AVIT
AARUPADAI VEEDU INSTITUTE OF TECHNOLOGY
VINAYAKA MISSION'S RESEARCH FOUNDATION
(Deemed to be University under section 3 of the UGC Act 1956)
Accredited by NAAC
Approved by AICTE

DEPARTMENT OF BIOTECHNOLOGY

M.TECH

MICROBIOLOGY LABORATORY
(481171L2)

HOD/BIOTECH

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481171L2-MICROBIOLOGY LAB

LIST OF EXPREIMENTS

Expt.No	Name of Experiment
1	Sterilization Techniques
2	Preparation Of Culture Media For Micro Organisms
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3	Culture of Micro organisms
	a. Pure culture techniques
	i) Streak plate
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4	Identification of Micro organisms
	a. Staining Techniques
	i) Simple staning
	ii) Gram staining
	iii) Endospore staining
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	v) Hanging drop
	b. Biochemical Testing
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5	Environmental Sample Analysis
	i) Isolation and enumeration of microbes from soil sample

6	**Food Microbiology** **i) Analysis of milk sample**
7	**Clinical Microbiology**
	i) Isolation of normal mouth flora
	ii) *In vitro* Determination of Anti Microbial Sensitivity

1. STERILIZATION TECHNIQUES

Exp: 1
Date:

Aim:

To sterilize the materials used for culturing microorganisms

Background:

It is the process to destroy all micro organisms without damaging or altering the substance being sterilized.

***Types*:**

***Dry heat*:**

Dry heat done using hot air ovens used to sterilize glassware, metal instruments and the materials that cannot be penetrated by steam and it was done at 180^{o} C for 2-3 hrs.

Moist heat:

Moist heat is done using autoclaves Steam is bactericidal and kills microbes by denaturing proteins present in the cell wall. Moist heat is used in sterilization of culture media and other liquids used.

Gaseous chemicals:

Highly effective for sterilization

Filter sterilization:

This type of sterilization used to sterilize solutions that are thermo labile. Function by entrapping micro organisms within the porous structure of the filter matrix. Ex: antibiotics, sugars, amino acids, vitamins.

Radiation:

Working area routinely sterilized using UV radiation and these lamps are called UV lamps, which emit radiation chiefly at wavelength at 2537 A.

Chemical disinfectants:

They are used to kill vegetative bacteria, fungi, viruses and occasionally spores. Example: phenolic compounds and hypo chlorides.

Procedure:

- Keep the glassware in the inoculation chamber and switch on UV light and let it glow for 30 minutes.
- Switch off the UV lamp.
- Check the sterility of working area by swabbing with sterile cotton plugs. The plates were filled with suitable agar and inoculated with swabs.
- Plates were incubated at 37^{o}C.

Observation:

Result:

2. PREPARATION OF CULTURE MEDIA FOR MICRO ORGANISMS

a) BROTH TYPE MEDIA

Exp:2
Date:

Aim:

To learn the principles, and techniques of preparing nutrient broth for the microbial culture.

Background:

A common medium (Nutrient broth) used for growing bacteria (non fastidious organism). It contains beef extract, peptone and NaCl as ingredients. This liquid medium is nutrient broth and can be supplemented with other substances like sugars, and other inorganic salts to meet the requirements of any particular organisms. These media are always kept sterile until they are used.

Requirements:

Beef extract, peptone, NaCl, agar, conical flask, cotton wool, measuring cylinder, pH meter etc.

Composition of Nutrient broth:

Beef extract	- 1g
Peptone	- 1g
NaCl	- 0.5 g
Distilled water	- 100 ml

Procedure:

- Hundred milliliter of distilled water is taken in a conical flask.
- Weigh the above ingredients and dissolve in distilled water.
- The pH of the medium is adjusted to 7.2 with the help of 0.1N sodium hydroxide.
- Distribute equal quantity of the nutrient broth in test tubes and plug the tubes with cotton.

- Sterilize the medium at 121^{o}C for 15 minutes in an autoclave.
- After 15 minutes remove the test tube and keep in a clean place.

Observation:

Result:

b) PREPARION OF SOLID TYPE MEDIA (AGAR SLANTS AND AGAR PLATES)

Exp:
Date:

Aim:

To prepare the solid agar medium for culturing the micro organisms.

Materials required:

Test tubes, Petri plates, measuring cylinder, pH meter, auto clave etc

Nutrient agar composition:

Beef extract - 1 g
Peptone - 1 g
NaCl - 0.5 g
Agar - 1.5 g
Distilled water - 100 ml

Procedure:

Agar slant preparation:

Allow the test tube containing the agar medium to cool down to about 45 – 50^{o} C and keep them in a slanting position by resting the plugged end over the glass rod on the table and leave the tubes in this position till the medium has cooled to room temperature and solidified.

***Agar plate preparation*:**

- Label the Petri dishes and organize them in inoculation chamber. Grasp the Erlenmeyer flask containing sterile agar medium.
- Turn your left hand palm side up and clamp the cotton plug between two fingers. Remove the plug and flasks the mouth of the Erlenmeyer flask.
- Use the hand holding cotton plug to lift the lid of the Petri dish. Now pour about 20 -25 ml of sterilized nutrient agar medium.

- Hold the lid so that it partially covers the bottom of the dish as you pour. This help to prevent microbes and air born dust dropping into your sterile plate and contaminating it.
- Immediately replace the lid, flame the mouth of the flask and pour the next plate in the same way.
- If air bubbles occur on the surface of the medium, break aseptically by quickly passing the Bunsen flame over the media surface.
- Allow the plates to cool at room-temperature and store the plates in sterile place.

Observation:

Result:

3.CULTURING OF MICROORGNAISMS

a) PURE CULTURE TECHNIQUES

Exp: 3
Date:

Aim :

To learn the pure culture techniques, and isolate pure culture of bacteria.

Back ground:

Micro organisms are ubiquitous in nature so it is necessary to isolate and culturing a pure culture of microorganisms in order to study the properties of a particular microorganisms.

Pure culture represent a population of organisms of a single species in the absence of living cells of any other species in this there are various techniques, where by the different species in a natural population can be isolated and grown as pure culture. They are

a) Streak plate culture
b) Pour plate culture
c) Spread plate culture

***Materials required*:**

Petri plates, test tubes, ‘ L’ shaped glass rod, Inoculation loop, 70% ethanol, nutrient broth, pipettes etc.

***i). Streak plate culture*:**

- Pour the autoclaved nutrient agar to the Petri plates under sterile condtions.
- Sterilize the inoculation needle, by flaming in red-hot and allow it to cool for 10 seconds.
- By holding the culture tube in your left hand near flame, remove cotton plug with your right hand and flame sterilize the mouth of the tube for few seconds.

- Touch the culture with the inoculation loop and take the culture.
- Plate it on agar plate and streak it on the plate once resterllize the loop and cool it as above and take the culture from on end of the plate to other end complete the streak as it.

***ii) Pour plate technique*:**

Liquefy the nutrient agar in the tubes by heating them in water bath cool the tubes to 45^{o}C and hold at this temperature until ready to pour into the plate.

Label the tubes and corresponding Petri dishes serially dilute the given mixture and from each dilution take 0.1ml of the sample.

Mix the sample with agar medium by gently rotating the tube between your plates. Pour the contents of the tubes into the corresponding labeled Petri dishes and allow solidifying and incubating the plates in inverted position at room temperature.

iii). Spread plate technique:

Use the nutrient agar plates as prepared earlier serially dilute the given culture and label the nutrient agar plates corresponding to the dilution.

Take 0.1ml from each dilution and pour over the surface of nutrient agar plates. Sterilize the 'L'- rod with alcohol and then by flaming it. Cool the rod and place the rod gently on the surface of the agar. Spread the sample over the surface of the agar medium by rotating the Petri plate manually or by placing on the rotating plate disc.

Incubate the plate in inverted position at the room temperature.

Observation:

Result:

4. IDENTIFICATION OF MICRO ORGANISMS

a) STANINING TECHNIQUES

i). SIMPLE STAINING

Exp: 4
Date:

Aim:

To learn the staining technique, for studying the morphology of microorganisms.

Principle:

Staining is the method of artificially producing colors in microbes to allow for the visualization under the microscope. Stains are employed not only to make the organisms visual but also understand their structure and chemical nature.

Dyes or stains are synthetic chemicals products of aniline type. They can be divided into acid dyes and basic dyes with respect to their color properly in the anionic acid cationic form. Acid dyes have shorter tendency to combine with the cytoplasm but basic dyes have greater affinity to the nuclear region of the cells. Basic dyes are generally used in bacteriological studies.

Simple stain contain only one dye may allow visualization of morphological characteristics of bacterial cells.

***Materials required*:**

24 hrs old bacterial culture, stain, clean glass slide, cover slips, microscope, Methylene blue, Carbol fuchsin, Crystal violet, Saffranin, Malachite green etc.

Procedure:

Clean the slides in any detergent solution. Rinse with tap water and wipe the slides. Dry with a clean cloth or tissue paper. There after handle the slides only by grasping them at the edges.

Flame the inoculation loop and the mouth of the culture tube containing microbial culture. Allow the loop to cool.

Pick up a loopful of bacterial colonies from the agar surface.

Transfer the loopful of suspension on to a clean slide and spread it onto a small area and air dry, thus making a smear.

Heat fixes the smear by passing the slide through the Bunsen flame two to three times so that the heat is just tolerable to the skin of the arm.

Flood the smear with any one of the dyes mentioned above for about 3 minutes. Wash the slide in a gentle steam of tap water to remove excess stain air dry. Examine the preparation with the oil immersion objective.

Observation **:**

Result:

ii). GRAM'S STAINING

Exp:

Date:

Aim:

To study and identify whether the given bacterial culture is gram positive of gram negative.

Principle:

Gram's stain was introduced in 1880 by the Danish bacteriologist Christian Gram. Gram's staining is employed to visualize and differentiate between organisms. It is useful of presumptive identification of organisms before carry out the other serological and biochemical test.

Bacteria can be classified into two classes: gram positive and gram negative. The bacteria, which retain the crystal violet, are termed as gram positive and the bacteria which lose the crystal violet are termed as gram negative.

The cell walls of negative bacteria are thinner and has high liquid content during the alcohol treatment it extracts the lipid this increases the porosity of cell. So the gram negative bacteria are decolorized and take up the color of the counter stain.

Iodine is used as a mordant, saffranin is used as a counter stain.

Materials required:

Crystal violet, gram's iodine, ethanol and saffranin, glass rod, hot plate, glass slides, 24 hrs old culture, microscope, distilled water etc.

Procedure:

Prepare a bacterial smear on the slide with a given bacterial culture as performed earlier. This smear is first treated with a basic dye crystal violet and allows it to stand for one or two minutes and then wash the slide with sterile water.

After washing, in second step, the smear is treated with iodine is added after waiting for 1 minute the slide is again washed with distilled water.

After treating with iodine solution decolorize with alcohol. Alcohol is added drop by drop on slide kept in staining position till all blue color was removed; wash it with distilled water and air dry.

Finally saffranin (Counter stain) was added and after waiting for 1 minute the slide is then wash with distilled water. Then slide is allowed to dry.

After completion of all four steps, the slide is examined that the stained smear in oil immersion objective were observed and determines whether the organism is gram positive of gram negative.

Observation:

Result:

iii). ENDOSPORE STAINING

Exp:
Date:

Aim:

To study and identify whether the given bacterial culture is gram positive of gram negative.

Principle:

Bacterial endospores are metabolically inactive, highly resistant structures produced by some bacteria as a defensive strategy against unfavorable environmental conditions. The bacteria can remain in this suspended state until conditions become favorable and they can germinate and return to their vegetative state.

In the Schaeffer-Fulton`s method, a primary stain-malachite green is forced into the spore by steaming the bacterial emulsion. Malachite green is water soluble and has a low affinity for cellular material, so vegetative cells may be decolourized with water. Safranin is then applied to counterstain any cells which have been decolorized. At the end of the staining process, vegetative cells will be pink, and endospores will be dark green.

Spores may be located in the middle of the cell, at the end of the cell, or between the end and middle of the cell. Spore shape may also be of diagnostic use. Spores may be spherical or elliptical.

Materials required:

Primary Stain: Malachite green (0.5% (wt/vol) aqueous solution)
0.5 gm of malachite green, 100 ml of distilled water

Decolorizing agent

Tap water or Distilled Water

Counter Stain: Safranin

Stock solution (2.5% (wt/vol) alcoholic solution),2.5 gm of safranin,100 ml of 95% ethanol.

Procedure:

1. Take a clean grease free slide and make smear using sterile technique.
2. Air dry and heat fix the organism on a glass slide and cover with a square of blotting paper or toweling cut to fit the slide.
3. Saturate the blotting paper with **malachite green** stain solution and steam for **5 minutes**, keeping the paper moist and adding more dye as required. Alternatively, the slide may be steamed over a container of boiling water.
4. Wash the slide in **tap water.**
5. Counterstain with **0.5% safranin** for **30 seconds**. Wash with tap water; blot dry.
6. Examine the slide under microscope for the presence of endospores. Endospores are bright green and vegetative cells are brownish red to pink.

Observation:

Result:

iv). ACID FAST STAINING

Exp:
Date:

Aim:

To differentiate bacteria into acid fast group and non-acid fast groups

Principle:

When the smear is stained with carbol fuchsin, it solubilizes the lipoidal material present in the Mycobacterial cell wall but by the application of heat, carbol fuchsin further penetrates through lipoidal wall and enters into cytoplasm. Then after all cell appears red. Then the smear is decolorized with decolorizing agent (3% HCL in 95% alcohol) but the acid fast cells are resistant due to the presence of large amount of lipoidal material in their cell wall which prevents the penetration of decolorizing solution.

The non-acid fast organism lack the lipoidal material in their cell wall due to which they are easily decolorized, leaving the cells colorless. Then the smear is stained with counterstain, methylene blue. Only decolorized cells absorb the counter stain and take its color and appears blue while acid-fast cells retain the red color.

Materials required:

Carbol fusion, acid alcohol, methylene blue, glass rod, hot plate, glass slides, 24 hrs old culture, microscope, distilled water etc.

Procedure:

1. Prepare bacterial smear on clean and grease free slide, using sterile technique.

2. Allow smear to air dry and then heat fix. Alcohol-fixation: This is recommended when the smear has not been prepared from sodium hypochlorite (bleach) treated sputum and will not be

stained immediately. M. tuberculosis is killed by bleach and during the staining process. Heat-fixation of untreated sputum will not kill *M. tuberculosis* whereas alcohol-fixation is bactericidal.

3. Cover the smear with carbol fuchsin stain.

4. Heat the stain until vapour just begins to rise (i.e. about 60 C). Do not overheat. Allow the heated stain to remain on the slide for 5 minutes. Heating the stain: Great care must be taken when heating the carbol fuchsin especially if staining is carried out over a tray or other container in which highly fiammable chemicals have collected from previous staining. Only a small fiame should be applied under the slides using an ignited swab previously dampened with a few drops of acid alcohol or 70% v/v ethanol or methanol. Do not use a large ethanol soaked swab because this is a fire risk.

5. Wash off the stain with clean water.
   Note: When the tap water is not clean, wash the smear with filtered water or clean boiled rainwater.

6. Cover the smear with 3% v/v acid alcohol for 5 minutes or until the smear is sufficiently decolorized, i.e. pale pink.
   Caution: Acid alcohol is fiammable, therefore use it with care well away from an open fiame.

7. Wash well with clean water.

8. Cover the smear with malachite green stain for 1–2 minutes, using the longer time when the smear is thin.

9. Wash off the stain with clean water.

10. Wipe the back of the slide clean, and place it in a draining rack for the smear to air-dry (do not blot dry).

11. Examine the smear microscopically, using the 100 X oil immersion objective.

Observation:

Result:

v). HANGING DROP TECHNIQUE

Exp:
Date:

***Aim*:**

To perform motility test of a bacteria, by hanging drop preparation, to find out whether it is motile or non-motile.

Principle:

A very small drop of bacteria suspension is hung from the center of a cover slip into the cavity of a cavity slide. The hanging drop is observed under a microscope using oil-immersion objective. If the bacteria are motile, its cells can be seen to have erratic movement in the surrounding medium.

In contrast, if it is non-motile, its cells remain static in the medium without any movement or may show brownian movement resulting from the bombardment by the water molecules in the medium, on the bacteria cells.

Material required:

Cavity slide, cover slip, petroleum jelly or Vaseline, immersion oil, 24-hour old broth culture of bacteria, loop and microscope (compound, dark-field or phase-contrast).

Procedure:

1. A cavity slide is cleaned properly under tap water, such that water does not remain as drops on its surface. A cavity slide is a glass slide with a small round depression at the center, into which a small drop of bacteria suspension can hang (Figure 5.3).

2. The slide is dried by wiping with bibulous paper and subsequently, moving it over flame or keeping it in the sun.

3. A ring of petroleum jelly (or vaseline) is applied around the cavity.

4. A loop is sterilised over flame and cooled. A lapful of bacteria suspension is taken from the 24-hour old broth culture aseptically. A small drop of the suspension is placed at the center of a cover slip. The broth culture should not be more than 24 hours old, because bacteria may lose their motility, as they grow older.

5. The cavity slide is inverted and placed on the cover slip, in such a way that, the cavity covers the drop.

6. The slide and cover slip are pressed together gently, so that the cavity is sealed. Care should be taken to see that no part of the cavity touches the drop.

7. The slide is inverted quickly, such that the drop hangs into the cavity without touching it.

8. The slide is clipped to stage of the microscope.

9. The edge of the drop is focused under low power objective.

The reasons for focusing the edge of the drop are as follows:

(a) Better contrast is obtained due to difference in the refractive index of the drop and the cover slip.

(b) As the drop hangs, it thins towards the edge, for which the edge contains less number of bacteria to be observed clearly for motility.

(c) Usually aerobic bacteria come towards the edge to get more oxygen for respiration, for which they can be observed on the edge.

10. A drop of immersion oil is put on the cover slip just above the hanging drop and the edge of the hanging drop is observed under oil-immersion objective of the microscope. Preferably, a phase-contrast or dark-field microscope should be used for clear observation.

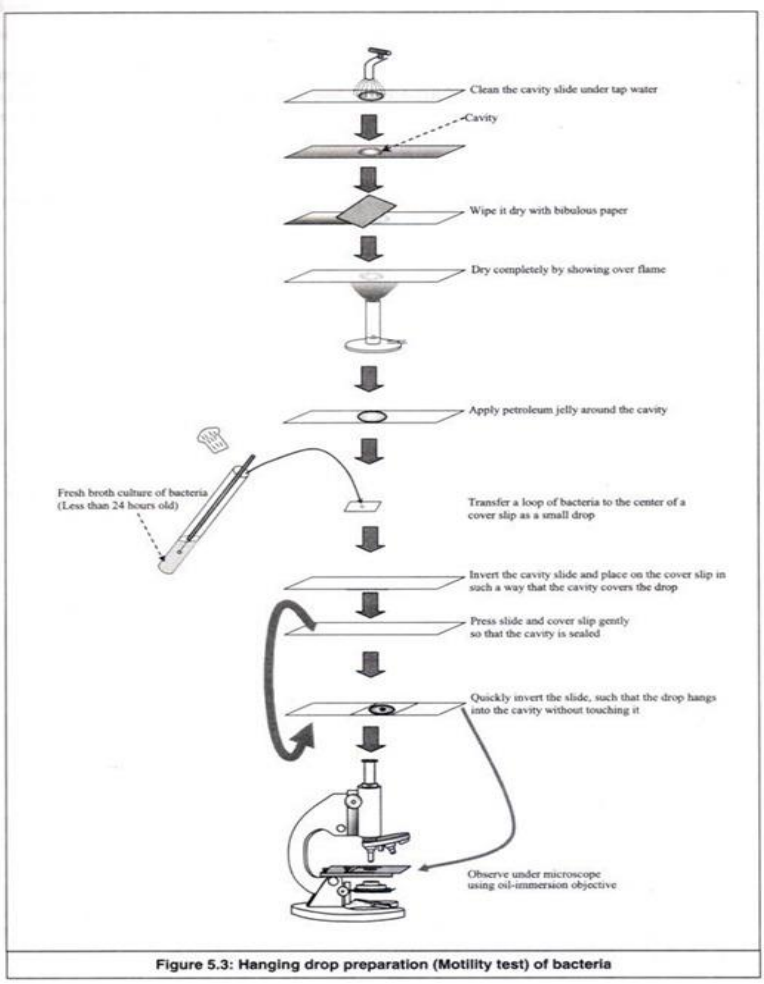


Figure 5.3: Hanging drop preparation (Motility test) of bacteria

Observation:

Result:

b) BIOCHEMICAL TESTING

i) INDOLE PRODUCTION TEST

Exp:
Date:

Aim:

To determine the ability of an organism to produce indole.

Principle:

Tryphotophan present in peptones of the culture media is acted upon by the enzyme tryptophanase an converted into indole, skatol and indole acetic acid. Indole reacts wilth aldehydes to produce a red colored product. So organisms are grown in tryptophan rich medium and tested for the presence of indole.

Materials required:

Tryptone broth, bacterial culture(*E.coli*, *Klebsiella pneumoniae*), Kovac's reagent, test tube, inoculation loop etc

Kovac's reagent:

Amyl or isoamyl alcohol	– 150ml
Paradimethyl aminobenzaldehyde	– 5.0 gm
Conc. HCL	- 25.0 ml

Procedure:

Indole agar containing test tubes are inoculated with test organism.

After inoculation the tubes are kept in incubator for 48 hrs at 37^{o}C.

Add 0.5 ml of kovac's reagent and shake gently and observe the result.

Observation:

Result:

ii) UREASE TEST

Exp:
Date:

***Aim*:**

To determine the ability of organism to ferment citrate as a sole carbon source.

Principle:

In the absence of fermentable glucose or lactose, some microorganisms are capable of using citrate as a carbon source for their energy. This ability depends on the presence of a citrate permease that facilitates the transport of citrate in the cell.

Citrate is the first major intermediate in the Krebs cycle and is produced by the condensation of active acetyl with oxaloacetic acid. Citrate is acted on by the enzyme citrase, which produce oxalic acid and acetate. These products are enzymatically converted to pyruvic acid and carbon dioxide. During this reaction the medium becomes alkaline- the carbondioxide that is generated combines with sodium and water to form sodium carbonate, an alkaline product.

The presence of sodium carbonate changes the bromothymol blue indicator incorporated into the medium from green to deep Prussian blue.

Material required:

Simmons citrate agar slants , Bunsen burner, inoculating needle, 24-48 hrs cultures of *E.coli , K. pnemoniae.*

Procedure:

1. Using sterile loop, inoculate test organism into its appropriately labeled tube by means of a stab and streak inoculation. The last tube will be serving as a control.

2. Incubate all cultures for 24 to 48 hrs at 37^0C and observe the result.

Observation:

Result:

5. ENVIRONMENTAL SAMPLE ANALYSIS

ISOLATION AND ENUMERATION OF BACTERIA FROM SOIL

Exp: Date: 5

Aim:

To enumerate the number of microorganisms present in the soil sample.

Principle:

Soil contains all source of microorganism (Bacteria, fungi, algae, protozoa). The amount the microorganism present in the soil can be quantified by serial dilution technique.

***Materials required*:**

Petri plates, test tubes, sterile tip box, 'L' rod, pipette, distilled water, conical flask, autoclave, nutrient agar, NaCl etc.

Procedure:

The analysis of soil sample involves the following steps:

Sample collection
Sample preparation (serial dilution)
Media preparation
Plating of sample in nutrient agar plate

Sample collection:

The soil sample was collected from the environment from which 1g was taken and suspended in 10 ml of sterile dis.water. It was allowed to stand on the worktable for 5 minutes. This is considered as 10^{-1} dilution.

Sample preparation:

Sample preparation was done using serial dilution technique. Sterile test tubes were arranged in a test tube stand which was cotton plugged and the test tube were labeled as 10^{-2},10^{-4},10^{-6} and 10^{-7}.

Nine ml of saline solution was distributed asepectically to each tube.

One ml of sample was taken from 10^{-1} dilution and transferred to second test tube and mixed well.

Take 1ml of sample from the 10^{-2} dilution test tube and transfer to next tube vice versa.

This kind of serial dilution from one test tube to next was repeated till the last tube. Finally, take 1ml of sample from the last test tube and discard the sample. Thus the sample dilution was prepared by serial dilution technique.

Media preparation

For bacterial enumeration nutrient agar was prepared in conical flask. Autoclave the nutrient agar containing flasks and Petri plates simultaneously.

After sterilization the media can be poured on the Petri plate and allow solidifying the media.

Plating of sample:

Pour plate technique:

0.1 ml of the diluted sample was transferred to the boiling tubes contain 20 ml of nutrient agar.(The sample should be add immediately after autoclaving around 37^{o}C and then the sample is transferred to the tubes).

The inoculated media transfer to the sterile Petri plates and allow solidifying.

After the media is solidified the plate are kept inverted position in incubator at 37^{o}C.

Spread plate technique:

The prepared nutrient agar containing plates was taken and kept in inoculation chamber.

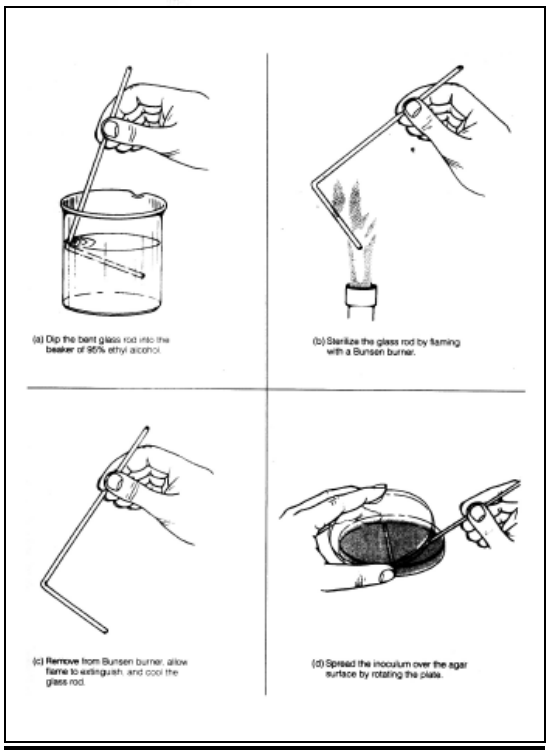
Take 0.1 ml of sample from diluted tube (using sterile tip) and pour over the surface of the nutrient agar.

Dip the 'L' rod in alcohol and flaming in Bunsen burner and allowed to cool for few seconds.

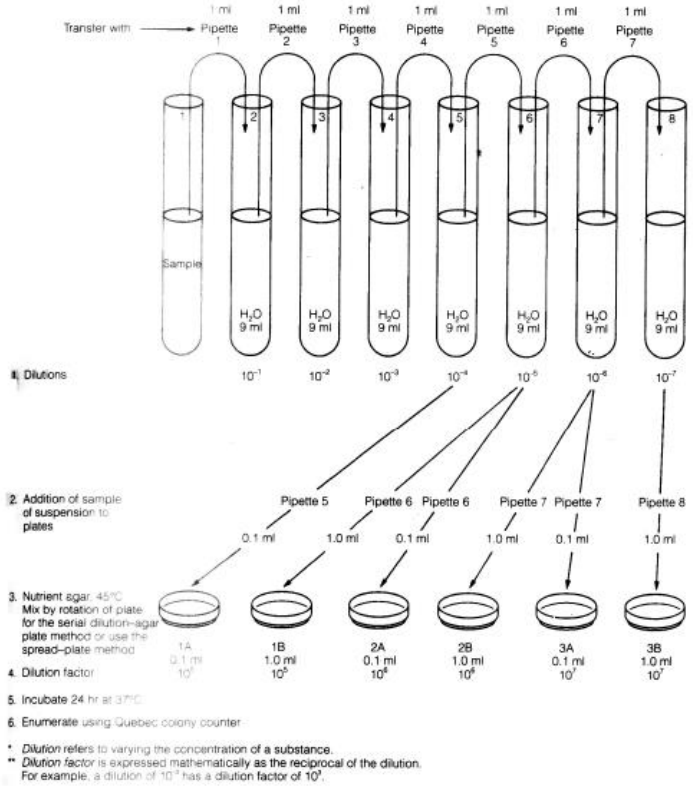
By using this sterile "L" rod evenly spread the sample over the surface of the agar medium. Then the plates allowed settling for few minutes and incubating in the incubator at 37^{o}C.

Observation:

Result:



SPREAD PLATE TECHNIQUE



SERIAL DILUTION TECHNIQUE

6. FOOD MICROBIOLOGY

ANALYSIS OF MILK SAMPLE

Exp: 6
Date:

Aim:

To examine the presence of microbes in the milk samples

Background:

Milk is made up of carbohydrates, proteins, fats, vitamins, minerals and water, which support the growth of microorganisms. Milk contains microorganism at the time it is drawn from the cow and it may be further contaminated in subsequent handling and processing.

Materials required:

Raw milk, pasteurized milk, nutrient agar, pipettes, test tubes, sterile screw cap tubes etc

Procedure:

a) ***Direct microscopic counting method:***

Spread a drop of milk sample onto an area of $1cm^2$ on a glass slide and dry gently with heat.

Remove the lipids by dipping the slide in xylene for 2 minutes.

Fix the slide by dipping in isopropyl alcohol for 2 minutes.

Stain for 2 minutes with methylene blue and wash with isopropyl alcohol until it becomes pale blue color.

Dry the slide and count the bacterial cells in each microscopic field.

b) ***Plate counting method:***

Dilute the milk sample by serial dilution technique.
Pipette 0.1ml of each dilution into labeled nutrient agar plates.
Incubate the inoculated plates for 48 hrs at 37^{o}C. And results were observed.

c)Methylene Blue Reductase Test (MBRT)

Principle

This reductase test is based on the oxidation-reduction activities of the bacteria present in the milk sample. The indicator used in the reaction is methylene blue which is color sensitive to oxygen concentration. The indicator is blue in the oxidized state and leuco or white in the reduced conditions. The speed of color disappearance of methylene blue is proportional to the microbial load in the milk sample. The more the bacteria present the faster will be the reduction.

The classification of milk as per methylene blue reductase test is as follows.

1. Class I- Excellent, not decolorized in 8 hours (<500 bacteria/ml)
2. Class II-Good, decolorized in less than 8 hours but not less than 6 hours (>500 bacteria/ml)
3. Class III-Fair, decolorized in less than 6 hours but not less than 2 hours (>40,00,000 bacteria/ml)
4. Class IV- Poor, decolorized in less than 2 hours. (> 2,00,00,000 bacteria/ml)

Materials Required

Milk sample, methylene blue solution, Mc Cartney bottles, pipettes, water bath set at 37^{o}C, distilled water, Bunsen burner.

Note

All the glass wares were sterilized before use.

Procedure

- Methylene blue solution was prepared by dissolving 1 mg methylene blue powder aseptically in 25 ml of distilled water.
- Transferred 10 ml of milk sample into sterile Mc Cartney bottle using sterile pipettes.

- Added 1 ml of methylene blue solution to the milk sample using a separate sterile pipette.
- The bottle was closed with the stopper.
- The contents of the tube were mixed by gently inverting it 2-3 times.
- Incubate the Mc Cartney bottle in a water bath at 37^{o}C for 6 hours.
- Controlled tubes containing 10 ml boiled milk and 1 ml of methylene blue was also incubated.

Recorded the time for discoloration

Observation:

Result:

7. CLINICAL MICROBIOLOGY

i) ISOLATION OF NORMAL FLORA FROM MOUTH

Exp: 7
Date:

Aim:

To determine the presence of various microorganisms present in human mouth.

Principle:

The human body provides a rich and inviting environment for many bacteria. The microorganisms that are permanently associated with a person are known as one's normal or indigenous flora. Typically, these bacteria help maintain the health of the individual but may under certain circumstances become opportunistic pathogens. There are numerous distinct sites or niches on or in the human body each harboring its own specialized microflora..

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Materials required:

Blood agar plate, Tongue depressor, Sterile swabs, Sterile saline etc.

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Procedure:

Preparation of Blood Agar Plate (BAP):

1. Prepare the blood agar base as instructed by the manufacturer. Sterilize by autoclaving at 121°C for 15 minutes.
2. Transfer thus prepared blood agar base to a 50°C water bath.
3. When the agar base is cooled to 50°C, add sterile blood aseptically and mix well gently. Avoid the formation of air bubbles. You must have warmed the blood to room temperature at the time of dispensing to molten agar base. *(**Note:** If you are planning to prepare a batch of blood agar plates, prepare few blood agar plates first to ensure that blood is sterile).*

4. Dispense 15 ml amounts to sterile petri plates aseptically
5. Label the medium with the date of preparation and give it a batch number (if necessary).
6. Store the plates at 2-8°C, preferably in sealed plastic bags to prevent loss of moisture. The shelf life of thus prepared blood agar is up to four weeks.

Sample collection and Inoculation :

1.Rotate a sterile swab over the surface of your tongue and gums.
2. Roll the swab over a small -cm square of surface of a blood agar plate, near but not touching one edge.. Rotate the swab fully in this area.
3. Discard the swab in a container of disinfectant.
4. Using an inoculating loop, streak the plate.
5. Incubate the plate (inverted) at 35°C

.***Observation:***

Result:

ii) *INVITRO* DETEREMINATION OF ANTI MICROBIAL SENSITIVITY(AGAR DIFFUSION METHOD)

Exp:
Date:

Aim:

To determine the antimicrobial agents against various micro organisms.

Principle:

Standard suspension of rapidly growing bacteria is inoculated on the surface of Muller Hinton agar plates. Filter paper discs containing specific concentration of antimicrobial agents are pressed on to the surface and incubated at 35^{o}C over night(18-24 hrs) zone of inhibition of growth around each disc is measured and the susceptibility determined.

Materials required:

Muller-Hinton agar, Bacterial strains, Discs containing anti microbial agents, sterile forceps, sterile buds etc.

Procedure:

Preparation of Muller- Hinton agar(MHA):

Weigh the ingredients, Prepare the medium in sterile conical flasks. Autoclave the medium containing flasks at 115^{o} C for 15 min and cool the medium in water bath at 45-50^{o} C.

Pour the plates up to a uniform thickness of approximately 4mm (25 ml /9 cm plates)

Allow the agar to set and store the plates at 4^{o} C. Use them with in one week.

Inoculation:

Inoculate the surface of the MHA plate with the swab. To ensure that the growth is uniform, pass the swab three times over the entire surface, by repeating the procedure.

***Application of anti microbial discs*:**

Arrange the discs containing antimicrobial agents on the surface of the inoculated plates by using sterile forceps. The distance between the discs should be at least 20 mm from one another.

Incubation:

Incubate the inoculated plates with the discs with in 15 min from inoculation. And keep the inoculated plate at 37^{o} C for 18 to 24 hrs and read the results.

Following incubation, the plates were examined for the presence of growth inhibition, which is indicated by a clear zone surrounding the disc. The susceptibility of an organism to a drug is determined by the size of this zone.

Observation:

Result: