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A BOOK ON EXPERIMENTS OF MICROBIOLOGY

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MEDIA PREPARATION

Experiment 1

Aim- To perform serial dilution for isolation of bacterial colonies

Quantitative analysis of bacterial population is necessary for successful isolation, identification and purification of the single colony. Analysis of concentration of particular colony is also necessary to estimate the bacterial population in any food and drug sample which indicate the pathogenic nature of given sample. For this purpose, microbiologists have employed serial dilution technique analyze the concentration of bacterial population in clinical, food, pharmaceutical, industrial and research samples.

Principle

Serial dilution is a systematic reduction of bacterial population from the original master sample into fixed volume. The master sample is diluted sequentially to get a particular systematic manner of microbial population. In the process of serial dilution, the master sample is first re-suspended as blank in water or saline solution and sometimes any growth medium. In serial dilution, the starting volume is diluted in an ordered reduced manner. As the sample is diluted more, the microbial population may also reduce accordingly. For example, in a stock solution of *E.coli,* 1 ml is taken and may be transferred to another 90 ml tube containing water making a dilution of $10^{-1.}$ Repeating this process by taking 1 ml of solution and adding to next 9 ml water solution is known as serial dilution process. In this example the dilution factor is 10 in each steps.

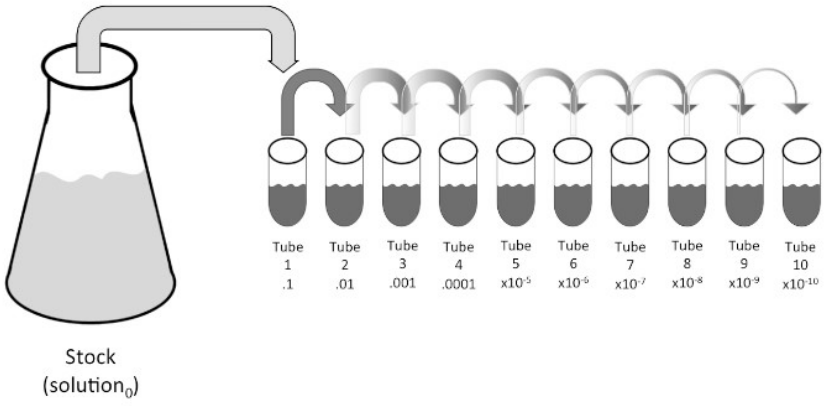


Figure 1: Serial dilution of a stock solution. 1 mL aliquot of the stock solution (In flask) is added to tube 1 which contains 9 mL distilled water, making the concentration to 1/10,

from tube 1, 1.0 mL of sample is taken and transferred tube 2 containing 9 mLdistilled water making the dilution 1/100. The process is repeated continuously to dilute the sample in sequence.

Materials Required

Test tube, Distilled water or any diluent, Sample, Pipette

Procedure

1. Prepare a set of ten test tube with 20 mL capacity and label them T1- T-10.
2. Pipette 9 mL of diluent (water or saline) into each of the 10 test tubes (These 10 tubes can be autoclaved if they are not previously autoclaved).
3. Now take the sample in flask and shake it vigorously.
4. Pipette out 1 mL of sample solution from flask and transfer into test tube labeled T1.
5. Take 1 mL of sample from test tube T1 and add it to test tube T2. Mix well.
6. Take 1 mL of sample from test tube T2 and add it to test tube T3. Mix well.
7. Repeat the step 5 and 6 until transfer into tube T10.
8. The sample present in the tube T1 to T10 can be transferred into nutrient media for the quantitative analysis of bacterial population. This serial dilution preparation can also be used for the purification of single colony from the mixed microbial population.

Experiment 2

AIM: To prepare Nutrient Agar media

A **growth medium** or **culture medium** is a solid, liquid or semi-solid chemical composition prepared to support the growth of microorganisms. Bacteria and fungi are grown on different types of nutrient media. The liquid culture media are termed as broth and solid media are known as agar base media. The selection of depend on microorganism that one is trying to isolate or identify. As humans, micro-organisms also have some nutrient requirements for their growth and proliferation. Different types of nutrients may be added to the growth medium such as carbohydrate source, organic matter source and proteins. Salt solution are also added to make the salt balance in the medium. Various pH indicators are often added for differentiation of microbes based on their biochemical reactions.

Nutrient Agar is a general purpose, nutrient medium used to support the growth of various types of bacteria. Nutrient agar is popular because it is available easily and is a low cost media, and contains many nutrients needed for the bacterial growth.

Materials and methods

A. Media composition for 1000 mL

Peptone – 5 gm

Beef extract – 3 gm

Agar – 15 gm

Distilled water – 1000 mL

B. Glassware and Instruments- Conical flask, Petri dishes, measuring ylinder, autoclave and Laminar air flow

Procedure

1. Weigh peptone, beef extract and agar and take in a flask. Add distilled water to it.
2. Now mix all the contents by gentle heating while stirring until all the contents dissolve.
3. Autoclave the dissolved mixture at 121 degrees Celsius for 15 minutes.
4. Once the nutrient agar has been autoclaved, transfer the media to Laminar Air flow. (The laminar Air flow must be cleaned using 70% ethanol before use).
5. Pour nutrient agar into each plate and leave plates on the sterile surface until the agar has solidified.
6. Replace the lid of each Petri dish and store the plates in a refrigerator.

Experiment 3

AIM: To prepare Potato Dextrose Agar media

Potato Dextrose Agar (PDA) is used for the growth of fungi. It is a general purpose medium for the growth and isolation purposes of yeasts and molds. It can also be supplemented with antibiotic to inhibit bacterial growth. Potato Dextrose Agar (PDA) is recommended for plate count methods for foods, dairy products and testing cosmetics. Potato infusion is used as nutrient to provide growth, sporulation and production spores in some fungi like dermatophytes.

Principle

Potato Dextrose Agar contains dehydrated Potato Infusion and Dextrose as its composition, that support fungal growth and sporulation. Agar is added as the solidifying agent. To inhibit the bacterial growth, a specified amount of sterile tartaric acid (10%) is added to medium to lower the pH of this medium. Chloramphenicol acts as a selective agent to inhibit bacterial overgrowth of competing microorganisms from mixed specimens, while permitting the selective isolation of fungi.

Materials and methods

A. Media composition for 1000 mL

Potato infusion	200 gm
Dextrose	20 gm
Agar	20 gm
Distilled water	1000 mL

(Chloremphenicol 25 mg/l or Tartaric acid 1.4 gm/l can also be added for antibacterial activity)

Note: 200 gm of potato infusion is equivalent to 4.0 gm of potato extract.

B. Glassware and Instruments- Conical flask, Petri dishes, measuring cylinder, autoclave and Laminar air flow

Procedure

1. To prepare potato infusion, boil 200 g sliced, unpeeled potatoes in 1 liter distilled water for 30 min.
2. Filter through cheesecloth, saving effluent, which is potato infusion (or use commercial dehydrated form).

3. Add Dextrose, Agar and Water to potato infusion and heat gently to dissolve the contents completely.
4. Sterilize media by autoclaving at 121ºC for 15 minutes.
5. Once the media has been autoclaved, transfer the media to Laminar Air flow. (The laminar Air flow must be cleaned using 70% ethanol before use).
6. Pour the media into each plate and leave plates on the sterile surface until the agar has solidified.
7. Replace the lid of each Petri dish and store the plates in a refrigerator.

Experiment 4

Aim: **Inoculation method for bacterial population**

For inoculation purposes, inoculating loop are used. Wire loops are sterilized using red heat in a Bunsen burner flame before and after use. They must be heated to red hot to make sure that any contaminating bacterial spores are destroyed. The handle of the wire loop is like a pen for holding it.

The common plating techniques employed in microbiology are Streak Plate Method, Spread Plate Method and Pour Plate Method.

1) Streak Plate Method

Streak Plate Method is employed for the isolation of bacteria in pure culture. In this technique, a sterilized inoculating loop or transfer needle is dipped into bacterial culture which is then streaked on the surface of an already solidified agar plate to make a series of parallel, non-overlapping streaks. The process is known as streaking and the plate so prepared is called a streak plate. The main objective of the streak plate method is to separate the colonies of bacteria from concentrated suspensions of cells. For inoculation, inoculating needle with a loop made up of either platinum or nichrome wire, is used for streaking.

One loopful of specimen is taken from the bacterial culture.The culture is transferred onto the surface of the agar plate in a sterile petridish and streaked across the surface in the form of a zig-zag line. This process is repeated thrice to streak out the bacteria on the agar surfacefor the separation ofindividual bacteria colony. After incubation of 24 hours, the first streak will contain a thick layer of microorganisms than the second streaking. Similarly, the second streaking should be more thick than the third and so on. The last streaks should thin out the culture sufficiently to give pure isolate colonies.

2) Spread Plate Method

The spread plate technique is used for the inoculation of a diluted microorganisms in liquid form so that individual colonies can be isolated. In this technique, a small volume of dilute microbial mixture is transferred to the center of an agar media plate and spread evenly over the surface with a sterile L-shaped bent glass rod. The Petri dishes may also be shaken slowly for uniform distribution of the microbial mixture.After inoculation, the media plates are incubated at 37ºC for 24 hours, in the inverted position. The dispersed cells will develop into isolated colonies. In the spread plate method, distribution of microbial population is equal on agar surface, so the bacterial colonies can be counted and the colony forming unit can be calculated. Spread plate technique is mostly used for the isolation of microorganisms from different sources.

3) Pour Plate Method

Pour plate method utilize the use of serial dilution method for the isolation and quantitative estimation of bacterial population. In pour plate method, diluted inoculum is added into sterile Petri dishes followed by melted agar medium. The plates are shaken slowly to mix the agar medium and inoculum and allowed to solidify. After incubation, the plates are incubated for at 37ºC for 24 hours, in the inverted position.

After incubation, the plates are examined for the presence of individual colonies. The pure colonies may be isolated and transferred into test tube culture media for making pure cultures. This technique is employed to estimate the viable bacterial count in a suspension.

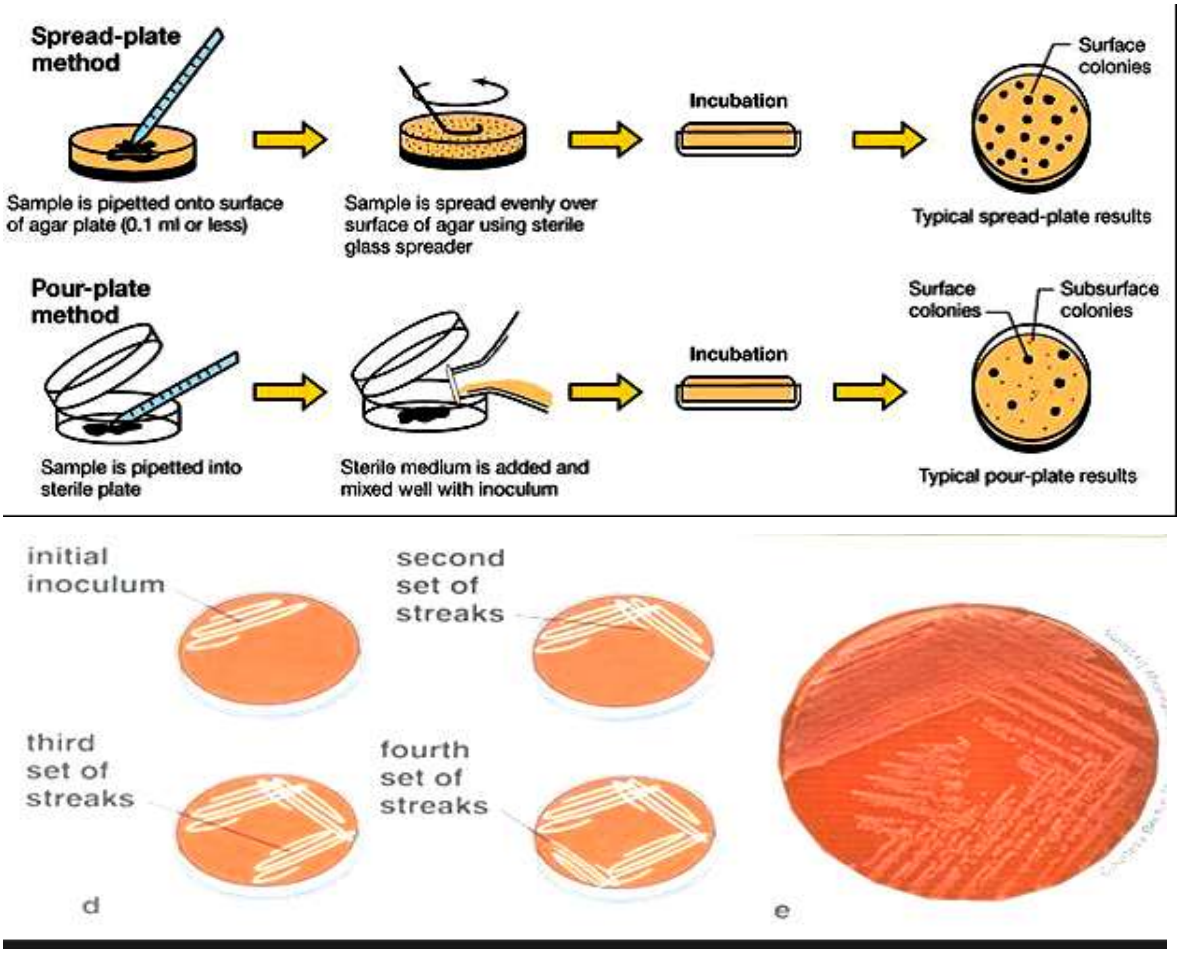


Figure:Plating Techniques

Experiment 5

Aim: **Preparation of bacterial smear for identification purposes**

Principle

Smear is a thin layer of any bacterial sample on a glass slide. A smear is prepared for identification of micro-organisms using a microscope. Smear of microbial specie is necessary to prepare because a thick layer of microbial colony on a glass slide may generate problem in identification of single type of specie because a single drop of culture may contain millions of microbial populations. So the smear is prepared along with serial dilution technique for the identification of purified and single type of microbial colony. Smear of bacteria, fungi or yeast is made on a microscope slide, fixed, stained, dried and examined with the aid of a microsope. Aseptic technique must be observed when taking samples of a culture for making a smear. Culture prepared from agar medium is much preferable to a liquid culture for making a smear. Smear makes ease in understanding shape and arrangement of cells to be clearly seen and ensures that the staining procedureis applied uniformly. There are two broad types of staining method:

Materials required

Microbial culture, glass slide, sterile water, inoculating loop, Bunsen burner

Procedure

1. Clean a plain microscope slide with 95% ethanol using lens tissue.
2. Label microscope slide with a marker pen with culture name.
3. Flame a wire loop to ensure that no culture remains from a previous experiment.
4. Transfer one or two loopfuls of sterile water on to the centre of the slide.
5. Flame loop and allow to cool.
6. Using aseptic technique, transfer a very small part of a single colony from a plate or slope of agar medium into the sterile water droplet on slide.
7. Spread the culture evenly over the slide using another slide as a smear.
8. Dry the suspension by warming gently a few times over a Bunsen burner flame for fixation of the suspension (this step is known as heat fixation and is not required for some heat sensitive micro-organisms).

EXPERIMENT 6

Aim: **Bacterial Identification by simple stainingprocedure**

Principle

In simple staining, bacterial smear is stained with a single type of staining reagent, which produces a distinctive contrast between the organism and its background. A simple stain that stains the bacteria is the direct stain. The purpose of simple staining technique is to determine cell shape, size arrangement of bacterial cells. In simple staining, basic stains are used which different exposure time (Crystal Violet 20-60 s, Carbol fuschin 15-30 s and Methylene blue 1-2 minutes).

Materials required

Bacterial culture, glass slide, inoculating loop, Bunsen burner, basic stain (any)

Procedure

1. Clean glass slide was taken and was washed and dried.
2. Bacterial smears were prepared from the bacterial cultures.
3. Put the slide with the fixed smear uppermost on a staining rack over a sink or staining tray.
4. Add few drops of stain and leave for 30 seconds. Pour off extra stain.
5. Hold the slide with forceps at a 45°angle over the sink wash the slide gently under slow running tap water.
6. Dry the slide using blotting paper.
7. Examine the slide under 10X, 45X and oil immersions objects respectively.

Observation

Mention the colour and shape of the colony appeared on microscope.

Experiment 7

Aim: Bacterial Identification by Gram's staining

Principle

The Gram stain, a differential stain was developed by Hans Christian Gram, a Danish physician, in 1884. Differential staining requires the use of at least 3 chemical staining reagents that are applied sequentially to a heat fixed smear. Its function is to impart its colour to all cells. The function of each chemical reagent may be different. They may provide colour different species. For example, the first reagent may give a colour contrast, the second reagent may be used is the decolorizing agent. The decolorizing effect of decolorizing agent may be positive or negative and depend on the chemical composition of cellular components. The final reagent is the counter stain. After discoloration, when the primary stain is washed, the counter stain can provide its colour to cells. In this way, cell type or their structure can be distinguished from each other. But if the primary stain is not washed out, the counter stain cannot be absorbed by the cells and cannot take the counterstain colour. On the basis of the colour retained by bacterial cell, the identification is confirmed by observing under microscope.

Gram staining classifies bacteria into 2 major groups, Gram positive and Gram negative bacteria. The Gram stain reaction is based on the chemical and physical composition of bacterial cell wall. Gram positive cells have a thick peptidoglycan layer, whereas peptidoglycan layer in Gram negative cells is much thinner and surrounded by outer lipid containing layer.

In Gram negative, the higher amount of lipid in the formation of large pores thus facilitating the leakage of crystal violet-iodine complex and resulting in the discoloration of the bacterium which later takes this complex counter stain. In contrast, the Gram positive cell wall are thick and composed mainly of proteins and cross linked mucopeptide complex, when treated with alcohol it causes dehydration and closure of the cell wall pores thereby not allowing the loss of complex and cell retains primary stain. The bacteria which retain the primary stain appear dark blue or violet are called Gram positive, where as those that lose the crystal violet and used counter stain, saffranin appear pink are called as Gram negative.

The Gram stain uses different reagents in the order, crystal violet, iodine solution, alcohol and saffranin.

Procedure

1. Prepare thin smear was prepared of the given bacterial species on a clean glass slide.
2. Let the smear dry.
3. Heat fixed smear.
4. Put the slide with the fixed smear uppermost on a staining rack over a sink or staining tray
5. Cover the smear with crystal violet for 1 minute.

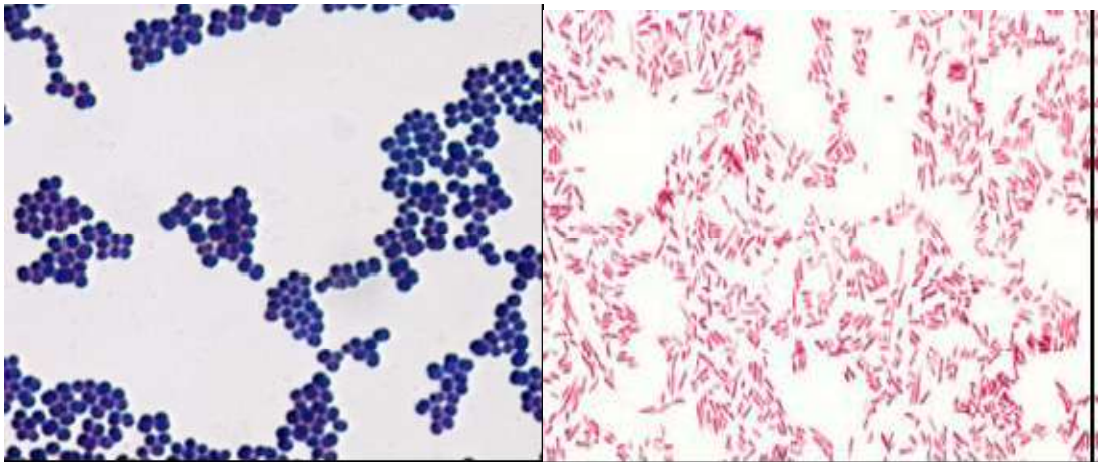
6. Washthe slide with distilled water for few seconds using wash bottles.
7. Cover the smear with Gram's iodine solution for 1 minute.
8. Wash the slide with distilled water using wash bottles.
9. Decolorized with 95% decolorizing agent.
10. Wash the slide with distilled water and drained.
11. Cover the slide with counter stain i.e. saffranin for 30 seconds.
12. Wash with distilled water and blot dried with absorbent paper.
13. Dry the stained slides and observe under the microscope.

Observation

Examine the slides microscopically using oil immersion objective.

Identify the Gram reaction of the given cultures. Observe the colour, morphology and arrangement of cells.

Those bacteria that appear blue are referred to as Gram positive and these appearing pink are described as Gram negative.



Experiment 8

Aim Hanging-Drop and Wet-Mount Preparations for bacterial identification

Procedure

a. Take a cover glass and clean it thoroughly, making certain it is free of grease (the drop to be placed on it will not hang from a greasy surface). It may be dipped in alcohol and polished dry with tissue, or washed in soap and water, rinsed completely and wiped dry.

b. Take one hollow-ground slide and clean the well with a piece of dry tissue. Place a thin film of petroleum jelly around (not in) the concave well on the slide.

c. Gently shake the broth culture of Proteus until it is evenly suspended. Using good aseptic technique, sterilize the wire loop, remove the cap of the tube, and take up a loopful of culture. Be certain the loop has cooled to room temperature before inserting it into the broth or it may cause the broth to "sputter" and create a dangerous aerosol. Close and return the tube to the rack.

d. Place the loopful of culture in the center of the cover glass (do not spread it around). Sterilize the loop and put it down.

e. Hold the hollow-ground slide inverted with the well down over the cover glass, and then press it down gently so that the petroleum jelly adheres to the cover glass. Now turn the slide over. You should have a sealed wet mount, with the drop of culture hanging in the well.

f. Place the slide on the microscope stage, cover glass up. Start your examination with the low-power objective to find the focus. It is helpful to focus first on one edge of the drop, which will appear as a dark line. The light should be reduced with the iris diaphragm and, if necessary, by lowering the condenser. You should be able to focus easily on the yeast cells in the suspension. If you have trouble with the focus, ask the instructor for help.

g. Continue your examination with the high-dry and oil-immersion objectives (be very careful not to break the cover glass with the latter). Although the yeast cells will be obvious because of their larger size, look around them to observe the bacterial cells.

h. Make a hanging-drop preparation of the Staphylococcus culture following the same procedures just described.

i. Record your observations of the size, shape, cell groups, and motility of the two bacterial organisms in comparison to the yeast cells.

j. Discard your slides in a container with disinfectant solution.

Experiment 9

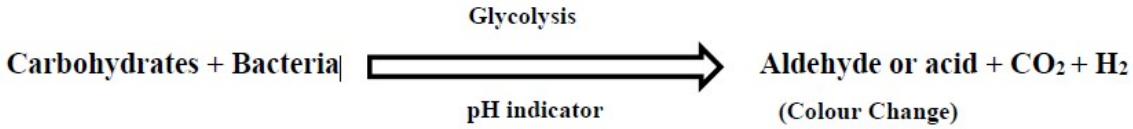
Aim

To determine the ability of microorganisms to ferment carbohydrate with acid and gas production

Principle

The carbohydrate fermentation test is used to determine the ability of some bacteria to ferment a specific carbohydrate. Carbohydrate fermentation test is useful in differentiating among bacterial groups or species. Some micro-organisms use carbohydrate differently depending on their enzymes components. In fermentation, substrate and alcohols undergo anaerobic dissimilation and produce organic acids like lactic acid, formic acid and acetic acid. The pH indicator Phenol Red, Andrade's solution, bromcresol purple (BCP) and bromothymol blue (BTB) are used to detect the production of acid. The production of the acid lowers the pH of the test medium, which is detected by the color change of the pH indicator. This indicates a positive reaction.Small inverted tubes called Durham tube is also immersed in the medium to test for the production of the gas (hydrogen or carbon dioxide). Durham tubes are inserted upside down in the test tubes to detect gas production. If the test organisms produce gas, the gas displaces the media present inside the tube and gets trapped producing a visible air bubble.

pH indicator	**Media characteristic**		**Acid production**		**Negative result**	
	pH	**Color**	**pH**	**Color**	**pH**	**Color**
Andrade's solution	7.1-7.2	Light pink	5.0	Pink red	12.0-14.0	Yellow, colorless
Bromcresol purple	7.4	Deep purple	5.2	Yellow	6.8	Purple
Bromothymol blue	7.0	Green	6.0	Yellow	7.6	Deep Prussian blue
Phenol red	7.4	Raddish orange	6.8	Yellow	8.4	Pink red



Materials Required

Test Tube, Durham's Tube, Phenol Red Indicator, Sugar (Glucose, Lactose, Sucrose), Bacterial culture, Inoculation loop

Fermentation Broth media composition:

1. Trypticase or protease peptone No. 3 — 10 g
2. Sodium Chloride (NaCl) — 5 g
3. Beef extract (optional) — 1 g
4. Phenol red (7.2 ml of 0.25% phenol red solution) — 0.018 g
5. Carbohydrate source — 10 g

Procedure:

I. Preparation of Carbohydrate Fermentation Broth

1. Weigh and dissolve trypticase, Sodium chloride, beef extract (optional) and Phenol red in 1000 ml distilled water and transfer into conical flasks. Heat gently to dissolve the contents.
2. Add 0.5% to 1% of desired carbohydrate into all flasks.
3. Insert Durham tubes in inverted positon into all tubes, the Durham tubes should be fully filled with broth.
   - Autoclave the prepared test media at 121°C for 15 minutes (*When using arabinose, lactose, maltose, salicin, sucrose, trehalose, or xylose as carbohydrate source, autoclave at 121°C for only 3 minutes as these carbohydrates are subject to breakdown by autoclaving*)
4. Transfer the sugar into screw capped tubes or fermentation tubes and label properly.

II. Inoculation of Bacterial Culture

1. Aseptically inoculate each labeled carbohydrate broth with test bacterial culture using an inoculating loop. One control should be kept in which inoculation is not done.
2. Incubate the tubes at 18-24 hours at 37°C.
3. Observe the reaction.

Observations and results

1. **Acid production:**
    1. **Positive:** After incubation the media turns yellow as indication of colour change by phenol red indicator. Fermentation of the carbohydrate lead to acid production which drop media Ph.
    2. **Negative:** The tube containing medium will remain red, indicating the bacteria cannot ferment that particular carbohydrate source present in the media.

2. **Gas Production**
    1. **Positive:** Bubble formation in the Durham tube indicate the gas production due to fermentation which indicate positive result.

    2. **Negative:** No bubble in the inverted Durham tube indicate that bacteria do not produce gas from the fermentation of that particular carbohydrate present in the media.

Experiment 10

Aim
To determine the oxidation fermentation characteristics of microorganisms

Principle
This method depends upon the use of semisolid tube medium containing the carbohydrate (Glucose) together with a pH indicator. The acid is produced only at the surface of medium where conditions are aerobic the attack on the medium where conditions are aerobic the attack on the sugar is oxidative. If acid is produced throughout the medium including lower layers and where the conditions are aerobic breakdown is fermentative.
Fermenting organism (*Enterobacteriaceace, Vibrio*) produce an acidic reaction throughout the medium in the covered (anaerobic) as well as open (aerobic) tube. Oxidizing organisms (*Pseudomonas)* produce an acidic reaction only in the open tube. Organisms that cannot breakdown carbohydrate aerobically/anaerobically (alkali genes faecalis) produce an alkaline reaction in the open tube and no change in the covered tube. This medium may be used for detecting gas production and motility.

Materials Required
Bacterial broth culture, D-F medium, liquid paraffinaol

Procedure
1. Using sterile technique, two tubes of medium were inoculated by stabbing with sterile urine.
2. Two inoculated tubes were used as control.
3. Liquid paraffin was poured over the medium to form a layer about 1cm in depth into one of the tube of each pair.
4. The tubes were incubated at 37°C for 24-48hrs was observed.

Observation
The tubes were observed for the colour of the medium and the type of metabolism was recorded

Experiment 11

AIM- To perform Indole Production Test for identification of bacterria

Aim

To determine the ability of microorganisms to decompose the amino acid tryptophan to indole

Principle

Tryptophan an essential amino acid oxidized by some bacteria by the enzyme *tryptophanase* resulting in the formation of indole, pyruvic acid and ammonia. In this experiment, the medium contains the substrate tryptophan which is utilized by the microorganisms.

This ability to hydrolyse tryptophan with the production of indole is not a characteristic of all microorganisms and therefore serves as a biochemical mask. The presence of indole is detected by adding Kovac's reagent, which produces a cherry red reagent layers. This colour is produced by the reagent which is composed of Paradimethyl aminobenzaldehyde yielding the cherry red colour

Indole Reaction with Kovac's Reagent

Culture producing a red reagent layers following addition of the Kovac's reagent are indole positive. The absence of red colouration demonstrates that the substrate tryptophan was not hydrolyzed and indicating indole negative reaction.

Another reagent used is Ehrlisch's reagent. It's believed to be more sensitive than Kovac's reagent and is recommend for the detection of indole production by anaerobic and non-fermentative Gram negative organism Kovac's reagent was used usually initially to classify the members of *Enterobacteriaceace* family.

Materials Required

15 ml test tubes, bacterial culture, peptone water, Kovac's reagent

Procedure

1. The peptone water tubes were inoculated with bacterial broth culture using sterile needle technique.
2. An uninoculated tube was kept as control.
3. Both tubes were incubated at 37°C for 24-48 hours.
4. After proper incubation, 1 ml of Kovac's reagent was added to both tubes including the control.
5. The tubes were shaken gently after an interval for 10 – 15 minutes.

Observation

The tubes were observed for the colour in the top reagent layer.

Note

Development of cherry red colour in the top layer of the tube is a positive test. Absence of red colouration is indole negative.

Examples

Positive: *E. coli, Proteus vulgaris*

Negative: *Klebsiella sp., Proteus mirabilis*

Experiment 12

Aim

To determine the ability of microorganism to oxidize glucose with the production and stabilization of high concentrations of acid end products

Principle

All enteric organisms oxidize glucose for energy production and the end products of this process will vary depending on the specific enzymatic pathway present in the bacteria. In this test, the pH indicator methyl red detects the presence of large concentrations of acidic red detects the presence of large concentrations of acidic products. The test can be used in differentiating *Escherichia coli* and *Enterobacter aerogenes* (both coliform bacteria) that are used as indicator of the sanitary quality of water, foods etc.

Both of these organisms initially produce organic acid end products during the early incubation period. The low acid end products produce acidic pH (4) which is stabilized and maintained by *E. coli* at the end of incubation. During the later incubation period *Enterobacter aerogenes* enzymatically converts these acids into nonacid end products such as 2,3 butanedial and acetyl methyl carbinol (pH 6).

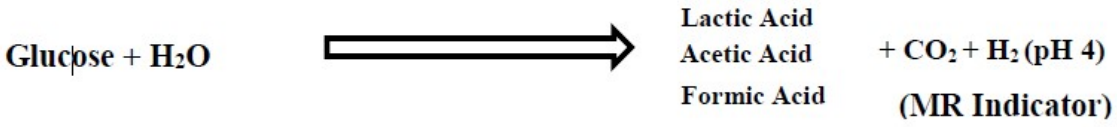


Figure. Methyl Red Test

At a pH of 4, Methyl red indicator will turn red throughout the tube, which is indicating of a positive test. At pH 6, still indicating the presence of acid but with a lower hydrogen ion concentration, the indicators turn Yellow, which is indicating the negative test.

Materials Required

MR broth, 24 hours broth cultures, Methyl red indicator, inoculating loop

Procedure

1. Using sterile technique experimental organisms were inoculated into appropriately labeled tubes containing MR broth by means of loop inoculation.
2. Uninoculated tube was kept as control
3. Both tubes were incubated at 37°C for 24-48 hours.
4. After proper incubation 5 drops of MR indicator was added to both tubes including control.
5. It was mixed well and colour was observed.

Observation

The tubes were observed for changes in the colour of Methyl Red.

Interpretation

The colour of MR reagents remaining red is a positive test and the colour turning to yellow is negative.

Experiment 13

Aim: Lipid Hydrolysis Test

INTRODUCTION

Lipids are high-molecular-weight compounds possessing large amounts of energy. Once assimilated into the cell, they are metabolized through aerobic respiration to produce cellular energy, adenosine triphosphate (ATP). The components may also enter other metabolic pathways for the synthesis of other cellular protoplasmic requirements.

However, before their assimilation by bacteria, they need to be degraded. The degradation of lipids such as triglycerides is accomplished by extracellular hydrolyzing enzymes, called lipases (esterases), that cleave the ester bonds in this molecule by the addition of water to form the building blocks glycerol (an alcohol) and fatty acids.

Objectives

1. To determine the ability of the organism to hydrolyse lipid.
2. To identify bacteria capable of producing the exoenzyme lipase.

Principle

In this experimental procedure, tributyrin agar is used to demonstrate the hydrolytic activities of the exoenzyme lipase. The medium is composed of nutrient agar supplemented with the triglyceride tributyrin as the lipid substrate. Tributyrin forms an emulsion when dispersed in the agar, producing an opaque medium that is necessary for observing exo- enzymatic activity. Following inoculation and incubation of the agar plate cultures, organisms excreting lipase will show a zone of lipolysis, which is demonstrated by a clear area surrounding the bacterial growth. This loss of opacity is the result of the hydrolytic reaction yielding soluble glycerol and fatty acids and represents a positive reaction for lipid hydrolysis. In the absence of lipolytic enzymes, the medium retains its opacity. This is a negative reaction.

Media:

Media:

Tributyrin agar:

Peptic digest of animal tissue	5.0 gm/L
Yeast extract	3.0 gm/L
Agar	15.0 gm/L
Final pH (at 25°C)	7.5±0.2

Method

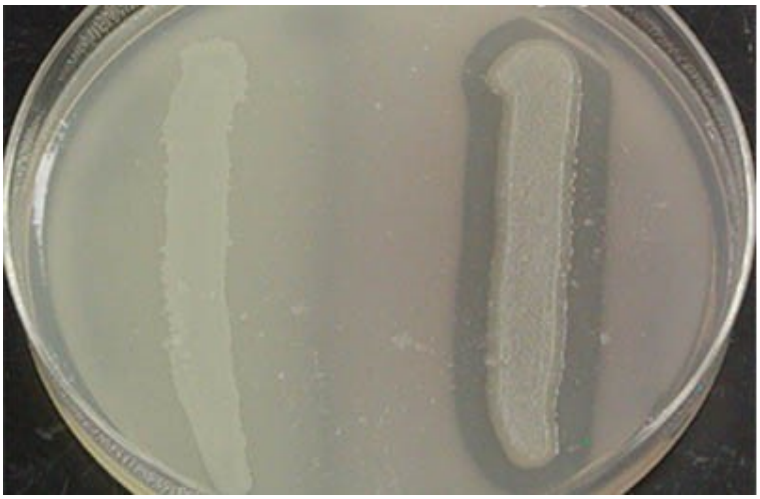
1. Prepare the tributyrin agar medium by mixing all the composition. Autoclave the media in Petri plates.

2. Inoculate the tributyrin agar medium with single line streaking of organism.

3. Incubate anaerobically in a gas pak jar immediately after streaking and transfer into the incubator maintained at 35-37^{o}C for 24-48 hours for anaerobes and for aerobes incubate the plate at 35-37^{o} C for 24-48 hours.

4. Examine the tributyrin agar plate cultures for the presence or absence of a clear area, or zone of lipolysis, surrounding the growth of each of the organisms.

Expected Results



Tributyrin agar plate. No Lipid hydrolysis on left; lipid hydrolysis on right.

- **Positive test:** Formation of a clear zone around the bacterial growth
- **Negative test:**No clear zone around growth.

Uses

- Helpful in identifying bacteria that secrete lipase, including members of Enterobacteriaceae, *Fusobacterium, Propionibacterium, Clostridium, Pseudomonas, Mycoplasma, Corynebacterium,* and *Staphylococcus*.
- For the detection and enumeration of lipolytic microorganisms in food and other material.

Limitations

- It is recommended that biochemical, immunological, molecular, or mass spectrometry testing be performed on colonies from pure culture for complete identification.

Experiment14

Objectivie: To count the bacterial number in a given food sample

Principle:

Many studies require the quantitative determination of bacterial populations. The two most widely used methods for determining bacterial numbers are the **standard**, or **viable**, **plate count method** and **spectrophotometric (turbidimetric)** analysis. Although the two methods are somewhat similar in the results they yield, there are distinct differences. For example, the standard plate count method is an indirect measurement of cell density and reveals information related only to live bacteria. The spectrophotometric analysis is based on turbidity and indirectly measures all bacteria (cell biomass), dead and alive.

The standard plate count method consists of diluting a sample with sterile saline or phosphate buffer diluent until the bacteria are dilute enough to count accurately. That is, the final plates in the series should have between 30 and 300 colonies. Fewer than 30 colonies are not acceptable for statistical reasons (too few may not be representative of the sample), and more than 300 colonies on a plate are likely to produce colonies too close to each other to be distinguished as distinct **colony-forming units (CFUs)**. The assumption is that each viable bacterial cell is separate from all others and will develop into a single discrete colony (CFU). Thus, the number of colonies should give the number of bacteria that can grow under the incubation conditions employed. A wide series of dilutions (e.g., 10^{-4} to 10^{-10}) is normally plated because the exact number of bacteria is usually unknown. Greater accuracy is achieved by plating duplicates or triplicates of each dilution, although we will not be doing that in this exercise.

Increased turbidity in a culture is another index of bacterial growth and cell numbers (biomass). By using a spectrophotometer, the amount of transmitted light decreases as the cell population increases. The transmitted light is converted to electrical energy, and this is indicated on a galvanometer. The reading, called **absorbance or optical density**, indirectly reflects the number of bacteria. This method is faster than the standard plate count but is limited because sensitivity is restricted to bacterial suspensions of 10^7cells or greater. **The procedure for the spectrophotometer use is at the end of this exercise**.

Why Is *E. coli* used in this exercise? When working with large numbers and a short time frame, one of the most reliable microorganisms is one that has been used in previous experiments, namely, *Escherichia coli*. *E. coli* has a generation time at 37ºC of 20 minutes. Thus, it reproduces very rapidly and is easy to quantify (i.e., the number (biomass) of viable *E. coli* cells in a bacterial culture can be easily determined by spectrophotometry).

MATERIALS NEEDED

- 24-hour 10ml nutrient broth culture of *Escherichia coli*
- 4 sterile 99ml saline bottles
- 1ml and 5ml pipets with pi-pumps (green for 5ml, blue for 1ml)
- 6 petri plates
- 6 agar pour tubes of nutrient agar (plate count agar)
- 48ºC to 50ºC water bath
- 6 micro-cuvettes and rack
- 1 SpectroVis micro-cuvette holder
- computer
- 4 tubes of 5ml nutrient broths

THE PROCEDURES

1. **Label** the bottom of **six petri plates 1-6**. Label four tubes of saline 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8}.
2. Using aseptic technique, the initial dilution is made by transferring 1 ml of ***E. coli*** sample to a 99ml sterile saline blank (figure below. This is a 1/100 or 10^{-2} dilution.
3. 10^{-2} dilution has been shaken, uncap it and aseptically transfer 1 ml to a second 99 ml saline blank. Since this is a 10^{-2} dilution, this second blank represents a 10^{-4} dilution of the original sample.
4. Shake the 10^{-4} dilution vigorously and transfer 1 ml to the third 99 ml blank. This third dilution represents a 10^{-6} dilution of the original sample. Repeat the process once more to produce a 10^{-8} dilution.
5. Shake the 10^{-4} dilution again and aseptically transfer 1.0 ml to one petri plate and 0.1 ml to another petri plate. Do the same for the 10^{-6} and the 10^{-8} dilutions.
6. Immediately after tha
7. Remove one agar pour tube from the 48º to 50ºC water bath. Carefully remove the cover from the 10^{-4} petri plate and aseptically pour the agar into it. The agar and sample are immediately mixed gently moving the plate in a figure-eight motion or a circular motion while it rests on the tabletop. Repeat this process for the remaining five plates.
8. After the pour plates have cooled and the agar has hardened, they are inverted and incubated at 25ºC for 48 hours or 37ºC for 24 hours.
9. At the end of the incubation period, select all of the petri plates containing between 30 and 300 colonies. Plates with more than 300 colonies cannot be counted and are designated **too many to count (TMTC).** Plates with fewer

than 30 colonies are designated **too few to count (TFTC).** Count the colonies on each plate. A Quebec colony counter should be used.

10. Calculate the number of bacteria (CFU) per milliliter or gram of sample by dividing the number of colonies by the dilution factor multiplied by the amount of specimen added to liquefied agar.

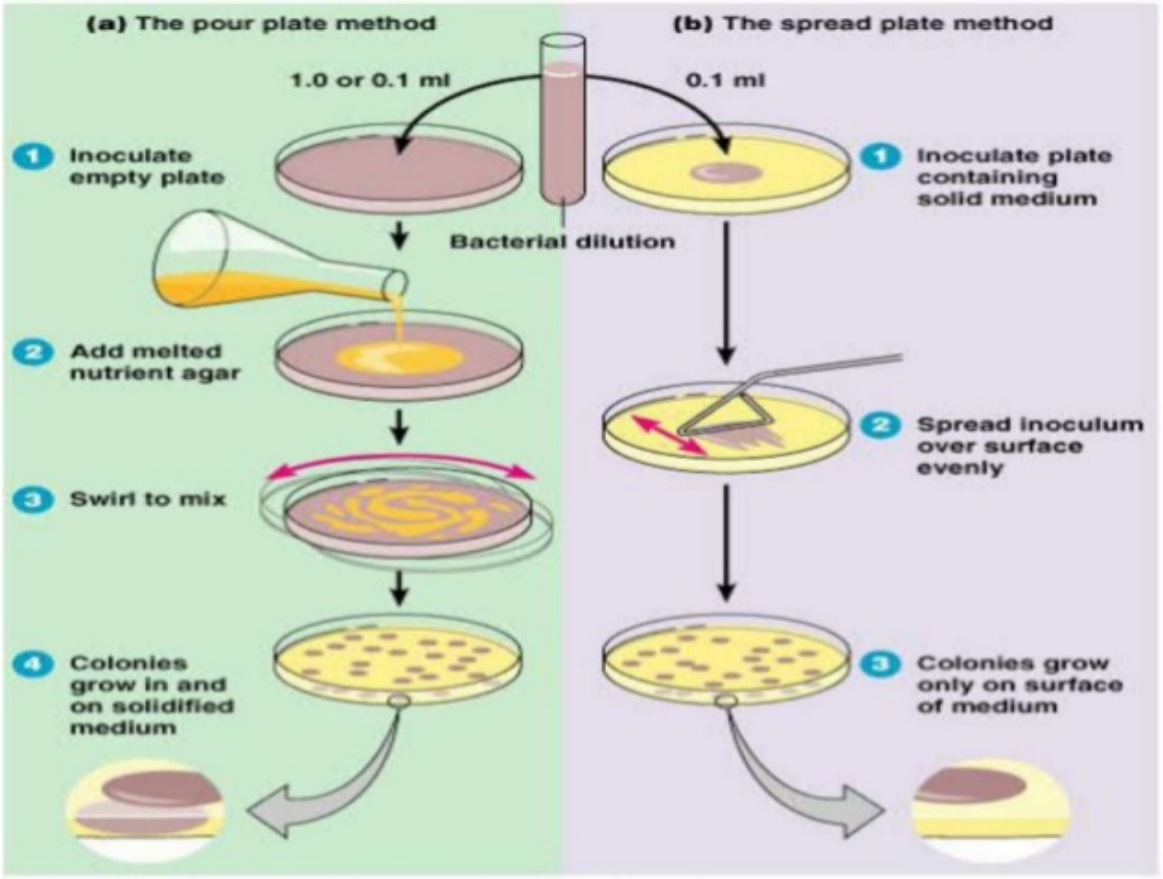
number of colonies (CFUs) = Number of bacteria/ ml

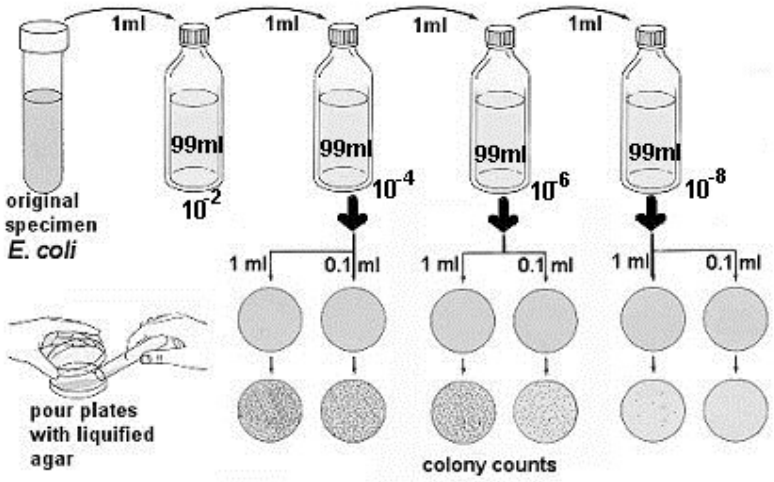
dilution X amount plated

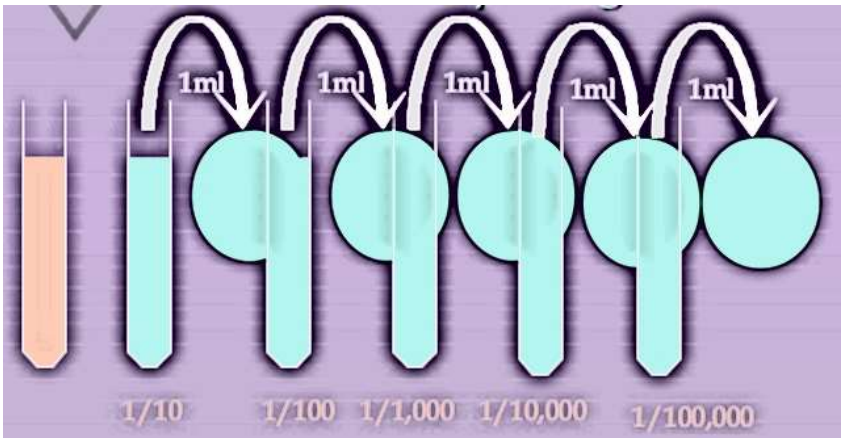
78/1/10000x1 = 780000 cfu/ml

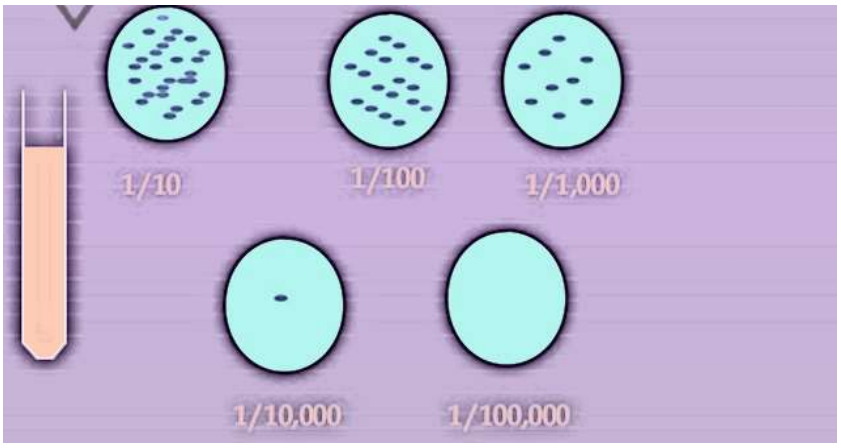
11. Record your results.

STANDARD PLATE COUNT









<u>number of colonies (CFUs)</u> = # of bacteria/ ml

dilution X amount plated

9/1/1000 X 1ML =9000 COLONIES/ML

8/1/1000X 1 ML = 8000 COLONIES /ML

9/1/1000 X 1ML = 9000 COLONIES/ML

9000+8000+9000/3= 8500 COLONIES/ML

Experiment 15

Objectivie: To count the bacterial number in a given food sample by turbidity method

Principle:

Many studies require the quantitative determination of bacterial populations. The two most widely used methods for determining bacterial numbers are the **standard**, or **viable**, **plate count method** and **spectrophotometric (turbidimetric)** analysis. Although the two methods are somewhat similar in the results they yield, there are distinct differences. For example, the standard plate count method is an indirect measurement of cell density and reveals information related only to live bacteria. The spectrophotometric analysis is based on turbidity and indirectly measures all bacteria (cell biomass), dead and alive.

The standard plate count method consists of diluting a sample with sterile saline or phosphate buffer diluent until the bacteria are dilute enough to count accurately. That is, the final plates in the series should have between 30 and 300 colonies. Fewer than 30 colonies are not acceptable for statistical reasons (too few may not be representative of the sample), and more than 300 colonies on a plate are likely to produce colonies too close to each other to be distinguished as distinct **colony-forming units (CFUs)**. The assumption is that each viable bacterial cell is separate from all others and will develop into a single discrete colony (CFU). Thus, the number of colonies should give the number of bacteria that can grow under the incubation conditions employed. A wide series of dilutions (e.g., 10^{-4} to 10^{-10}) is normally plated because the exact number of bacteria is usually unknown. Greater accuracy is achieved by plating duplicates or triplicates of each dilution, although we will not be doing that in this exercise.

Increased turbidity in a culture is another index of bacterial growth and cell numbers (biomass). By using a spectrophotometer, the amount of transmitted light decreases as the cell population increases. The transmitted light is converted to electrical energy, and this is indicated on a galvanometer. The reading, called **absorbance or optical density**, indirectly reflects the number of bacteria. This method is faster than the standard plate count but is limited because sensitivity is restricted to bacterial suspensions of 10^7cells or greater. **The procedure for the spectrophotometer use is at the end of this exercise**.

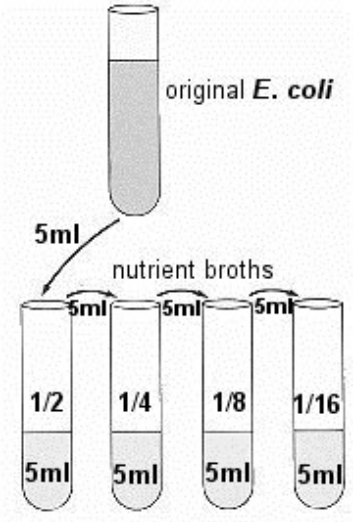
Why Is *E. coli* used in this exercise? When working with large numbers and a short time frame, one of the most reliable microorganisms is one that has been used in previous experiments, namely, *Escherichia coli*. *E. coli* has a generation time at 37ºC of 20 minutes. Thus, it reproduces very rapidly and is easy to quantify (i.e., the number (biomass) of viable *E. coli* cells in a bacterial culture can be easily determined by spectrophotometry).

MATERIALS NEEDED: per table (exercise performed by table)

- 24-hour 10ml nutrient broth culture of *Escherichia coli*
- 4 sterile 99ml saline bottles
- 1ml and 5ml pipets with pi-pumps (green for 5ml, blue for 1ml)
- 6 petri plates
- 6 agar pour tubes of nutrient agar (plate count agar)
- 48ºC to 50ºC water bath
- 6 micro-cuvettes and rack
- 1 SpectroVis micro-cuvette holder
- computer
- 4 tubes of 5ml nutrient broths

PROCEDURE

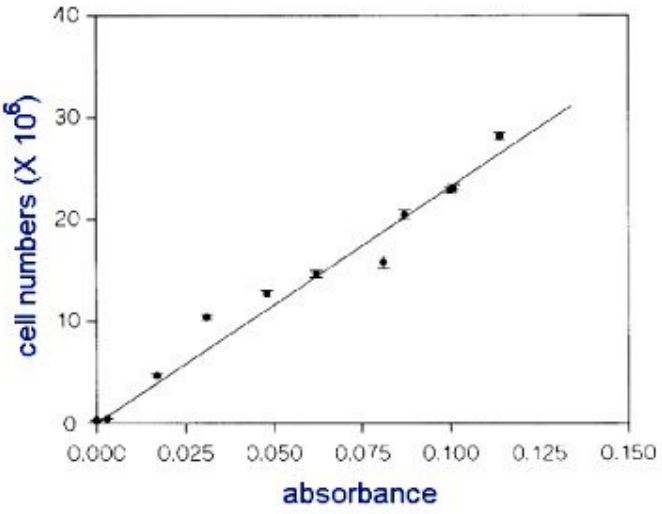
1. Place the **ORIGINAL tube of *E. coli*** and four tubes of the sterile NB in a test-tube rack. Each tube of NB contains 5 ml of sterile broth. Use four of these tubes (tubes 2 to 5) of broth to make four serial dilutions of the culture.
2. Transfer 5ml of *E. coli* to the first tube of NB, thoroughly mixing the tube afterwards. Transfer 5ml from that tube to the next tube, and so on until the last of the 4 tubes has 5ml added to it. These tubes will be 1/2, 1/4, 1/8, and 1/16 dilutions.
3. **The directions for spectrophotometer use are BELOW.**
4. Record your values, along with the dilutions that they came from. Using the plate count data, calculate the colony-forming units per milliliter for each dilution.



DATA COLLECTION

dilutions	absorbance (X)	# of bacteria (Y)
original *E. coli*		
1/2	0.8	80
1/4	0.4	40
1/8	0.2	20
1/16	0.1	10

1. Fill in your absorbance values for the 5 tubes read in the spectrophotometer.
2. Calculate the number of bacteria in the original tube of E. coli, and place that value in the top right cell of the table. This is done AFTER THE PLATES HAVE INCUBATED.
3. Calculate the approximate numbers of bacteria in the 1/2, 1/4, 1/8, and 1/16 by **halving the number in the cell above**.
4. Plot these 5 coordinates on a graph, **using EXCEL software** (it is available in the computer labs). The **DIRECTIONS** on howto use the software is **at end of exercise**.
5. Here is an example of a graph.



Experiment 16

Aim: Antibacterial activity by disc diffusion method.

Principle: This method is based on the principle that antibiotic-impregnated disk, placed on agar previously inoculated with the test bacterium, pick-up moisture and the antibiotic diffuse radially outward through the agar medium producing an antibiotic concentration gradient. The concentration of the antibiotic at the edge of the disk is high and gradually diminishes as the distance from the disk increases to a point where it is no longer inhibitory for the organism, which then grows freely. A clear zone or ring is formed around an antibiotic disk after incubation if the agent inhibits bacterial growth.

Materials:

1. Media (Mueller-Hinton Agar)
2. Tryptic soy broth
3. Ethanol
4. Petri dishes
5. wire loop
6. MacFarland standard
7. cotton swab

Preparation of agar medium

1. Prepare MHA from the dehydrated medium according to the manufacturer's instructions. Media should be prepared using distilled water or deionized water.
2. Heat with frequent agitation and boil to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 min.
3. Ceck the pH of each preparation after it is sterilized, which should be between 7.2 and 7.4 at room temperature. This is done by macerating a small amount of medium in a little distilled water or by allowing a little amount of medium to gel around a pH meter electrode.
4. Cool the agar medium to 40-50°C. Pour the agar into sterile glass or plastic petri dish on a flat surface to a uniform depth of 4 mm.
5. Allow to solidify.
6. Prior to use, dry plates at 30-37°C in an incubator, with lids partly ajar, for not more than 30 minutes or until excess surface moisture has evaporated. Media must be moist but free of water droplets on the surface. Presence of water droplets may result to swarming bacterial growth, which could give inaccurate results. They are also
   easily contaminated.
7. Plates are not to be immediately used, they may be stored in the refrigerator inside airtight plastic bags at 2-8°C for up to 4 weeks.
8. Unpoured media may be stored in airtight screw-capped bottles under the conditions specified by the manufacturer.

Inoculumpreparation:

1. From a pure bacterial culture (not more than 48 hours, old except for slow growing organisms), take four or five colonies with a wire loop.
2. Transfer colonies to 5 ml of Trypticase soy broth or 0.9% saline.
3. Incubate the broth at 30°C or at an optimum growth temperature until it achieves or exceeds the turbidity of 0.5 MacFarland standard (prepared by adding 0.5 ml of 0.048 M $BaCl_2$ to 99.5 ml of 0.36 NH_2SO_4 commercially available).
4. Compare the turbidity of the test bacterial suspension with that of 0.5 MacFarland (vigorously shaken before use) against a white background with contrasting black line under adequate light. Arrow points to tube with correct turbidity.
5. Reduce turbidity by adding sterile saline or broth.

Inoculation of plate

1. Dip a sterile cotton swab into the standardized bacterial suspension.
2. Remove excess inoculum by lightly pressing the swab against the tube wall at a level above that of the liquid.
3. Inoculate the agar by streaking with the swab containing the inoculum.
4. Rotate the plate by 60° and repeat the rubbing procedure. Repeat two times. This will ensure an even distribution of the inoculum.
5. Allow the surface of the medium to dry for 3-5 minutes but not longer than 15 minutes to allow for absorption of excess moisture.

Antimicrobial Disks applications:

1. Using sterile forceps or disk dispenser, place antibiotic disk on the surface of the inoculated and dried plate.
2. Immediately press it down lightly with the instrument to ensure complete contact between the disk and the agar surface. Do not move a disk once it has come into contact with the agar surface since some diffusion of the drug occurs instantaneously.
3. Position disks such that the minimum center - center distance is 24 mm and no closer than 10 to 15 mm from the edge of the petri dish. A maximum of six disks may be placed in a 9-cm petri dish and 12 disks on a 150 mm plate. Reduce the number of disks applied per plate if overlapping zones of inhibition are encountered.

Incubation:

1. Incubate plates in an inverted position at 30°C or at an optimum growth temperature.
2. Observe for the zone of inhibition after 16 to 18 hours. Slow growing organisms may require longer incubation period.

Reading and measurement of zones of inhibition:

1. The zone of inhibition (arrow) is the point at which no growth is visible to the naked eye.
2. Record the presence of individual colonies (arrow) within zones of inhibition.
3. Record occurrence of fuzzy zones (arrow). In measuring the zone diameter, the fuzzy portion of the zone should be ignored as much as possible. The zone limit is the inner limit of the zone of normal growth.

Reading

1. Read and record the diameter of the zones of inhibition using a ruler graduated to 0.5 mm.
2. Round up the zone measurement to the nearest millimeter.

Experiment 17

Aim:To isolate pure strain of E.coli. by the technique of streaking plate method.

(i) Serial dilution of soil sample

(ii) Media preparation

(iii) Streaking plate.

Materials required:

1) A source of bacteria (soil)
2) Inoculation loop,
3) A striker/lighter
4) Bunsen burner,
5) Lysol (10%v/v)
6) Agar plate (Nutrient agar or any other agar medium)
7) Autoclave

Procedure:

Serial dilution of soil sample:

1) Weigh 1gm of soil and dissolve in 10ml of distilled water in a test tube marked as 1.
2) In another test tube take 1ml of this solution and makeup it 10ml with distilled water, mark the test tube as 2.
3) Repeat step 2 and make total 10 test tubes labeled 1 to 10.

Media Preparation:

4) Take 0.4gm Nutrient agar (NA) in 10ml of sterile distilled water.
5) Warm it to dissolve.
6) Autoclave it.
7) Pour the moderately warm NA media in a petri plate.

Streaking plate:

8) Wait till the media solidify.
9) Sterilize the inoculating loop in the bunsen burner by putting the loop into the flame until it is red hot.
10) Allow it to cool.
11) Dip in the 10^{th} test tube, which have the highest dilution.
12) Immediately streak the inoculating loop very gently over agar media.
13) Tape the plate closed and incubates the plate in an inverted position in an incubator for 24 hours.
14) Observe the colonies next day.

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APPENDIX-I

MEDIA PREPARATION

1. Peptone Broth

Peptone : 10g
NaCl : 5g
Distilled water : 1000ml

2. Nutrient Agar

Peptone : 5g
NaCl : 5g
Beef extracts : 3g
Agar : 20g
Distilled water : 1000ml
The ingredients are dissolved in warm water and pH adjusted to 7.2-7.6. Autoclaved at 121°C, 15 lbs for 15 minutes.

3. Nutrient Broth

Peptone : 5g
NaCl : 5g
Beef extracts : 3g
Distilled water : 1000ml
The ingredients are dissolved in warm water and pH adjusted to 7.2-7.6. Autoclaved at 121°C, 15 lbs for 15 minutes.

4. Mac Conkey Agar

Peptone : 20g
NaCl : 5g
Bile salt : 1.5g
Lactose : 10g
Neutral red solution : 10ml
Crystal violet : 0.001g
Agar : 13.5g
Distilled water : 1000ml

5. ***Sabouraud's Dextrose Agar (SDA)***

Peptone : 10g
Dextrose : 40g
Chloramphenicol : 0.05g
Agar : 15g
Distilled water : 1000ml

6. Sabouraud's Dextrose Broth

Peptone : 10g
Dextrose : 40g
Chloramphenicol : 0.05g
Distilled water : 1000ml

7. Mueller – Hinton Agar

Beef infusion form : 300g
Acid hydrolysate of casein : 17.5g
Agar : 17g
Starch : 1.5g

8. Lactose Broth
Peptone : 5g
Beef extract : 3g
Lactose : 5g
Distilled water : 1000ml

9. EMB (Eosin Methylene Blue) Agar

Peptone : 10g
Lactose : 5g
Sucrose : 5g
Dipotassium hydrogen phosphate : 2g
Eosin Y : 0.40g
Methylene blue : 0.065g
Agar : 13.50g
Distilled water : 1000ml

10. Methylene Blue Solution (1:25,000)

Methylene blue dye : 1mg
Distilled water : 25ml
Dissolved the methylene blue in distilled water and was dispensed into regular staining bottles.

11. Carbohydrate Fermentation

Peptone : 1g
Carbohydrates : 10g
NaCl : 5g
Phenol red indicator : 10ml (0.1g in 10ml ethanol)
Distilled water : 1000ml
Mix all the ingredients, except phenol red indicator. Adjust pH to 7. Then add phenol red indicator. Dispense the medium in 8ml test tubes containing the Durham's tubes. Sterilize the medium at 10lbs for 20 minutes.

12. Oxidation – Fermentation

Peptone : 20g
Dipotassium hydrogen phosphate : 2g
NaCl : 5g
Bromothymol blue : 3ml (1% aqueous solution)
Agar : 13.50g
Distilled water : 1000ml
Mix all the ingredients, expect Bromothymol blue indicator. Adjust pH to 7.1. Then add Bromothymol blue indicator. The medium is poured into the tube to a depth of about 4cm. sterilized by autoclaving at 121°C for 20 minutes at 10 lbs. it was then allowed to set.

13. Voges – Proskauer

Reagents: Barrett's A
α – naphthol : 5g
Ethanol : 100ml
Dissolve α – naphthol in small amount of alcohol and then add remaining alcohol to 100ml. Store in brown bottle at 4°C.
Barrett's B
Potassium hydroxide : 40g
Distilled water : 100ml

Cool the volumetric flask in cold water with 80ml water, add KOH crystals, dissolve and make up to100ml. Store in polyethene bottles at 4°C.

14. Citrate Utilization

MgSo4 : 0.2g
Ammonium dihydrogen phosphate : 1g
Dipotassium phosphate : 1g
Sodium citrate : 2g
Sodium chloride : 0.5g
Bromothymol blue : 0.08g
Agar : 15g
Distilled water : 1000ml
Dissolve the ingredients in 1000ml distilled water. Dispense in tubes and sterilize by autoclaving at 121°C for 20 minutes at 10 lbs.

15. Nitrate Broth

Beef extract : 3g
Peptone : 5g
Potassium Nitrate : 1g
Distilled water : 1000ml
Dissolve all the ingredients and sterilize by autoclaving at 121°C for 20 minutes at 15 lbs.
Reagents: Sulphanlic acid
Dissolve 8g of sulphanlic acid in 1 l of acetic acid.
α- Naphthol amines
Dissolve 5g of α-Naphthol amines in 1 l of acetic acid. Immediately before use, mix equal volumes of Sulphanlic acid and α- Naphthol amines to give the test reagent.

16. Urease Test

Peptone : 1g
Phenol red : 0.012g
Dextrose : 1g
NaCl : 5g
Disodium phosphate : 1.2g
Mono potassium phosphate : 0.8g
Agar : 15g
Distilled water : 1000ml
Dissolve ingredients in 950ml distilled water. Sterilize by autoclaving at 10lbs for 20 minutes. Cool to 58°C and aseptically add 50ml of 40% urea. Sterilize the urea

solution by autoclaving at 10lbs for 15 minutes, mix well and add the Phenol red indicator. Dispense into sterilized test tubes and allow to set in a slanting position.

17. Mannitol Motility Test

Peptone : 20g
NaCl : 5g
Potassium Nitrate : 2g
Mannitol : 64g
Agar : 6g
Distilled water : 1000ml
Phenol red : 4ml (1g in 100ml ethanol)
Mix all the ingredients, expect phenol red indicator. Adjust pH to 7. Then add phenol red indicator. Dispense in tubes. Sterilize the medium at 10lbs for 20 minutes.

18. Triple Sugar Iron Agar Test

Peptone : 20g
Yeast extract : 3g

Beef extract : 3g
Lactose : 10g
Sucrose : 10g
Glucose : 10g
Sodium chloride : 5g
Ferrous sulphates : 0.2g
Sodium thiosulphate : 0.3g
Agar : 12g
Distilled water : 1000ml
Phenol red : 0.024g
Mix all the ingredients, expect phenol red indicator. Adjust pH to 7. Then add phenol red indicator. Sterilize by autoclaving at 121°C for 20 minutes. Allow the medium to set in slopped form with a butt about 1 inch long. The medium is used in the form a butt and slant.

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