microwaved to liquefy the media so as to do pouring on Petri dishes in lamina flow to maintain sterility. After pouring the media into the Petri dishes the media is left to solidify. Once it solidifies the petri dish cover is placed on it and taped to it. Again the media name/individual's name/ date is written under it.

Figure 2: LB Broth and LB Media preparation

III. SERIAL DILUTION METHOD

Serial dilution is the process of stepwise dilution of a solution with an associated dilution factor

Or

In biology, serial dilution is often associated with reducing the concentration of cells in a culture to simplify the operation.

Objectives of serial dilution

The objective of the serial dilution method is to estimate the concentration (number of organisms, bacteria, viruses, or colonies) of an unknown sample by the enumeration of the number of colonies cultured from serial dilutions of the sample.

- In serial dilution, the density of cells is reduced in each step so that it is easier to calculate the concentration of the cells in the original solution by calculating the total dilution over the entire series.
- Serial dilutions are commonly performed to avoid having to pipette very small volumes (1-10 µl) to make a dilution of a solution.
- By diluting a sample in a controlled way, it is possible to obtain incubated culture plates with an easily countable number of colonies (around 30–100) and calculate the number of microbes present in the sample.

Serial Dilution Formula and Calculation

- Serial dilution involves the process of taking a sample and diluting it through a series of standard volumes of sterile diluent, which can either be distilled water or 0.9 % saline.
- Then, a small measured volume of each dilution is used to make a series of pour or spread plates.
- Depending on the estimated concentration of cells/organisms in a sample, the extent of dilution is determined. For e.g., if a water sample is taken from an extremely polluted environment, the dilution factor is increased. In contrast, for a less contaminated sample, a low dilution factor might be sufficient.
- The dilution factor in a serial dilution can be determined either for an individual test tube or can be calculated as a total dilution factor in the entire series.

- The dilution factor of each tube in a set:

$$\frac{volume\ of\ sample}{volume\ of\ sample + volume\ of\ diluent}$$

For a ten-fold dilution, 1 ml of sample is added to 9 ml of diluent. In this case, the dilution factor for that test tube will be:

$$\text{Dilution factor} = \frac{1\ ml}{1\ ml + 9\ ml} = \frac{1}{10} = 10^{-1}$$

- After the first tube, each tube is the dilution of the previous dilution tube.

Now, for the total dilution factor,

Total dilution factor for the second tube = dilution of first tube × dilution of the second tube.

Example:

For the first tube, dilution factor = 10^{-1} (1 ml added to 9 ml)
For the second tube, dilution factor = 10^{-1} (1ml added to 9 ml)
Total dilution factor = previous dilution × dilution of next tube

= total dilution of $10^{-1} \times 10^{-1} = 10^{-2}$

Procedure of Serial Dilution

The following is the procedure for a ten-fold dilution of a sample to a dilution factor of 10^{-6}:

1. The sample/culture was taken in a test tube and six test tubes, each with 9 ml of sterile diluents, which can either be distilled water or 0.9% saline, were taken.

2. A sterile pipette was taken.

3. 1 ml of properly mixed sample/culture was drawn into the pipette.

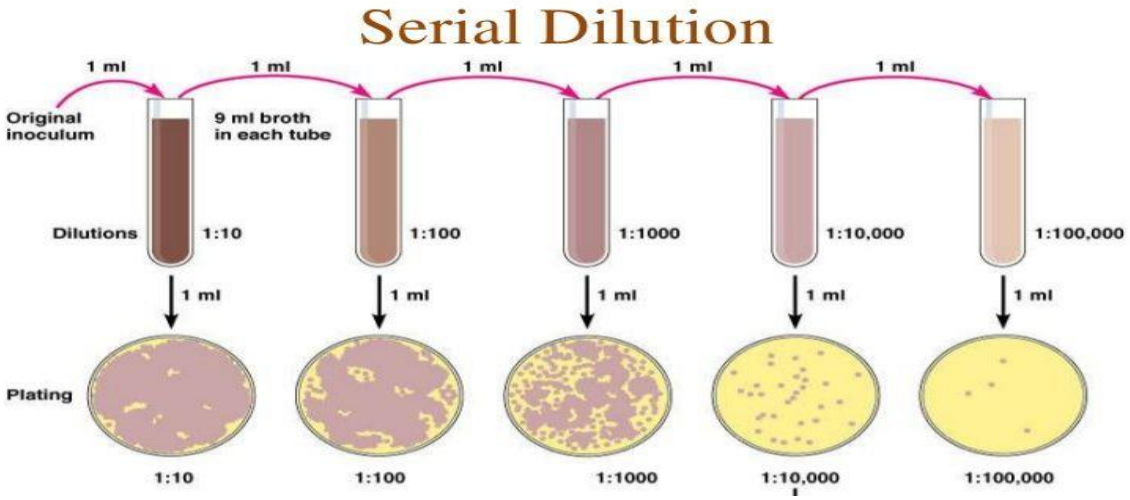
4. The sample was then added to the first tube to make the total volume of 10 ml. This provides an initial dilution of 10^{-1}.

5. The dilution was thoroughly mixed by emptying and filling the pipette several times.

6. The pipette tip was discarded, and a new pipette tip was attached to the pipette.

7. Now, 1 ml of mixture was taken from the 10^{-1} dilution and was emptied into the second tube. The second tube now had a total dilution factor of 10^{-2}.
8. The same process was then repeated for the remaining tube, taking 1 ml from the previous tube and adding it to the next 9 ml diluents.

9. As six tubes were used, the final dilution for the bacteria/cells will be 10^{-6} (1 in 1,000,000).



Serial Dilution Application/Uses

Serial dilution is performed in a number of experimental sciences like biochemistry, pharmacology, physics, and homeopathy.

1. Serial dilution is used in microbiology to estimate the concentration or number of cells/organisms in a sample to obtain an incubated plate with an easily countable number of colonies.

2. In biochemistry, serial dilution is used to obtain the desired concentration of reagents and chemicals from a higher concentration.

IV. ISOLATION OF BACTERIA

In order to isolate bacteria from different habitats following methodologies were used.

Laminar flow

Laminar flow/ Laminar flow cabinet is a carefully enclosed bench designed to prevent contamination of biological samples , semiconductor wafers or any particle sensitive material. In this air is passed through HEPA(High Efficiency Particle Air)filter which removes all airborne contamination to maintain sterile condition.

Components of laminar cabinet

A cabinet

Blower

High efficiency Performance Air (HEPA) filter

A plenum chamber(pressurized housing containing air at positive pressure

Fluorescent light and UV light

Based on the direction in which the air moves the laminar flow cabinets are classified into two types:

1.Vertical laminar flow cabinet

In which the air flows from the upper part of the cabinet towards the lower part in cabinet.

2.Horizontal laminar flow cabinet

In which air flows from behind the bench and carried by the blower towards the HEPA filter.

Figure: Vertical Laminar Flow

Streaking Method for pure culture

The streak plate method is a technique of isolating pure cultures, and getting well-isolated colonies of bacteria from a mixed population.

Principle

It is essentially a dilution technique that involves spreading a loopful of culture over the surface of an agar plate. That result in individual colonies in agar plate .

Procedure

Give uv treatment to laminar flow hood .

Sterilized the working platform with 70%alcohol.

Heat the loop till red hot and touch in side of the plate allow it to cool.

Streak on the 1st quadrant of agar plate .

Heat it again, streak on 2nd quadrant and so on 3rd and final quadrant.

Plates were closed and left in incubator overnight at 37°C.

Spreading Method for pure culture

It is a technique to plate a liquid sample containing bacteria so that the bacteria are easy to count and isolate.

Principle

When a diluted liquid specimen containing one or more microorganisms is spread over a suitable solid agar media, each of the viable microorganisms will multiply forming a separate colony.

These colonies can be counted and expressed in terms of the CFU/ml.

Procedure

Inside the laminar flow LB plate is opened.

1micro ltr of bacterial culture was paced using micropipette into plate.

The culture was spread using spreader.

Plates were sealed and left in incubator overnight at 37°C.

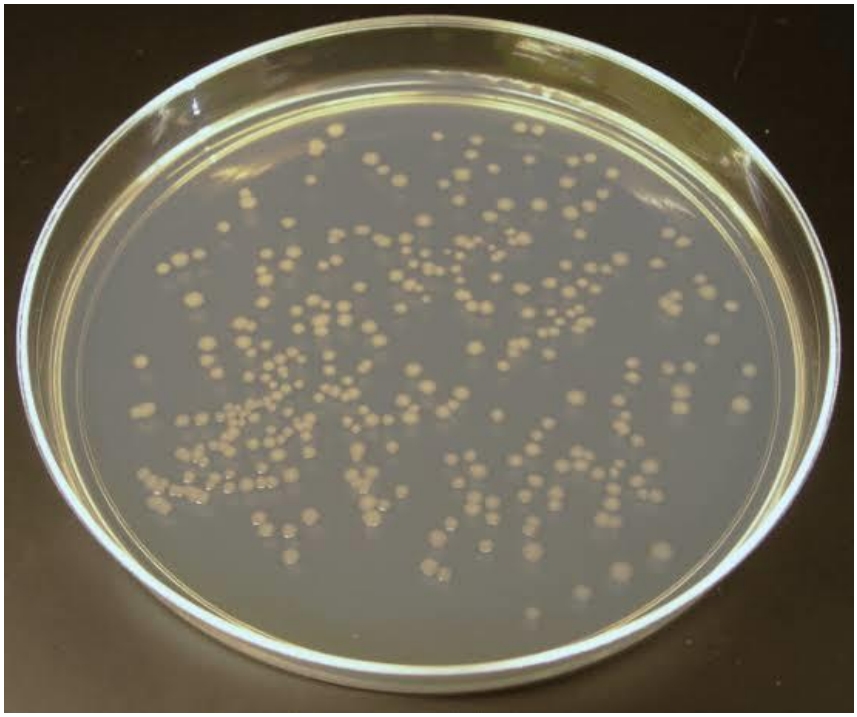


Figure : Isolated colonies of Bacteria

V. GROWTH CURVE OF BACTERIA

Growth is the orderly increase in the quantity of cellular constituents. It depends on the ability of cell to form new protoplasm from nutrients available in the environment. For unicellular organisms like bacteria growth can be measured in terms of: Change in cell mass and change in cell number

Growth Curve is a curve in graph form that shows the change in the number of bacteria (single celled organism) in an experimental culture at different times. The various phases of growth in culture medium are:

1) LAG PHASE

   It is the initial phase of bacterial growth when bacteria are transferred from one medium to another. Lag in division is associated with a physiological adaptation to the new growth environment, by the cells, prior to their resumption in division. Cells may increase in the size during the time, becoming physiologically very active and synthesizing new protoplasm, enzymes and coenzymes but simply do not undergo division. This phase is also called as Adaptation phase.

2) LOG PHASE/ EXPONENTIAL PHASE

   This follows the lag phase and bacterial cell division occurs in this phase. This is called log phase because rate of increase in cell number is a multiplicative function. We can observe the maximum growth rate in this phase. This phase is mathematically used to

   calculate the generation time of bacteria. During this phase the growth curve is straight line.

3) STATIONARY PHASE

   This is a steady state equilibrium where the rate of cell growth is exactly balanced by rate of cell death. When there is lack of nutrients in microbial culture and growth

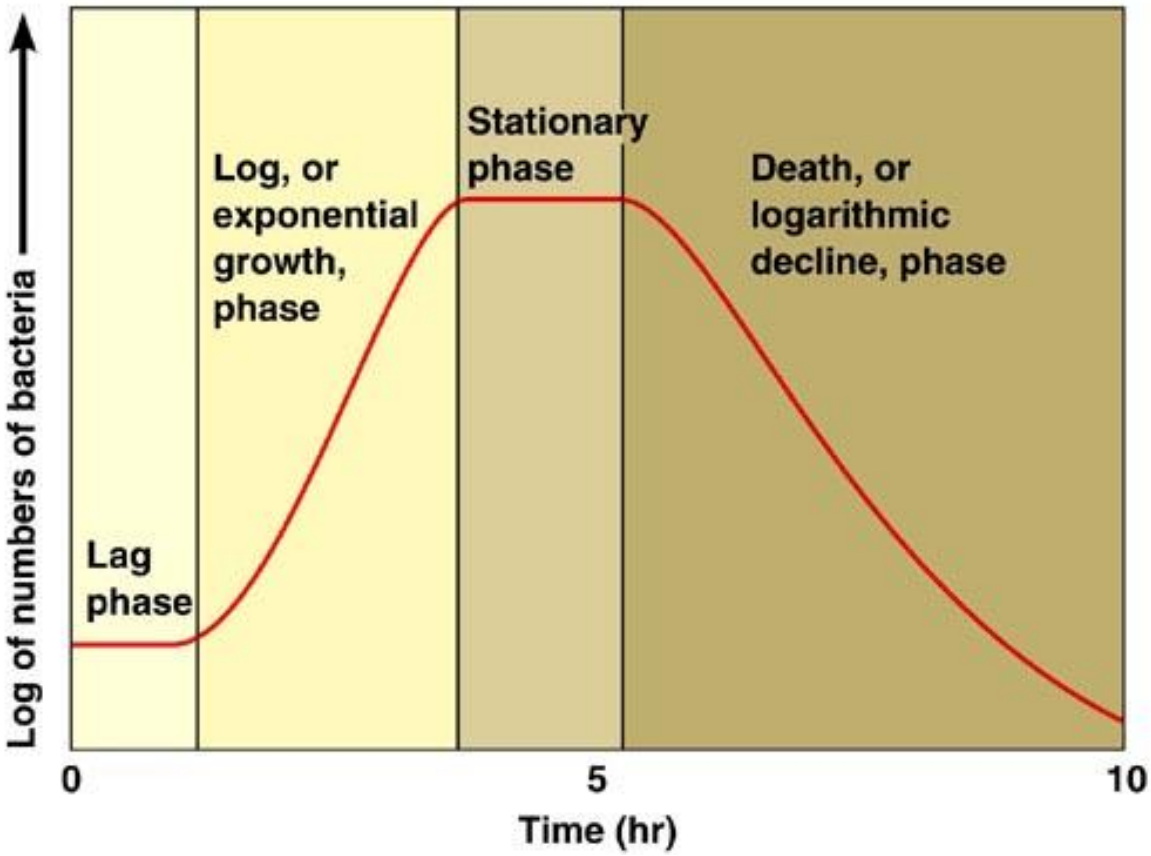
of bacteria is inhibited by waste products then the bacteria enter in stationary phase. During this phase growth curve become horizontal.

4) DEATH/ DECLINE PHASE

It follows the stationary phase because the cells cannot remain in stationary phase for a long time. In this phase cell death occur and cell is unable to resume division followed by their transfer to new environments.

Generation Time: The amount of time needs for bacteria to perform one complete cell division is called as generation time. It is calculated form the bacterial growth curve.

The growth curve of a particular species of bacteria is obtained by growing a pure culture of that bacteria in a liquid culture at a constant temperature. After that samples of bacteria are taken from that culture after fixed intervals of time and no. of bacteria is determined from it. After that the recorded data is plotted on the logarithm graph paper where logarithm of number of bacteria is plotted against time.


Log of numbers of bacteria
Lag phase
Log, or exponential growth, phase
Stationary phase
Death, or logarithmic decline, phase
0
5
10
Time (hr)

VI . ANTIBIOTICS ASSAY

The bioactivity of different antibiotics is measured using the antibiotic assay, also known as the bio potency assay. Analytical microbiology is a subfield of analysis as a whole and not just a small subfield of bacteriology. The bacterium is used as reagents and are typically the element of the processes that are most reliable. Relative tests, such as antibiotic assays, measure responses to a sample, are contrasted with answers to a standard. This relativity establishes specific if the assays are to be accurate, the system must be constrained. The necessity for the qualitative identification of standard and sample solutions and of their treatments is the main restriction. These criteria for validity state that the standard and sample must be chemically equivalent, dissolved in the same solvents (often aqueous solutions with the same pH and buffer capacity), and processed in the assay in an indistinguishable way.

The susceptibility of various substances is measured for a number of reasons, including antibiotic-resistant microbiological species. One is to gather information to direct clinical applications. Another is a tool to help choose the right assay organisms. Some bacteria are used in tube procedures, whereas others are used in diffusion experiments. *Sarcina lutea, Bacillus subtilis,* and *Staphylococcus aureus* are the most widely used test organisms for diffusion experiments. On rare occasions, *Salmonella gallinarium*, Escherichia coli, *Klebsiella pneumoniae, Photobacterium* fished, and *Pseudomonas sp*. are employed for particular antibiotics.

The assay reveals the antibiotic's effectiveness in eradicating germs, and this is done by expressing it as a ratio of the dose. Biopotency testing is essential for ensuring the quality of antibiotics. All antibiotics must pass a potency test in order to be sold.

Antibiotics assay is done generally by two methods: the cylinder-plate (or cup-plate) method and the turbidimetric (or tube assay) method. The cylinder-plate method (Method A) relies on the antibiotic diffusing from a vertical cylinder through a layer of solidified agar in a Petri dish or plate to the point where the added microorganism's growth is completely inhibited in a zone surrounding the cylinder containing the antibiotic solution. The turbidimetric method (Method B) relies on the suppression of microbial culture growth in a fluid medium that is conducive to its rapid development in the absence of the antibiotic.

By separating relatively significant sources of potential bias and error through appropriate experimental designs, microbial assays are significantly more precise. The crucial comparisons in a cylinder plate assay are only made between zone diameter measurements taken from different plates, without taking into account differences in plate preparation and handling. It is necessary to perform a turbid metric assay with greater uniformity in the environment created for the tubes through tighter thermostatic control of the incubator and to prevent systematic bias by randomly placing replicate tubes in different tube racks, each of which contains one full set of treatments, in order to ensure that the observed turbidity differences reflect variations in antibiotic concentration. The most important contrasts are then limited to correlations between the measured turgidities within racks. Two alternative designs are advised within these constraints: a 3-level (or 2-level) factorial assay, or a 1-level test with a standard curve. On the day of the assay, make solutions of 3 or 2 corresponding test dilutions for the standard and the unknowns, as indicated under the preparation of the standard and the samples. Prepare solutions of five test dilutions of the standard instead for a 1-level assay with a standard curve and a solution of one median test level for the unknown, as stated in the same sections. Consider an assay to be preliminary if, using either design, its computed potency is less than 60% or greater than 150% of the amount estimated while making the stock solution of the unknown. Repeat the assay after making the necessary adjustments to the anticipated potency. Two or more independent tests are necessary for an accurate estimation of the potency of a given assay preparation or unknown because microbial estimates of potency are prone to both intra- and inter-assay factors. On a different day, repeat the assay of the supplied unknown, starting with separately produced stock solutions and test dilutions of both the standard and unknown. Conduct one or more additional experiments if the calculated standard error shows that the estimated potency of the second assay differs significantly from the estimated potency of the first. A more accurate estimate of potency than one from a single large assay with the same total number of plates or tubes comes from the sum of a number of smaller, independent assays conducted over a number of days.

Preparation of media is required to be done to conduct the test on the inoculated organism from the ingredients: peptone, yeast extract and glucose. It is acceptable to make minor adjustments to the individual components or utilise reconstituted dehydrated media as long

as the resulting media have comparable standard curve responses and equally good or better growth-promoting qualities. The ingredients are dissolved in sufficient water to produce 1000 ml.

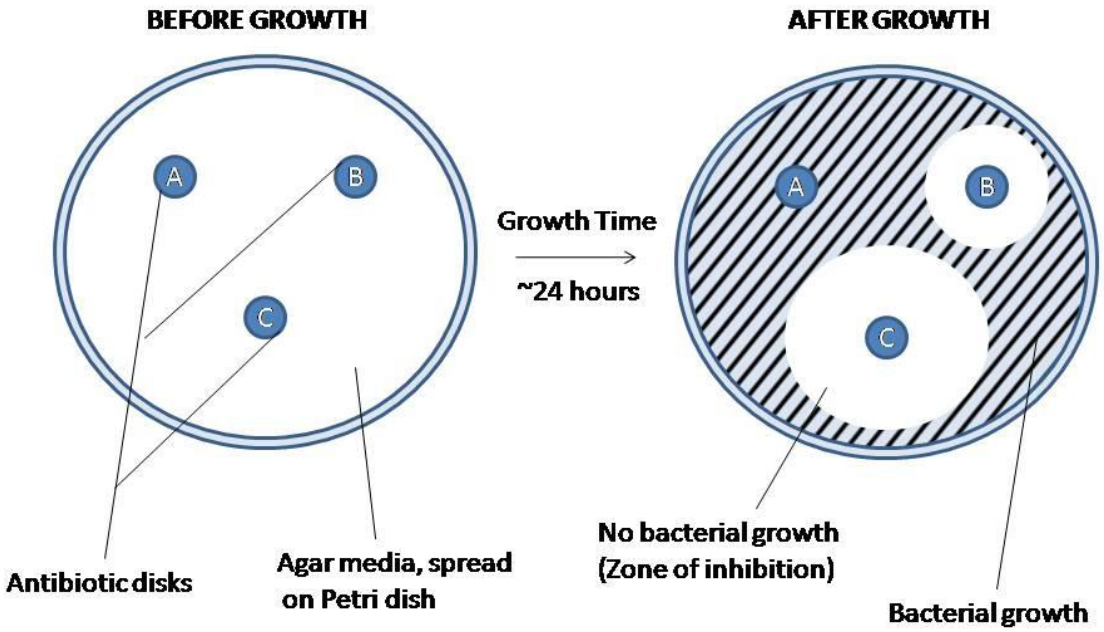
An authentic sample of the suitable antibiotic that has had its potency accurately determined by comparison to the relevant international standard is referred to as a Standard Preparation. It is possible to express the potency of the standard preparation in international units or in grams per milligram of the pure antibiotic. A working standard created by any laboratory can take the place of a standard preparation and should be compared to the standard at set intervals under different circumstances. In order to help doctors choose the best course of therapy for their patients, the Kirby-Bauer disc diffusion susceptibility test evaluates the sensitivity or resistance of pathogenic microorganisms to various antibiotic drugs. The pathogenic organism is cultured on Mueller-Hinton agar with filter paper discs that have been impregnated with antibiotics. An indirect indicator of a compound's effectiveness to suppress an organism is the presence or lack of growth surrounding the discs. This is followed by the preparation of buffer solutions and then finally assaying the organism by the virtue of following methods.

METHODS

- **Cylinder-plate or Cup-plate method**

  Based on the diffusion of the antibiotic throughout solid culture media, the Diffusion (Cylinder-Plate) technique. A solid agar growing medium containing a target bacterium is placed on top of a metal cylinder or paper disc holding an antibiotic. The minimum effective concentration can be ascertained by running this test at various concentrations. Following incubation, the target organism's growth is evaluated. An antibiotic should prevent the target organism's growth.

  In contrast to chemical assays, which merely offer a concentration, one significant benefit of microbiological potency testing is that the effect of the antibiotic is directly assessed on a living bacteria, providing a true estimate of the antibiotic action.



- **Turbid metric or Tube assay method**

Antigen concentration in a solution can be determined using turbid metric assays, which are sensitive tests. The assay principle, which is typically employed in clinical laboratories, is based on the creation of antigen-antibody complexes that scatter light in their route, with decreased light transmission being linked with higher antigen concentrations.

Hospital laboratories and laboratories offering healthcare services frequently use turbid metric assays. Similar to the ELISA technique, this assay's format uses tiny particles instead of coating the wells with capturing antibodies (latex, gold etc.). This shortens the assay time by enabling faster binding of the antibodies and antigen. By examining the amount of visible light transmission, the assay is measured. More antigen-antibody complexes will develop as the antigen concentration in the solution rises, which in turn enhances light absorption.

Preparation of media is required to be done to conduct the test on the inoculated organism from the ingredients: peptone, yeast extract and glucose. It is acceptable to make minor adjustments to the individual components or utilise reconstituted dehydrated media as long as the resulting media have comparable standard curve responses and equally good or better growth-promoting qualities. The ingredients are dissolved in sufficient water to produce 1000 ml.

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VII. PRESERVATION TECHNIQUES OF BACTERIA

Bacteria are the most widely studied organisms amongst all the microorganisms. These single celled prokaryotes are used in the Biotechnology sector for various applications benefitting the humankind. Bacterial cultures are prepared for various strains; and stock cultures are maintained in order to keep bacteria in a viable condition for further subculturing into fresh medium.

Preservation of culture aims at preserving integrity and functionality of cells, keeping the organism alive, uncontaminated and preventing any changes in its genotype and unique characteristics. Depending upon the type of bacteria and period of storage, different preservation techniques are used.

These techniques can be classified into following types on the basis of **Duration of Preservation**:

SHORT TERM STORAGE

i. Refrigeration- This technique is used for the strains which are used on a regular basis, and stores pure cultures at 0-4 °C in a refrigerator/cold room. At low temperatures, metabolic activities of bacteria significantly slow down but do not cease, thus after some time the cell dies due to nutrient shortage and waste accumulation. Therefore bacteria cannot be preserved for long here.

   DURATION – 2-3 weeks

ii. Preservation in Glycerol – Here pure cultures are grown on a solid media, culture is scrapped off and the small clumps of culture are suspended in sterile neutral glycerol. It is then stored at -20 °C.

DURATION – 12-18 months

iii. Agar Slant Culture – This is one of the simplest and most economical technique to preserve bacteria. Slants are incubated for 24 hours and then stored in a refrigerator at 4 °C. Slant surface provides greater surface area for the growth of bacteria.

DURATION – 4-6 weeks

iv. Cooked Meat Medium – This technique is mostly used for cultivation and maintenance of anaerobic bacteria. It is a liquid, non-selective medium which after incubating with the desired strain, can be stored at room temperature.

DURATION – 2 months

v. Agar Stab Culture – This technique is used mainly for preserving fastidious organisms (like *Staphylococci* and Enterobacteriaceae) at 4 °C. Here the tubes are prepared with Tryptic soy agar, bacteria is stabbed into agar and the incubated at 35°C overnight, then cork is put over and sealed by molten paraffin wax.

DURATION – 1 year

vi. Saline Suspension – Bacteria are suspended in 1% salt solution (in screw cap tubes to prevent evaporation), and tubes are stored at room temperature. Sodium chloride inhibits its growth, and the stock culture can be transferred for use whenever required.

DURATION – few days

- ❖ Heterotrophic bacteria can be preserved in dried gelatin drops or by drying on sterile silica gel granules.

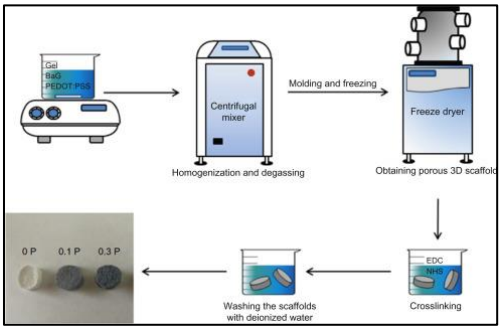
LONG TERM STORAGE

Techniques for long term storage permit intervals of months or years between subcultures, keeping cells in dormant state.

i. Freezing – Aerobes and anaerobes are stored at -70 °C by freezing technique. The low temperature drastically decreases enzymatic activity and metabolic processes of bacteria. Bacteria are revived by thawing at 37°C and bacterial stocks can remain viable for several decades.

ii. Cryopreservation – Bacteria are rapidly frozen in Liquid Nitrogen at -196 °C, in the presence of stabilizing agents (like Glycerol) to prevent cell damage by ice crystals formation. It promotes cell survival and is useful to keep various species viable for 10-30 years.

iii. Lyophilization – Also known as Freeze-drying, this is the most effective technique for long term bacterial preservation. It removes water and other solvents from the frozen products via sublimation, thus arresting microbes' biological processes and transforming cells to dry dormant pellet stage. Culture viable for more than 30 years.



Lyophilization

iv. Paraffin technique – This is a simple and economical technique involving preservation by overlaying culture medium with mineral oil. Sterile liquid paraffin is poured over the culture slant, providing anaerobic conditions and preventing medium's dehydration. Bacteria can remain viable for long duration (months to years).

v. Microencapsulation – Herein the bacterial cells are trapped in a matrix which shields cells and increases its stability during storage.

vi. Electrospinning and Electrospraying – In electrospinning the bacterial strains are trapped in nanofibers while in electrospraying bacteria are trapped in droplets. This technique preserves viability of Sensitive probiotic bacteria.

The aforementioned preservation techniques of Bacteria are practiced in microbiology laboratories to store variety of bacterial strains for future use, along with ensuring their overall safety and reducing time, energy and material costs of their maintenance.

(End of Report)