KARPAGAM ACADEMY OF HIGHER EDUCATION

(Established Under Section 3 of UGC Act 1956)

Coimbatore – 641 021.

(For the candidates admitted from 2017 onwards)

DEPARTMENT OF MICROBIOLOGY

CLASS: I M.Sc (MB) **BATCH – 2019 -2021**

SUBJECT NAME: Basic Practical I **SUB.CODE:19MBP111**

SEMESTER: I **4H – 2C**

SYLLABUS

Instruction Hours / week: L: 0 T:0 P:4 **Marks:** Internal: **40** External: **60** Total:**100**

End Semester Exam: 9 Hours

COURSE OBJECTIVES

This course is put forward with the objectives of equipping the candidates with practical knowledge on basic techniques involved in the isolation, characterization and identification of different types of microorganism.

COURSE OUTCOME

1. A student able to skillfully isolate and identify the microorganisms using different techniques which is the needed skill in medical laboratories and researchsectors.

EXPERIMENTS

1. Micrometry
2. Measurement of pH
3. Staining techniques: Simple, Gram, Negative and Endospore
4. Motility determination - Hanging drop and SIM inoculation
5. Cultivation of anaerobic microorganisms – Wrights tube – McIntosh anaerobic jar - roll tube methods.
6. Lactophenol cotton blue mounting of fungi - *Aspergillus*sp, *Mucor*sp, *Rhizopus*sp, *Fusarium*sp, *Penicillium* sp
7. Measurement of microbial growth – Viable count – Direct count – Turbidity methods
8. Biochemical characterization

a) Indole Test
b) Methyl RedTest
c) Voges ProskauerTest
d) Citrate utilizationTest
e) TSITest
f) CatalaseTest
g) OxidaseTest
h) UreaseTest
i) NitrateTest
j) Carbohydrate fermentationTest
k) Amino acid utilizationTest
l) Hydrolysis of polymers- Starch, Lipid, Casein,Gelatin.

SUGGESTED READINGS

Aneeja, K.R. (2001). *Experiments in Microbiology, Plant Pathology, Tissue Culture and Mushroom Production Technology*, (3rd ed.)., New Age International (P) Limited Publishers, NewDelhi.
Cappucino, J.G. and Sherman, N., (2001). *MicrobiologyA Laboratory Manual*, (6thed.). Benjamin Cummings, NewYork.
Dubey, R.C., and Maheshwari, D.K., (2002). *Practical Microbiology*, (1sted.). S. Chand and Company Ltd, NewDelhi.
Gunasekaran, P. (1996). *Lab Manual in Microbiology*, (1sted.). New Age International (P) Ltd, Publishers , NewDelhi.

MICROMETRY

Aim

To became familiar with the calculation of microscopic field. To perform an experimental procedure is the measurement of microorganisms.

Principles

Measurement of the dimensions of microorganisms is done under microscope with the help of two micro-scales called 'micrometers'. Both the micrometers have microscopic graduations etched on their surfaces. One of them, the 'ocular micrometer' is a circular glass disc, which fits into the circular shelf inside the eyepiece.

It has arbitrary graduations etched on its surface. However, the distance between the etched graduations is constant for a particular ocular micrometer. The other micrometer, called 'stage micrometer', is a special glass slide, which is clipped to the stage of the microscope. It has standard graduations etched on its surface, which are 10 µ apart.

Calibration:

Using the required objective, the graduations on the ocular micrometer are calibrated against the standard graduations on the stage micrometer. Calibration is required, because the distance between ocular graduations varies depending on the objective being used, which determines the size of the field.

For calibration, both the micrometer etchings are superimposed by rotating the eyepiece. The number of ocular divisions (O.D.) coinciding with the number of stage divisions (S.D.) is found out.

From this, the calibration factor for one ocular division (O.D.) is calculated as follows:

If 10 O.D. coincide with 2 S.D., then

10 O.D. = 2 S.D. = 2 x 10 µ = 20 µ (… 1 S.D. = 10 µ)

1 O.D. = 20/10 µ = 2 µ

Thus, the distance between two adjacent ocular etchings is 2 µ (i.e. calibration factor is 2 µ).

Measurement:

After calibration, the stage micrometer is removed and the microbe, whose dimensions are to be measured, is placed on the stage on a slide and focused. Now, the number of ocular divisions occupied by the microorganism is counted. Then, by multiplying this number of divisions with the calibration factor, the size of the microbe is determined as follows.

If the microbe occupies 6 O.D. in length, then length of microbe = 6 x calibration factor = 6 x 2 = 12 µ.

It is possible to measure the size of microbes by directly observing them on a stage micrometer. This would avoid calibration and calculations. But the stage micrometer is a standard scale, which is costly because of the micro etchings on it. If used frequently for direct observation, its etchings get worn away.

Moreover, the etchings on the stage micrometer are more widely spaced (about 10 times) than those on the ocular micrometer. Thus, the dimensions cannot be precisely determined with a stage micrometer. That is why; it is never used for direct measurement.

Materials Required

1. Ocular micrometer
2. Stage micrometer
3. Clean glass slides
4. Microbial culture
5. Bunsen burner

6. Microscope
7. Lens paper and lens cleaner
8. Immersion oil

Procedure

1. The eyepiece is removed from the microscope, and its top lid is unscrewed. The lid is removed. Carefully, the eye lens is removed. The ocular micrometer (a circular etched glass piece, which slips into the eye piece) is placed carefully into the eyepiece. The eye lens is placed back and the top lid is screwed to its original condition. The eyepiece is placed back in the microscope.
2. Measurement of the Size of Microbes by Micrometry
3. The stage micrometer is clipped to the stage and the etchings centered by moving the mechanical stage.
4. The low power objective is taken to position.
5. The eyepiece is rotated till the etchings on both the micrometers superimpose.
6. The required objective is taken to position. The required objective is that, using which the whole microorganism can be viewed and it covers the microscopic field to the maximum possible extent.
7. With the required objective in position (for oil-immersion objective, a drop of oil is put on the stage micrometer) the mechanical stage is moved, so that a line on the stage micrometer coincides with a line on the ocular micrometer. Then, another line is searched on the ocular micrometer, which coincides with another line on the stage micrometer. The number of divisions between the coinciding lines is counted for both the micrometers.
8. The calibration factor for the objective used is calculated.
9. In a similar way, calibration factors are calculated for the other objectives.
10. The stage micrometer is removed.
11. The slide containing the microbe to be observed is placed on the stage and focused.
12. The number of ocular divisions covered by the microbe is counted by viewing through the eyepiece.
13. The size of the microorganism is determined by multiplying the number of ocular divisions covered by the microbe with the calibration factor.

Result

The length of the given organism is identified as 0.69µm.

Diagramatic representation of Micrometry Procedure

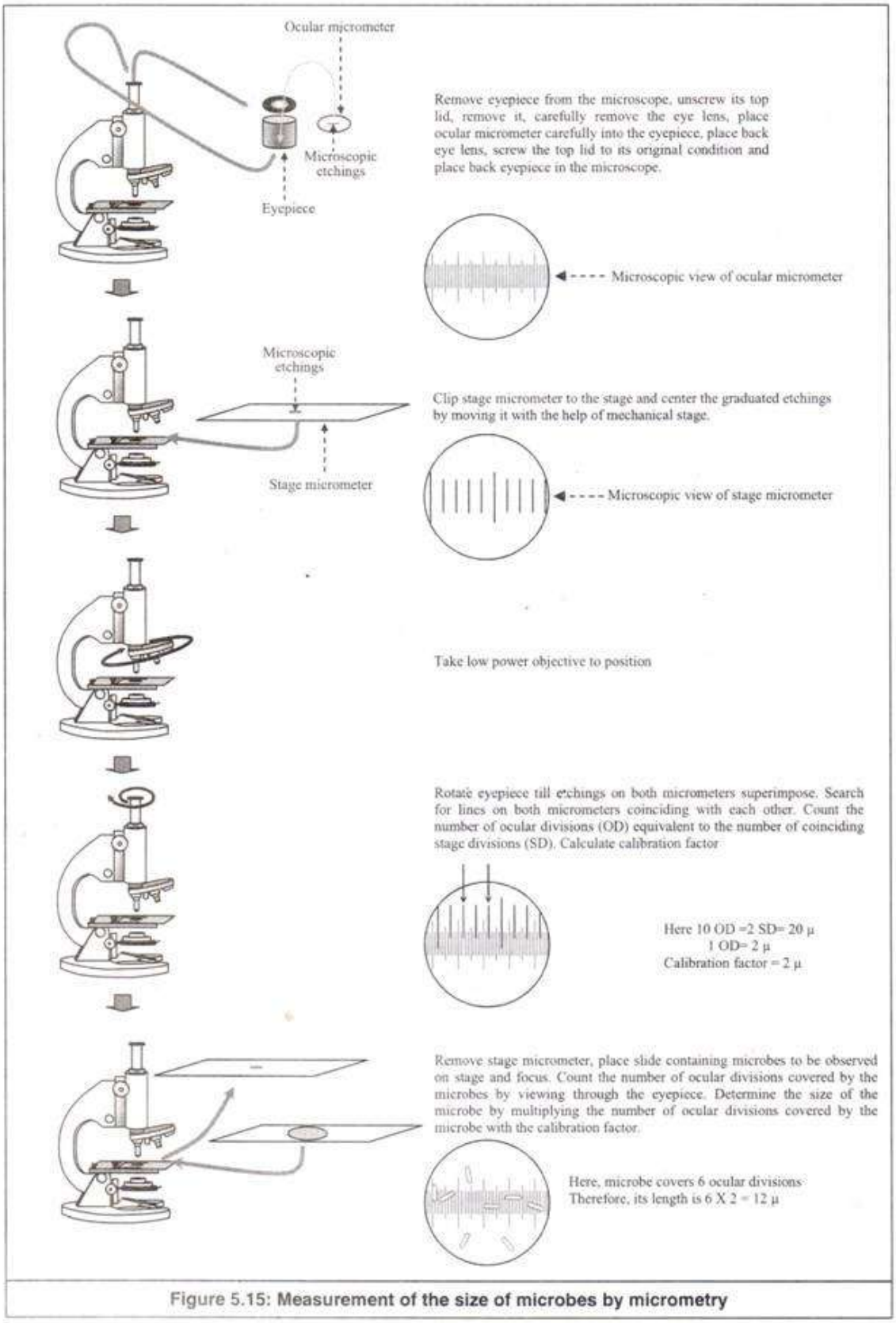


Figure 5.15: **Measurement of the size of microbes by micrometry**

MEASUREMENT OF pH

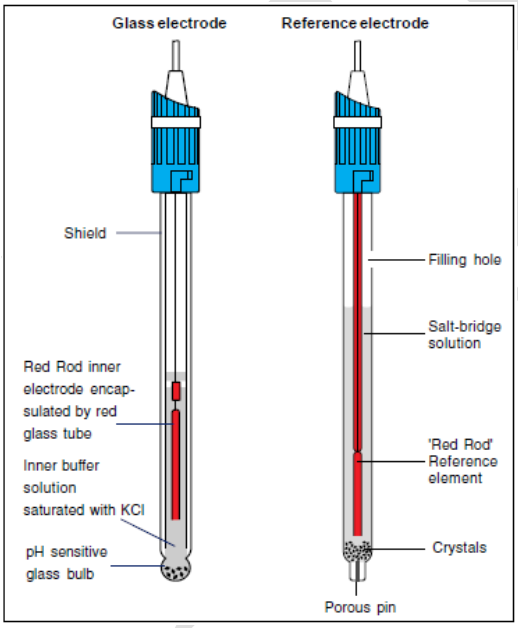
Introduction

pH is an abbreviation of "pondus hydrogenii" and was proposed by the Danish scientist S.P.L. Sørensen in 1909 in order to express the very small concentrations of hydrogen ions. In 1909, pH was defined as the negative base 10 logarithm of the hydrogen ion concentration. However, as most chemical and biological reactions are governed by the hydrogen ion activity, the definition was quickly changed. As a matter of fact, the first potentiometric methods used actually resulted in measurements of ion activity. The definition based on hydrogen ion activity is the definition we use today:

$$pH = - \log 10 [H^+]$$

To became familiar with the calculation of microscopic field. To perform an experimental procedure is the measurement of microorganisms.

The construction of glass indicator electrodes and reference electrodes can be made in various ways. A typical glass electrode and a typical calomel reference electrode are shown below.

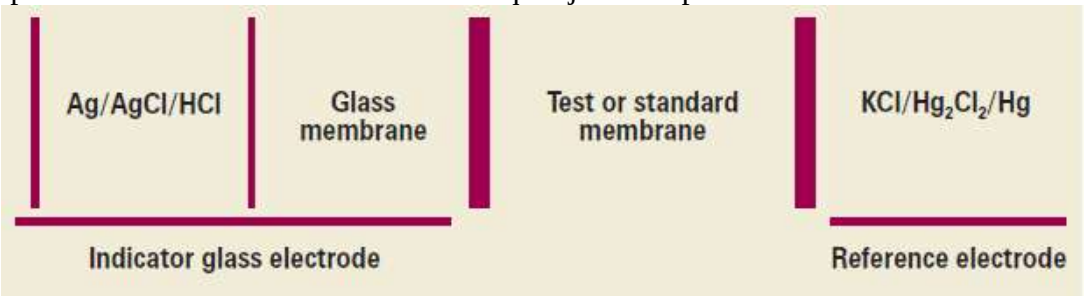


Both the composition of the **glass electrode's** pH-sensitive glass and the composition of the glass electrode's inner solution have an influence on the potential which will develop. The response of the electrode is the voltage developed between the inside and outside of the membrane. This voltage is proportional to the difference in pH in the inner solution and in the sample. The response is caused by an exchange at both surfaces of the swollen membrane between the ions of the glass and the H+ ions of the solution - an ion exchange which is controlled by the concentration of H+ in both solutions. As the structure of the glass membrane may not be uniform, an asymmetry potential may develop even if pH is the same on both sides.

The **reference electrode** shown on the previous page is a saturated silver/ silver chloride electrode (Ag/AgCl) where the two components and the KCl are encapsulated in a red tube. The red tubing affords protection from the harmful effects of light. Contact with the chamber is made by means of a platinum wire and the Ag/AgCl is surrounded by a saturated solution of KCl. The liquid junction, i.e. contact to the measuring solution, is achieved through a

porous ceramic pin. The potential which occurs is determined by the solubility product of the silver chloride and the concentration of the KCl solution and is therefore constant.

A similar electrode construction can be made using mercury and mercurous chloride (calomel) instead. Such electrodes are not suitable for varying temperatures or temperatures above 60°C. The potential of the reference electrode should be independent of the sample solution. This ideal situation will occur if all transport in the porous pin only involves the K+ and Cl- ions, and if they move at the same speed. This is the case in most samples in the pH range 1 to 13 and when a saturated or 3 M KCl salt-bridge solution is used. Deviation from this optimal situation creates the so-called liquid junction potential.



In order to measure an electrode potential, another voltage source (such as another metal/solution interface) is needed to measure it against. Each of the electrodes is called a half-cell. The two half-cells arranged together constitute an electrochemical cell, in which one of the half-cells maintains a constant voltage. The electrode in the halfcell with the constant voltage is called the **reference electrode**, whereas the variable voltage portion is termed the **indicator electrode**. It is possible to measure the potential difference between these two electrodes and calculate the concentration of ions in the solution of the indicator (measuring) electrode.

For example, if a silver wire is immersed in a solution of silver chloride, ionisation of the silver metal occurs, with the formation of silver ions (Ag+) and electrons. An electric potential now exists between the wire and the solution. If two half-cells are used, each with a silver wire or foil immersed in a different silver solution, and the two solutions are connected through a meter, a difference in potential can be detected between them.

As the potential of each solution depends on the concentration of silver ions in it, the concentration of ions in one solution can be predicted if the value for the other solution and the difference in potential between them are known. A temperature difference between the two half-cells would affect the reproducibility of measurement. Other minor technical factors, such as coating (eg protein), will affect the measurements. Potentiometric methods are based on the quantitative relationship between the potential of a cell as given by the following distribution of potential:

Ecell = Ereference + Eindicator + Ejunction

Because the reference and junction potentials are constant, the indicator potential can be determined. The potential of the indicator electrode can then be related to concentration. Electrode systems used in biomedical laboratories have precalibrated read-out devices that give results in concentration units.

The **cell potential** is related to concentration through the **Nernst equation**, which, in its simple form, is E=KT log (C1/C2) where K = Constant 2.3 (R/zF) and if either C1 or C2 is known then the concentration can be calculated from the measurement of the potential developed.

Procedure for Measurement of pH

1. The electrode was rinsed with distilled water and wiped gently with tissue paper.

2. The electrode was dipped into a standard buffer solution when pH is accurately known without touching the side and bottom of the beaker. The pH meter was then adjusted to the standard pH using the pH adjusting knob.
3. The standard buffer system was removed and the electrode was again rinsed with distilled water and copied with tissue paper.
4. The electrode was dipped in the solution where pH was to be determined.
5. The pH of the test solution was read directly by the dial.
6. The electrode was again washed and rinsed in distilled water

Result

The pH of the given solution was found to be 7.2.

STAINING TECHNIQUES - SIMPLE STAINING

Aim

To gains expertise in using some simple stains to observe the morphology and characteristics of four different bacteria

Principles

While negative staining is satisfactory when making simple observations on bacterial morphology and size, more specific stains are necessary if bacterial detail is to be observed. One way of achieving this detail involves smear preparation and simple staining. A **bacterial smear** is a dried preparation of bacterial cells on a glass slide. In a bacterial smear that has been properly processed, (1) the bacteria are evenly spread out on the slide in such a concentration that they are adequately separated from one another, (2) the bacteria are not washed off the slide during staining, and (3) bacterial form is not distorted.

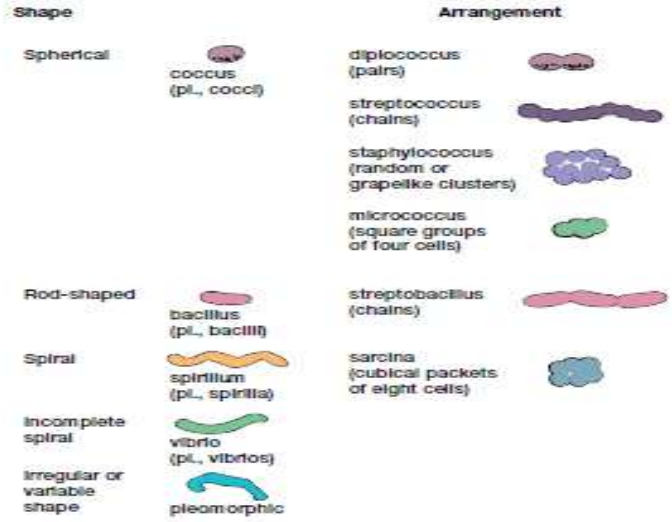
In making a smear, bacteria from either a broth culture or an agar slant or plate may be used. If a slant or plate is used, a *small* amount of bacterial growth is transferred to a drop of water on a glass slide and mixed. The mixture is then spread out evenly over a large area on the slide. One of the most common errors in smear preparation from agar cultures is the use of too large an inoculum. This invariably results in the occurrence of large aggregates of bacteria piled on top of one another. If the medium is liquid, place one or two loops of the medium directly on the slide and spread the bacteria over a large area. Allow the slide to air dry at room temperature. After the smear is dry, the next step is to attach the bacteria to the slide by **heat-fixing.** This is accomplished by gentle heating, passing the slide several times through the hot portion of the flame of a Bunsen burner. Most bacteria can be **fixed** to the slide and killed in this way without serious distortion of cell structure.

The use of a single stain or dye to create contrast between the bacteria and the background is referred to as **simple staining.** Its chief value lies in its simplicity and ease of use. Simple staining is often employed when information about cell shape, size, and arrangement is desired. In this procedure, one places the heatfixed slide on a staining rack, covers the smear with a small amount of the desired stain for the proper amount of time, washes the stain off with water for a few seconds, and, finally, blots it dry. Basic dyes such as **crystal violet** (20 to 30 seconds staining time), **carbolfuchsin** (5 to 10 seconds staining time), or **methylene blue** (1 minute staining time) are often used. Once bacteria have been properly stained, it is usually an easy matter to discern their overall shape. Bacterial morphology is usually uncomplicated and limited to one of a few variations.

Materials Required

1. 24- to 48-hour tryptic soy broth or agar slants of *Staphylococcus aureus*, *Bacillus cereus* and *E.coli*
2. Microscope
3. Clean microscope slides
4. Blotting paper
5. Inoculating loop and needle
6. Sterile distilled water
7. Bunsen burner
8. Crystal violet (1% aqueous solution)
9. Immersion oil
10. Lens paper and lens cleaner
11. Slide holder or clothespin
12. Slide warmer

Common Bacterial Shapes

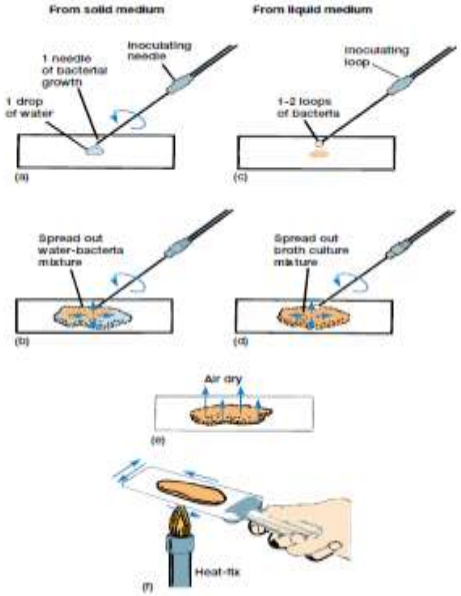


Procedure

Smear Preparation

1. With the wax pencil, mark the name of the bacterial culture in the far left corner on each of three slides.
2. For the broth culture, shake the culture tube and, with an inoculating loop, aseptically transfer 1 to 2 loopfuls of bacteria to the center of the slide. Spread this out to about a d-inch area. When preparing a smear from a slant or plate, place a loopful of water in the center of the slide. With the inoculating needle, aseptically pick up a *very small* amount of culture and mix into the drop of water. Spread this out as above.
3. Allow the slide to air dry, or place it on a slide warmer.
4. Pass the slide through a Bunsen burner flame three times to heat-fix and kill the bacteria.

Bacterial Smear Preparation



Simple Staining

1. Place the three fixed smears on a staining loop or rack over a sink or other suitable receptacle.
2. Stain one slide with alkaline methylene blue for 1 to 1d minutes; one slide with carbolfuchsin for 5 to 10 seconds; and one slide with crystal violet for 20 to 30 seconds.
3. Wash stain off slide with water for a few seconds.
4. Blot slide dry with bibulous paper. Be careful not to rub the smear when drying the slide because this will remove the stained bacteria.
5. Examine under the oil immersion lens and complete the report for this exercise.
6. You may want to treat smears of the same bacterium with all three stains in order to compare them more directly. It is also instructive to cover bacterial smears for varying lengths of time with a given stain in order to get a feel for how reactive they are and the results of overstaining or understaining a slide preparation.

Simple Staining Procedure

RESULT

The stained organism was appeared violet in colour. The results were tabulated

S.No	Organism	Arrangements
1	*Staphylococcus aureus*	Cocci in Cluster
2	*Bacillus cereus*	Long rod arrangement in cluster
3	*E.coli*	Rod shaped bacilli

STAINING TECHNIQUES - GRAM STAINING

Aim

1. Understand the biochemistry underlying the Gram stain
2. Understand the theoretical basis for differential staining procedures
3. Differentiate a mixture of bacteria into gram-positive and gram-negative cells

Principles

Simple staining depends on the fact that bacteria differ chemically from their surroundings and thus can be stained to contrast with their environment. Bacteria also differ from one another chemically and physically and may react differently to a given staining procedure. This is the principle of **differential staining.** Differential staining can distinguish between types of bacteria. The **Gram stain** (named after Christian Gram, Danish scientist and physician, 1853–1938) is the most useful and widely employed differential stain in bacteriology. It divides bacteria into two groups— **gram negative** and **gram positive.**

The first step in the procedure involves staining with the basic dye crystal violet. This is the **primary stain.** It is followed by treatment with an iodine solution, which functions as a **mordant;** that is, it increases the interaction between the bacterial cell and the dye so that the dye is more tightly bound or the cell is more strongly stained. The smear is then decolorized by washing with an agent such as 95% ethanol or isopropanol-acetone. Gram-positive bacteria retain the crystal violet-iodine complex when washed with the decolorizer, whereas gram-negative bacteria lose their crystal violet-iodine complex and become colorless.

Finally, the smear is **counterstained** with a basic dye, different in color than crystal violet. This counterstain is usually safranin. The safranin will stain the colorless, gram-negative bacteria pink but does not alter the dark purple color of the gram-positive bacteria. The end result is that gram-positive bacteria are deep purple in color and gram-negative bacteria are pinkish to red in color.

The Gram stain does not always yield clear results. The Gram stain does not always yield clear results. Species will differ from one another in regard to the ease with which the crystal violet-iodine complex is removed by ethanol. Gram-positive cultures may often turn gram negative if they get too old. Thus, it is always best to Gram stain young, vigorous cultures rather than older ones. Furthermore, some bacterial species are **gram variable.** That is, some cells in the same culture will be gram positive and some, gram negative.

Therefore, one should always be certain to run Gram stains on several cultures under carefully controlled conditions in order to make certain that a given bacterial "strain" is truly gram positive or gram negative. Indistinct Gram-stain results can be confirmed by a simple test using KOH. Place a drop of 10% KOH on a clean glass slide and mix with a loopful of bacterial paste. Wait 30 seconds, and then pull the loop slowly through the suspension and up and away from the slide. A gram-negative organism will produce a mucoid string; a gram-positive organism remains fluid.

Materials Required

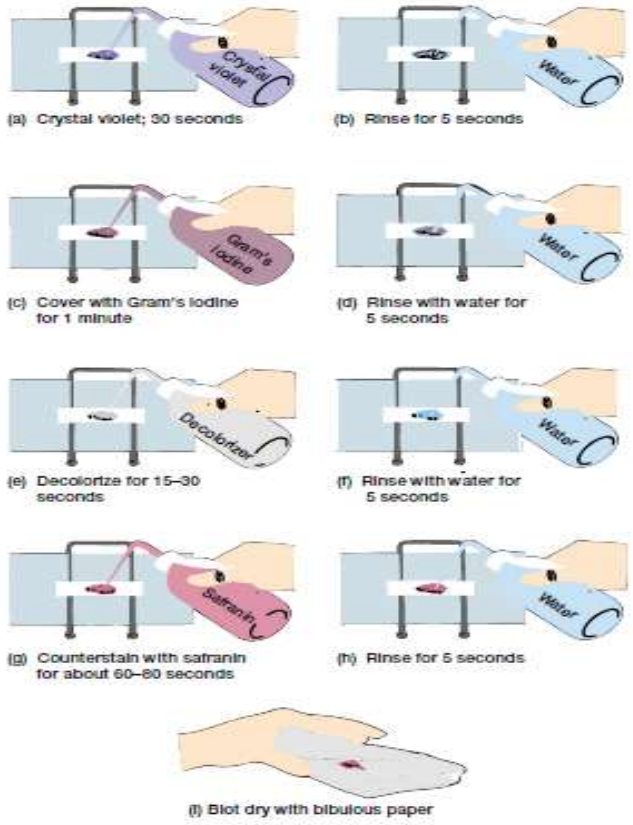
9. 18- to 24-hour tryptic soy broth cultures of Staphylococcus *aureus, Escherichia coli* and a mixture of *S. aureus and E. coli*
10. Solutions of crystal violet
11. Gram's iodine (2 g potassium iodide in 300 ml distilled water plus 1 g iodine crystals)
12. 95% ethanol and/or isopropanol-acetone mixture (3:1 v/v)
13. Safranin
14. Clean glass slides
15. Inoculating loop
16. Bunsen burner

17. Bibulous paper
18. Microscope
19. Lens paper and lens cleaner
20. Immersion oil

Procedure for Gram-Stain Technique

14. Prepare heat-fixed smears of *E. coli, S. aureus*, and the mixture of *E. coli* and *S. aureus* .
15. Place the slides on the staining rack.
16. Flood the smears with crystal violet and let stand for 30 seconds.
17. Rinse with water for 5 seconds.
18. Cover with Gram's iodine mordant and let stand for 1 minute.
19. Rinse with water for 5 seconds.
20. Decolorize with 95% ethanol for 15 to 30 seconds.
21. Do not decolorize too long. Add the decolorize drop by drop until the crystal violet fails to wash from the slide. Alternatively, the smears may be decolorized for 30 to 60 seconds with a mixture of isopropanol-acetone (3:1 v/v).
22. Rinse with water for 5 seconds.
23. Counterstain with safranin for about 60 to 80 seconds. Safranin preparations vary considerably in strength, and different staining times may be required for each batch of stain
24. Rinse with water for 5 seconds
25. Blot dry with bibulous paper and examine under oil immersion. Gram-positive organisms stain blue to purple; gram-negative organisms stain pink to red. There is no need to place a coverslip on the stained smear.

Gram-stain Procedure



Result

Purple colour cells were observed. Purple colour cells identified as a gram positive where as pink colour cells were identified as gram negative.

STAINING TECHNIQUES - NEGATIVE STAINING

Aim

1. Understand the reason for the negative staining procedure
2. to observe the morphology of given organism

Principles

Sometimes it is convenient to determine overall bacterial morphology without the use of harsh staining or heat-fixing techniques that change the shape of cells. This might be the case when the bacterium does not stain well (e.g., some of the spirochetes) or when it is desirable to confirm observations made on the shape and size of bacteria observed in either a wet-mount or hanging drop slide. Negative staining is also good for viewing capsules.

Negative, indirect, or **background staining** is achieved by mixing bacteria with an acidic stain such as nigrosin, India ink, or eosin, and then spreading out the mixture on a slide to form a film. The above stains will not penetrate and stain the bacterial cells due to repulsion between the negative charge of the stains and the negatively charged bacterial wall. Instead, these stains either produce a deposit around the bacteria or produce a dark background so that the bacteria appear as unstained cells with a clear area around them.

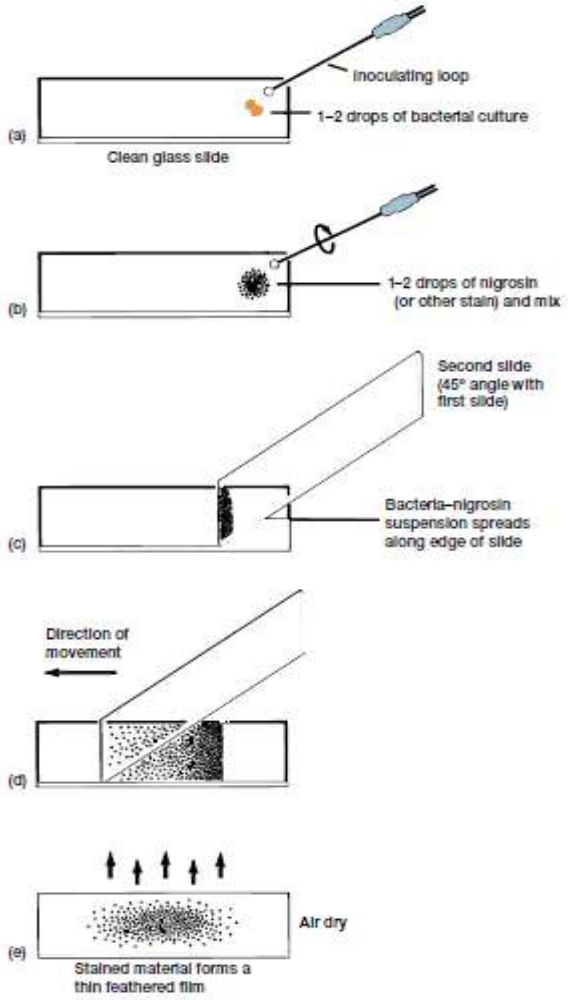
Materials Required

1. 24- to 48-hour tryptic soy broth cultures of *Bacillus subtilis, Klebsiella*
2. Dorner's nigrosin solution, India ink, or eosin blue
3. Clean microscope slides
4. Inoculating loop
5. Immersion oil
6. Microscope
7. Lens paper and lens cleaner
8. Marker pen
9. Bunsen burner

Procedure

1. With a wax pencil, label the left-hand corner of three glass slides with the names of the respective bacteria.
2. Use an inoculating loop to apply a small amount of bacteria to one end of a clean microscope slide.
3. Add 1 to 2 loops of nigrosin, India ink, or eosin solution to the bacteria and mix thoroughly.
4. Spread the mixture over the slide using a second slide. The second slide should be held at a 45° angle so that the bacteria-nigrosin solution spreads across its edge. The slide is then pushed across the surface of the first slide in order to form a smear that is thick at one end and thin at the other. This is known as a thin smear.
5. Allow the smear to air dry. **Do not heat-fix**.
6. With the low-power objective, find an area of the smear that is of the optimal thickness for observation.
7. Use the oil immersion lens to observe and report the morphology of given bacterial species

Diagramatic representation of Negative Staining and Thin Smear Preparation



Result:

The morphology of the given bacterial species was observed under oil immersion.

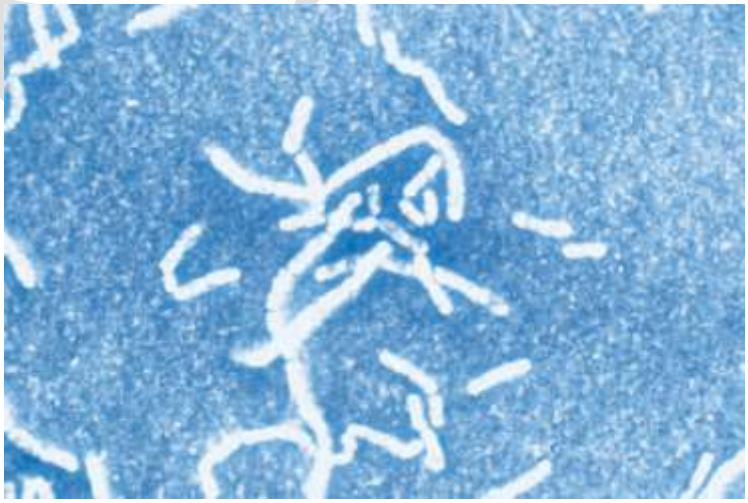


Fig: India ink Stain of *Bacillus* (×1,000).
Notice the dark background around the clear bacterial cells.

STAINING TECHNIQUES - ENDOSPORE STAINING

Aim:

1. Understand the biochemistry underlying endospore staining
2. Perform an endospore stain
3. Differentiate between bacterial endospore and vegetative cell forms

Principles

Bacteria in genera such as *Bacillus* and *Clostridium* produce quite a resistant structure capable of surviving for long periods in an unfavorable environment and then giving rise to a new bacterial cell. This structure is called an **endospore** since it develops within the bacterial cell. Endospores are spherical to elliptical in shape and may be either smaller or larger than the parent bacterial cell. Endospore position within the cell is characteristic and may be central, subterminal, or terminal.

Endospores do not stain easily, but, once stained, they strongly resist decolorization. This property is the basis of the **Schaeffer-Fulton** (Alice B. Schaeffer and MacDonald Fulton were microbiologists at Middlebury College, Vermont, in the 1930s) or **Wirtz-Conklin method** (Robert Wirtz and Marie E. Conklin were bacteriologists in the early 1900s) of staining endospores. The endospores are stained with malachite green. Heat is used to provide stain penetration. The rest of the cell is then decolorized and counterstained a light red with safranin.

Materials per Student

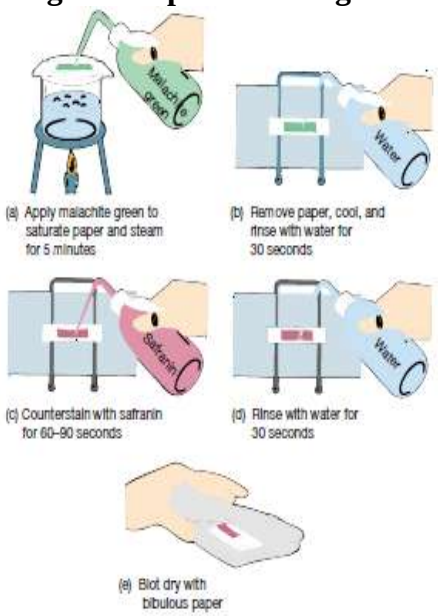
1. 24- to 48-hour nutrient agar slant cultures of *Bacillus.*
2. Clean glass slides
3. Microscope
4. Immersion oil
5. Marker pen
6. Inoculating loop
7. Hot plate or boiling water bath with staining rack or loop
8. 5% malachite green solution
9. Safranin
10. Blotting paper
11. Lens paper and lens cleaner
12. Slide warmer
13. Forceps

Procedure

1. With a wax pencil, place the names of the respective bacteria on the edge of four clean glass slides.
2. As shown in figure 14.3, aseptically transfer one species of bacterium with an inoculating loop to each of the respective slides, air dry (or use a slide warmer), and heat-fix. 3.
3. Place the slide to be stained on a hot plate or boiling water bath equipped with a staining loop or rack. Cover the smear with paper toweling that has been cut the same size as the microscope slide.
4. Soak the paper with the malachite green staining solution. Gently heat on the hot plate (just until the stain steams) for 5 to 6 minutes after the malachite green solution begins to steam. Replace the malachite green solution as it evaporates so that the paper remains saturated during heating. Do not allow the slide to become dry.

5. Remove the paper using forceps, allow the slide to cool, and rinse the slide with water for 30 seconds.
6. Counterstain with safranin for 60 to 90 seconds.
7. Rinse the slide with water for 30 seconds.

Fig: Endospore Staining Procedure.



Result:

Green round spore surrounded by pink coloured vegetative cells were observed.

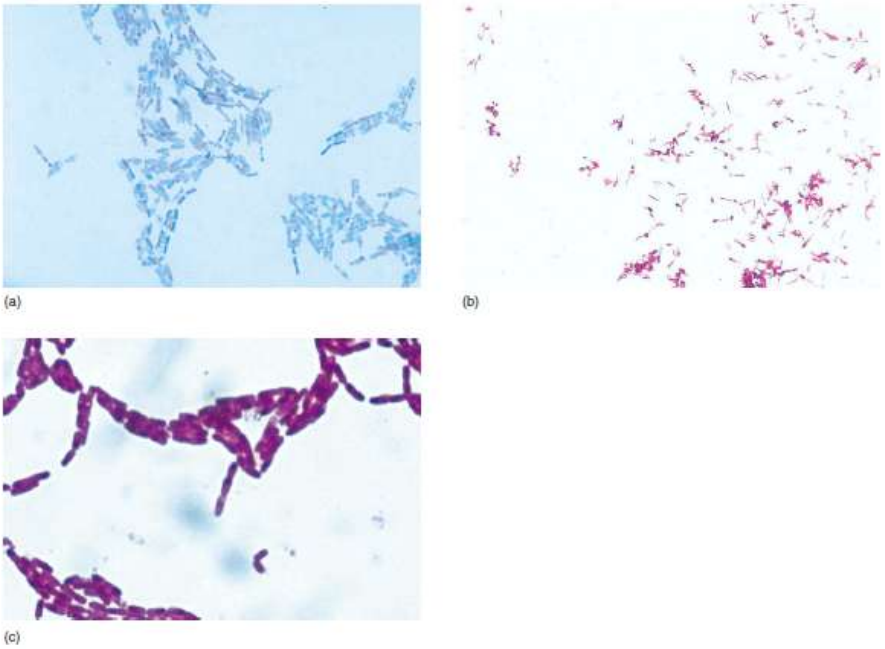


Fig: Examples of Endospores. (**a**) Central spores of *Bacillus* stained with malachite green and counterstained with safranin (×1,000). Notice that the cells are rod-shaped and straight, often arranged in pairs or chains, with rounded squared ends. The endospores are oval and not more than one spore per cell. (**b**) *Clostridium tetani* showing round, terminal spores that usually distend the cell (×1,000). Notice that the cells are rod-shaped and are often arranged in pairs or short chains with rounded or sometimes pointed ends. (**c**) *Bacillus megaterium* showing short oval to elongate spores.

MOTILITY DETERMINATION - HANGING DROP METHOD

Aim

To gain expertise in making hanging drop slides and observing the motility of living bacteria

Principle

Many bacteria show no motion and are termed nonmotile. However, in an aqueous environment, these same bacteria appear to be moving erratically. This erratic movement is due to Brownian movement. Brownian movement results from the random motion of the water molecules bombarding the bacteria and causing them to move. True motility (self-propulsion) has been recognized in other bacteria and involves several different mechanisms. Bacteria that possess flagella exhibit flagella motion. Helical-shaped spirochetes have axial fibrils (modified flagella that wrap around the bacterium) that form axial filaments. These spirochetes move in a corkscrew- and bending-type motion. Other bacteria simply slide over moist surfaces in a form of gliding motion. The above types of motility or non motility can be observed over a long period in a hanging drop slide. Hanging drop slides are also useful in observing the general shape of living bacteria and the arrangement of bacterial cells when they associate together. A ring of Vaseline around the edge of the coverslip keeps the slide from drying out.

Materials Required

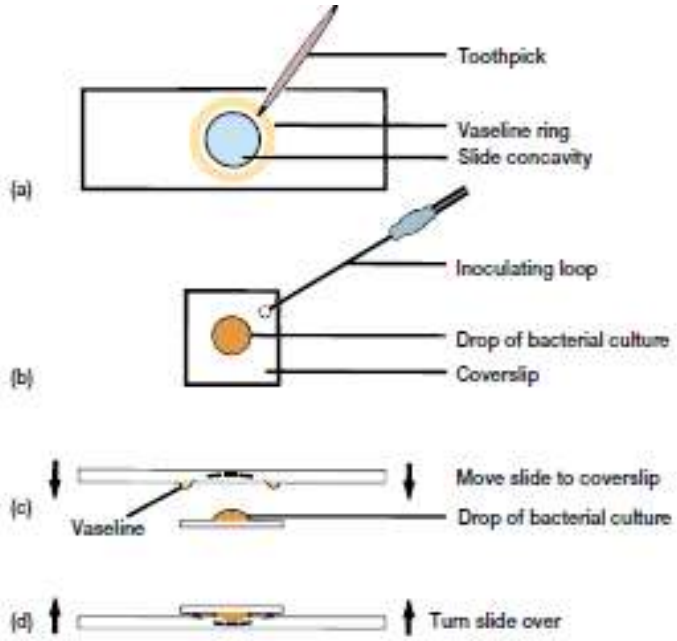
1. 24- to 48-hour tryptic soy broth cultures of *Pseudomonas aeruginosa*, *Bacillus cereus* and *Escherichia coli*
2. Microscope
3. Lens paper and lens cleaner
4. Immersion oil
5. Clean depression slides and coverslips
6. Petroleum jelly (Vaseline)
7. Inoculating loop
8. Toothpicks
9. Bunsen burner

Procedure

1. With a toothpick, spread a small ring of Vaseline around the concavity of a depression slide. Do not use too much Vaseline.
2. After thoroughly mixing one of the cultures, use the inoculating loop to aseptically place a small drop of one of the bacterial suspensions in the center of a coverslip.
3. Lower the depression slide, with the concavity facing down, onto the coverslip so that the drop protrudes into the center of the concavity of the slide. Press gently to form a seal.
4. Turn the hanging drop slide over and place on the stage of the microscope so that the drop is over the light hole.
5. Examine the drop by first locating its edge under low power and focusing on the drop. Switch to the high-dry objective and then, using immersion oil, to the 90 to 100× objective.
6. In order to see the bacteria clearly, close the diaphragm as much as possible for increased contrast. Note bacterial motility. Be careful to distinguish between motility and Brownian movement.

7. Discard your coverslips and any contaminated slides in a container with disinfectant solution.

Preparation of a Hanging Drop Slide



Result

Active motility of motile cells and stable non motile cells were observed.

MOTILITY DETERMINATION - SIM INOCULATION METHOD

Aim

To determine the motility of living bacteria by using SIM inoculation method

Principles

The SIM medium (named after J. S. Simmons in 1926) contains peptones and sodium thiosulfate as substrates, and ferrous ammonium sulfate, $Fe(NH_4)SO_4$, as the H_2S indicator. Cysteine is a component of the peptones used in SIM medium. Sufficient agar is present to make the medium semisolid. Once H_2S is produced, it combines with the ferrous ammonium sulfate, forming an insoluble, black ferrous sulfide precipitate that can be seen along the line of the stab inoculation. If the organism is also motile, the entire tube may turn black. This black line or tube indicates a positive H2S reaction; absence of a black precipitate indicates a negative reaction.
SIM agar may also be used to detect the presence or absence of motility in bacteria as well as indole production. Motility is present when the growth of the culture is not restricted to the stab line of the inoculation. Growth of nonmotile bacteria is confined to the line of inoculation. One can also use semisolid media (motility test medium deeps) to determine whether a bacterial strain is motile. During growth, motile bacteria will migrate from the line of inoculation to form a dense turbidity in the surrounding medium; nonmotile bacteria will grow only along the line of the inoculation.

Materials Required

1. 24- to 48-hour tryptic soy broth cultures of *Klebsiella* sp, *Proteus* sp, and *Salmonella* sp
2. Bunsen burner
3. Inoculating needle
4. Test-tube rack
5. 3 SIM (sulfide-indole-motility) agar deeps
6. Incubator set at 35°C
7. Marker pens

Procedure

First Period

1. Label each of the SIM agar deep tubes with the name of the bacterium to be inoculated, your name, and date.
2. Using aseptic technique, inoculate each tube with the appropriate bacterium by stabbing the medium f of the way to the bottom of the tube.
3. Incubate the cultures for 24 to 48 hours at 35°C.

Second Period

1. Examine the SIM cultures for the presence or absence of a black precipitate along the line of the stab inoculation. A black precipitate of FeS indicates the presence of H_2S.
2. Based on your observations, determine and record in the report for exercise 27 whether or not each bacterium was capable of H_2S production, and the presence (+) or absence (–) of motility.

Result

S.NO	Organism	Motility
1	*Klebsiella* sp	Non Motile
2	*Proteus* sp	Motile
3	*Salmonella* sp	Motile

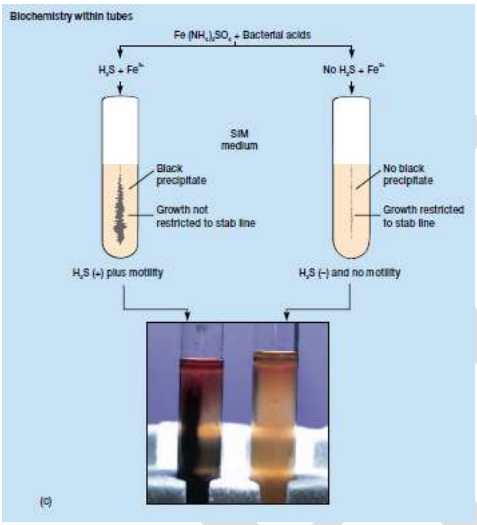


Fig: Motility test using SIM agar. It should be noted that not all bacteria are either H2S positive with motility or H2S negative and no motility. Many other possible combinations exist.

CULTIVATION OF ANAEROBIC MICROBES

Respiration is an energy yielding process in which electrons from oxidisable substrate are transferred via series of oxidation-reduction reaction to an exogenous terminal electron transport chain. The principle gases that effect bacterial growth are oxygen and carbon dioxide. Microbes are buffer on their ability to use oxygen for cellular respiration. Hence it is convenient to divide into 4 groups:

1: Obligate aerobes need oxygen because they cannot ferment or respire anaerobically. They gather at the top of the tube where the oxygen concentration is highest.

2: Obligate anaerobes are poisoned by oxygen, so they gather at the bottom of the tube where the oxygen concentration is lowest.

3: Facultative anaerobes can grow with or without oxygen because they can metabolise energy aerobically or anaerobically. They gather mostly at the top because aerobic respiration generates more adenosine triphosphate (ATP) than either fermentation or anaerobic respiration.

4: Microaerophiles need oxygen because they cannot ferment or respire anaerobically. However, they are poisoned by high concentrations of oxygen. They gather in the upper part of the test tube but not the very top.

5: Aerotolerant organisms do not require oxygen as they metabolise energy anaerobically. Unlike obligate anaerobes however, they are not poisoned by oxygen. They can be found evenly spread throughout the test tube.

Aerobes can survive in the presence of oxygen only by virtue of an elaborate system of defenses. Without these defenses, key enzyme systems in the organisms fail to function and the organisms die. Obligate anaerobes, which live only in the absence of oxygen, do not possess the defenses that make aerobic life possible and therefore cannot survive in air.
During growth and metabolism, oxygen reduction products are generated within microorganisms and secreted into the surrounding medium. The superoxide anion, one oxygen reduction product, is produced by univalent reduction of oxygen:

$$O_2 \xrightarrow{e^-} O_2^-$$

It is generated during the interaction of molecular oxygen with various cellular constituents, including reduced flavins, flavoproteins, quinones, thiols, and iron-sulfur proteins. The exact process by which it causes intracellular damage is not known; however, it is capable of participating in a number of destructive reactions potentially lethal to the cell. Moreover, products of secondary reactions may amplify toxicity. For example, one hypothesis holds that the superoxide anion reacts with hydrogen peroxide in the cell:

$$O_2^- + H_2O_2 \rightarrow OH^- + OH^{\cdot} + O_2$$

This reaction, known as the Haber-Weiss reaction, generates a free hydroxyl radical (OH·), which is the most potent biologic oxidant known. It can attack virtually any organic substance in the cell. A subsequent reaction between the superoxide anion and the hydroxyl radical produces singlet oxygen (O_2*), which is also damaging to the cell:

$$O_2^- + OH \rightarrow OH + O_2^*$$

The excited singlet oxygen molecule is very reactive. Therefore, superoxide must be removed for the cells to survive in the presence of oxygen.

Most facultative and aerobic organisms contain a high concentration of an enzyme called superoxide dismutase. This enzyme converts the superoxide anion into ground-state oxygen and hydrogen peroxide, thus ridding the cell of destructive superoxide anions:

$$2O_2^- + 2H^+ \xrightarrow{\text{Superoxide Dismutase}} O_2 + H_2O_2$$

The hydrogen peroxide generated in this reaction is an oxidizing agent, but it does not damage the cell as much as the superoxide anion and tends to diffuse out of the cell. Many organisms possess catalase or peroxidase or both to eliminate the H_2O_2. Catalase uses H_2O_2 as an oxidant (electron acceptor) and a reductant (electron donor) to convert peroxide into water and ground-state oxygen:

$$H_2O_2 + H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

Peroxidase uses a reductant other than H_2O_2:

$$H_2O_2 + H_2R \xrightarrow{\text{Peroxidase}} 2H_2O + R$$

One study showed that facultative and aerobic organisms lacking superoxide dismutase possess high levels of catalase or peroxidase. High concentrations of these enzymes may alleviate the need for superoxide dismutase, because they effectively scavenge H_2O_2 before it can react with the superoxide anion to form the more active hydroxyl radical. However, most organisms show a positive correlation between the activity of superoxide dismutase and resistance to the toxic effects of oxygen.

In another study, facultative and aerobic organisms demonstrated high levels of superoxide dismutase. The enzyme was present, generally at lower levels, in some of the anaerobes studied, but was totally absent in others. The most oxygen-sensitive anaerobes as a rule contained little or no superoxide dismutase. In addition to the activity of superoxide dismutase, the rate at which an organism takes up and reduces oxygen was determined to be a factor in oxygen tolerance. Very sensitive anaerobes, which reduced relatively large quantities of oxygen and exhibited no superoxide dismutase activity, were killed after short exposure to oxygen. More tolerant organisms reduced very little oxygen or else demonstrated high levels of superoxide dismutase activity.

The continuous spectrum of oxygen tolerance among bacteria appears to be due partly to the activities of superoxide dismutase, catalase, and peroxidase in the cell and partly to the rate at which the cell takes up oxygen. Clearly, other factors influence tolerance: the location of protective enzymes in the cell (surface versus cytoplasm), the rate at which cells form toxic oxygen products (e.g., the hydroxyl radical or singlet oxygen), and the sensitivities of key cellular components to the toxic oxygen products.

CULTIVATION OF ANAEROBIC MICROBES - WRIGHT'S TUBE METHOD

Aim

To cultivate anaerobic microorganism using Wright's tube method

Principle

Wright's tube method is a simple method which does not employ the use of sealed jar. Anaerobic microorganism can be cultivated by this method using solid media in the form of slant. This method pyrogallic acid was used where the crystals combines with few drops of NaOH to evaluate the residual oxygen inside the tube. Then the cotton plug is dipped in the liquid paraffin to avoid further entry of oxygen.

Materials Required

1. Anaerobic media in slant
2. Pyrogallic acid
3. 4% NaOH
4. Liquid paraffin

Procedure

1. Agar slants were prepared and inoculated with the test organism by streaking of the agar slope
2. The cotton plug was cut right on to the mouth of the test tube and pushed further into the tube with the glass rod. Until it nearly touched the slant.
3. A pinch of pyrogallic acid was added and add 2ml of 4% NaOH added on the surface of the mouth of the test tube was closed with a rubber stopper tightly.
4. The tube was inverted to seal the mouth of the tube by dipping in molten paraffin wax.
5. The tubes were reinverted by and incubated for 24-48 hour at 37^{o}C.

Result

Few colonies were appeared on the slant they may be anaerobic organism.

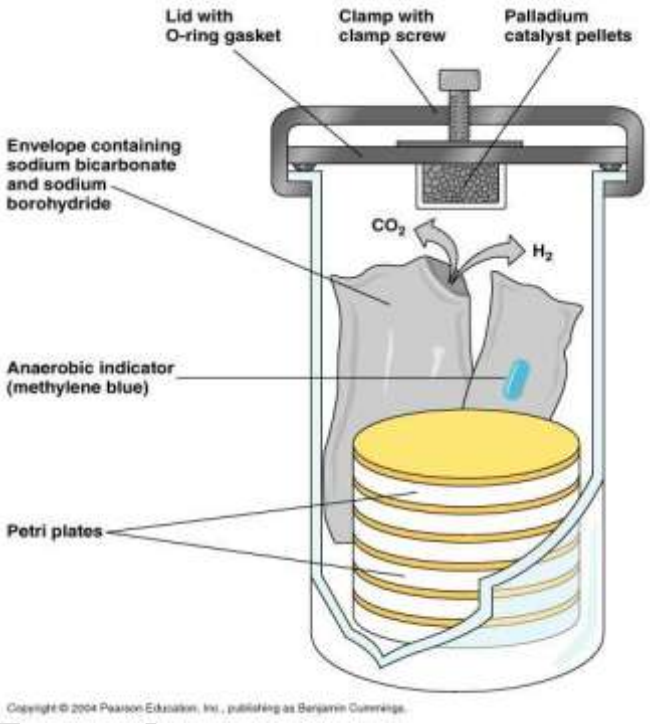
CULTIVATION OF ANAEROBIC MICROBES - Mc INTOSH ANAEROBIC JAR METHOD

Aim

To cultivate anaerobic microorganism using Mc Intosh Jar method

Principle

Mc Intosh jar is widely used for thr cultivation of anaerobes in the presence of a gas pack system. The culture plates are placed in the jar and oxygen is removed by the following process. A packet of chemicals [Na_2CO_3] and sodium borohydridein the jar is moistened by adding few ml of water and the jar is sealed. H and CO_2 are produced by the reaction of chemical with water. Palladium catalysis in the jar combines the oxygen with hydrogen produced by the chemical reaction and water is formed. As a result the oxygen disappears quickly moreover the CO_2 produced aids in the growth of many anaerobic bacteria.



Materials Required

1. Anaerobic media in petri plate
2. Gas packfor generation of H and CO_2
3. Methylene blue

Procedure

1. The organism to be cultivated was streaked on the plate and kept inside the jar.
2. The corner of the gas pack was cut off and placed inside the jar.
3. Few ml of redox indicator methylene blue was poured to the tube which was located on the side of the jar.

4. 10ml of water was added to the gas pack using a pipette the chamber was sealed quickly with lid.
5. The sealed jar was placed inside an incubator at 37°C for 24-48 hours. The tube containing methylene blue was observed for colour change from blue to colourless which is indicative of anaerobic condition.

Result

Few colonies were appeared on the plate they may be anaerobic microorganism.

CULTIVATION OF ANAEROBIC MICROBES - ROLL TUBE METHOD

Obligate anaerobe can be defined as microorganisms which are unable to utilize molecular oxygen for growth. A further differentiation is possible based on their relationship in the presence of oxygen. Aerotolerant anaerobes are only slightly inhibited by significant level of oxygen in atmosphere. Strict anaerobes die or immediately stop growing even on a brief exposure to low level of oxygen. It is there are important to retain anoxic condition during all steps in handling of these microorganisms. Most strict anaerobe requires not only the absence of oxygen to initiate growth but also redox potential below 500mv, which can be only achieved, by the supplementation of media with reducing agent.

Although techniques for growing anaerobes vary in detail among laboratories, these procedures follow the legacy of Robert Hungate who perfected the preparation on pre-reduced media. He developed methods for the exclusion of oxygen in the preparation and sterilized of anoxic media as well as methods for the aseptic inoculation and transfer of anaerobic microbes in media were on O/R potential below (-) 330mvwas maintained. Roll tube method introduced by Hungate is often used instead of conventional plate for isolation and culture of strict anaerobes. In roll tube method the agar medium was distributed as thin layer over the internal surface of test tubes changed with an aerobic atmosphere for the isolation of obligate anaerobic bacteria of the rumen. In the roll tube method, exposure of bacteria and culture media is avoided by displacing the air in the culture vessel with an oxygen free gas such as CO_2, H, N and mixture of these gases. CO_2 is the gas of choice because it is heatier than air; relatively cheap and valuable in buffering vessels are stoppered in conditions preventing access of air the culture require no special incubates and can be removed and examined with no anaerobic precaution if kept stoppered. If opened, anaerobic can be continuously maintained during necessary manipulation and the culture again closed without exposure to oxygen.

Media Preparation

Composition of the media depends upon the kind of anaerobe to be cultivated. The majorirty of anaerobic microorganism in fastidious and requires complex media with many supplements. Three essential steps are (a) use of nature's buffer of CO_2 – bicarbonate carbonate to maintain a pH near neutrality, (b) use of cysteine and sodium sulphide as reducing agents and (c) use of resazurine as an O/B indicator ie., reddish at (-) 330mv with rubber stopper and screw caps with holes. The media were flushed with oxygen free gas $N_2/H_2/CO_2$ or a combination of 2 gases of atleast 30 mins, until the redox indicator (resazurine) turned colourless using his procedure. The Hungate technique is based on the use of passing camular usually, several cannules are connected by acetyl rubber tubing to a manifold supplying oxygen free gas with an over pressure that should be adjusted to appear 0.5 at least two chamber are needed one for vessel to be inoculated or filled with medium to be dispersed when an aseptic gassing of media or culture is necessary a barrel of a glass syringe is packed with counter and fitted between the gassing needle and the butyl rubber tubing.

Steps involved in roll tube Hungate Method

1. Exclusion of oxygen by flushing the tube with the desired gas [$N_2/H_2/C_2$] or mixture of two gases

2. Addition of pre-reduced anaerobic agar medium into tube
3. Sealing the tube with the butyl rubber and stopper and screw cap
4. Autoclaving the tube
5. Inoculation using syringe
6. Preparation on roll tube spinner
7. Inoculation in water bath

LACTOPHENOL COTTON BLUE MOUNTING OF FUNGI

Aim

To stain fungal cells by using lactophenol cotton blue

Principles

The lactophenol cotton blue (LPCB) wet mount preparation is the most widely used method of staining and observing fungi and is simple to prepare. The preparation has three components:

1. Phenol: kills any live organisms;
2. Lactic acid: It preserves fungal structures, and
3. Cotton blue: It stains the chitin in the fungal cell walls.

Lactophenol Cotton Blue Solution is a**mounting medium** and **staining agent** used in the preparation of slides for microscopic examination of fungi. Fungi are eukaryotic organism and they are classified into two main groups that is yeast and molds.Its cell wall is made up of chitin.Fungal cells have both macroscopic as well as microscopic structure. This microorganism is useful as well as harmful to human being useful because it produces many antibiotics,natural products as well as used in industrial fermentation process.It is harmful in the sense as it causes human diseases,produce toxic substances as well as harm important crops. So it is very important to study fungal species.The branch of science that deals with study of fungal species is called as Mycology.Staining of these fungal cells is a very important step.These are some examples of fungi *Rhizopus, Aspergillus, Penicillium, Fusarium, Candida, Mucor* etc. Here we are going to stain fungal cells by using Lactophenol cotton blue method this staining technique is also called as mounting of fungus.

Materials per Student

1. Fungal Cultures of *Aspergillus* sp, *Mucor* sp, *Rhizopus* sp, *Fusarium* sp, *Penicillium* sp
2. Glass Slide and Cover slip
3. Lactophenol Cotton Blue Solution
4. Bunsen burner
5. Inoculating needle or loop
6. Marker pen
7. Blotting paper
8. disposable gloves

Procedure

1. Take a clean grease free slide.
2. Add a drop of Lactophenol cotton blue solution on the slide
3. Using a sterile inoculation loop or needle, transfer the fungal myellicial growth to the stain and gentle tease using teasing needle.
4. take a clean coverslip and with the help of a forcep place the coverslip on the Stain with fungal myecellium
5. With the help of blotting paper, remove the excess stain.
6. Observe the preparation under the low and high power objective.

Result

***Rhizopus* Species:**

They are rapidlygrowing white coloured fungus that swarms over entire plate. It has the aerial mycelium. Its spores are oval, colourless or brown non septate mycelium give rise to straight sporangiophores that tuminate with black sporangium containing a columella root like hyphae (rhizoids) penetrate the medium.

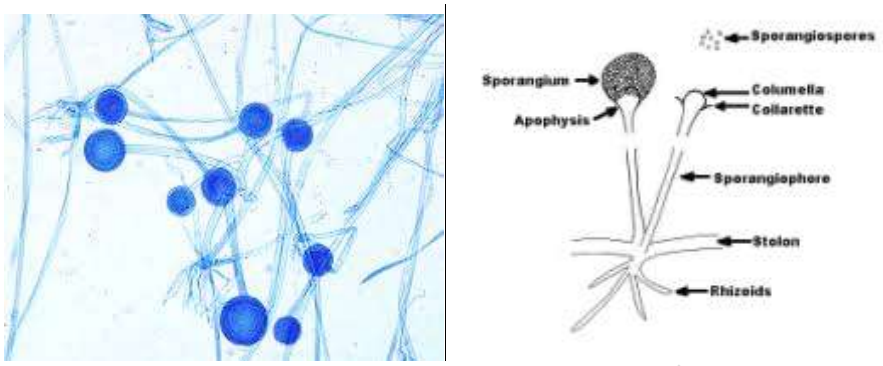


Fig: *Rhizopus* species

***Mucor* Species:**

Rapidly growing white colouredfungus swarms over entire plate cottony and fuzzy spore4 to single sporangiophores with globular sporangium containing a columella. There are no rhizoids.

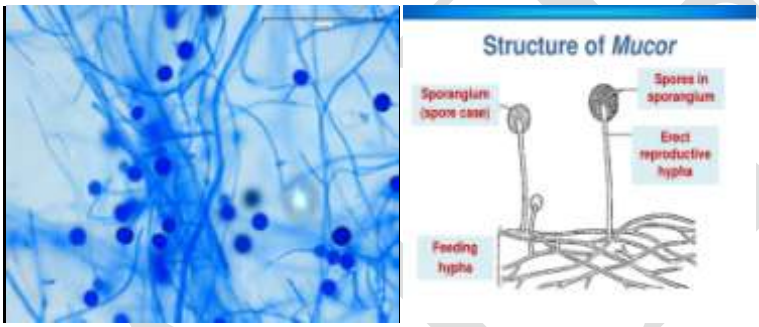


Fig: *Mucor* species

***Aspergillus* Species:**

The colonies are white in colour when young becomes greenish, blue, black or brown as the culture ages. Coinidia is chainsdevelops at the end of the stigma arising from the terminal bulb of the conidiophore, branching arise from a septate mycellium.

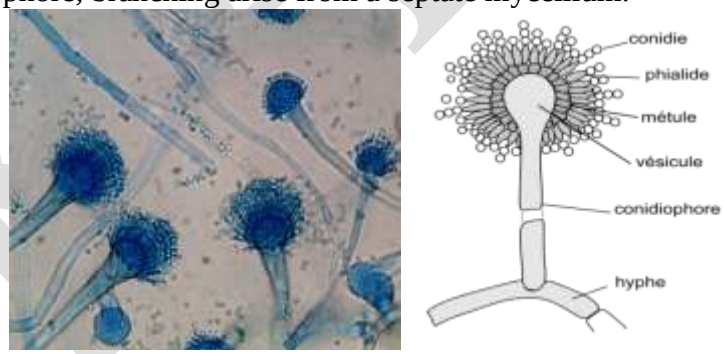


Fig: *Aspergillus* Species

***Penicillium* Species:**

Matured culture usually are greenish or blue green. Conidia in chain develop at the end of the sterigmata arising from the metulla of the conidiophore, branching arise from a septate mycellium.

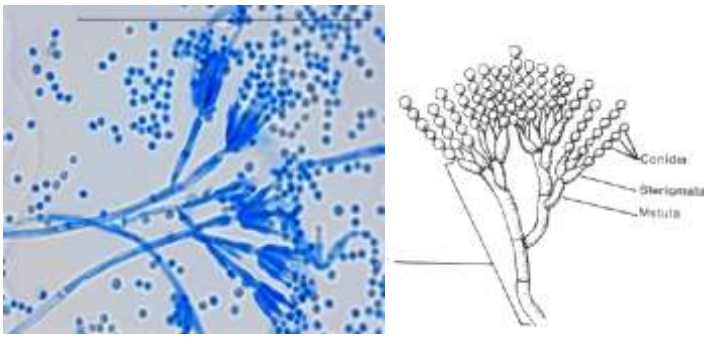


Fig: *Penicillium* species

***Fusarium* Species:**

The fusaria are imperfect filamentous fungi belonging to the class Deutromycetes. Thus genus contains 50-70 species many of which are phytopathogenic causing crop disease. Some species are involved in the type 1 allergy immunocompromised individuals.

Colories are cottony or flaky and generally bright coloured white to cream yellow, brown, pink, red or purple. The conidiophores are grouped into sporodaetia with relatively elongated philalides pear shaped void micro conidia [1-2 cells] and fusiform septate, curved, cresent - shaped macro conidias with a pedicellote fasal cell. They produce mycotoxins such as trichothceae zearalenon etc.

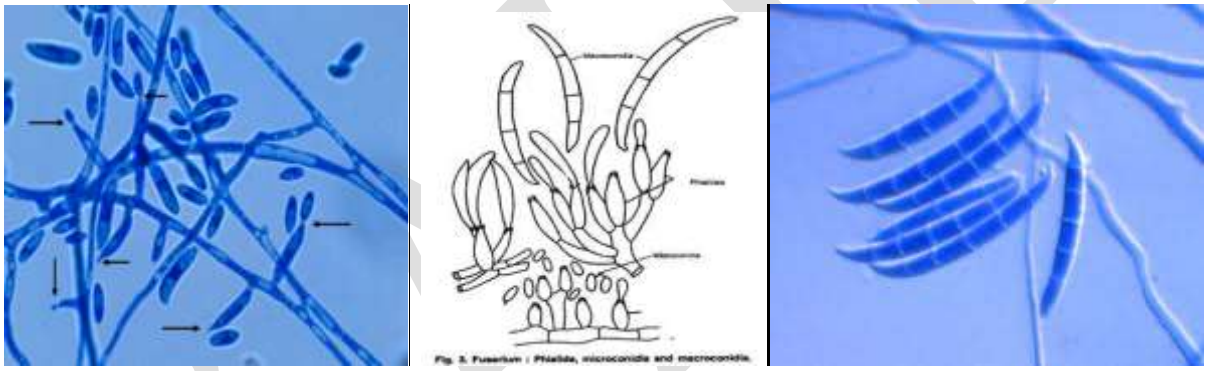


Fig: *Fusarium* species

MEASUREMENT OF MICROBIAL GROWTH

Introduction

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, 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, 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 is normally plated because the exact number of bacteria is usually unknown. Increased turbidity in a culture is another index of bacterial growth and cell numbers (biomass). 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 10^7 cells or greater. The determination of group requires a quantitative measurement of incubation and again after incubation period growth can be determined by the following methods:

1. Direct Count of Cells
   Direct Count Using a Counting Chamber, Direct Count Using Fluorescent Dyes
2. Indirect Count of Cells
   Viable Count, The Most Probable Number (MPN)
3. Direct Measurement of Microbial Biomass
4. Indirect Measurement of Microbial Biomass

MEASUREMENT OF MICROBIAL GROWTH - VIABLE COUNT

Aim

To enumerate bacterial cells by viable count

Principle:

The most common procedure for the enumeration of bacteria is the viable plate count. In this method, serial dilutions of a sample containing viable microorganisms are plated onto a suitable growth medium. The suspension is either spread onto the surface of agar plates (spread plate method), or is mixed with molten agar, poured into plates, and allowed to solidify (pour plate method). The plates are then incubated under conditions that permit microbial reproduction so that colonies develop that can be seen without the aid of a microscope. It is assumed that each bacterial colony arises from an individual cell that has undergone cell division. Therefore, by counting the number of colonies and accounting for the dilution factor, the number of bacteria in the original sample can be determined.

There are several drawbacks to the viable count method. The major disadvantage is that it is selective and therefore biased. The nature of the growth conditions, including the composition and pH of the medium used as well as the conditions such as temperature, determines which bacteria in a mixed population can grow. Since there is no universal set of conditions that permits the growth of all microorganisms, it is impossible to enumerate all microorganisms by viable plating. This same disadvantage, however, becomes advantageous when one is interested in only a specific microbial population. For example, we can design selective procedures for the enumeration of coliforms and other physiologically defined microbial groups.

The viable count is an estimate of the number of cells. Because some organisms exist as pairs or groups and because mixing and shaking of the sample does not always separate all the cells, we actually get a count of the "colony forming units". One cell or group of cells will produce one colony, therefore when we record results for a viable count, it is customary to record the results as colony forming units per ml (cfu/ml) or per gram (cfu/g) of test material.

Because we generally have no idea of how many bacteria are in a sample, it is almost always necessary to prepare a dilution series to ensure that we obtain a dilution containing a reasonable number of bacteria to count. Dilutions in the range 10-1 (1/10) to 10-8 (1/100,000,000) are generally used, although with particular types of samples the range of dilutions can be restricted. For example, for water that is not turbid, the maximal dilution needed is 10-6 because we know that if there were 107 or more bacteria per milliliter, the water would be turbid.

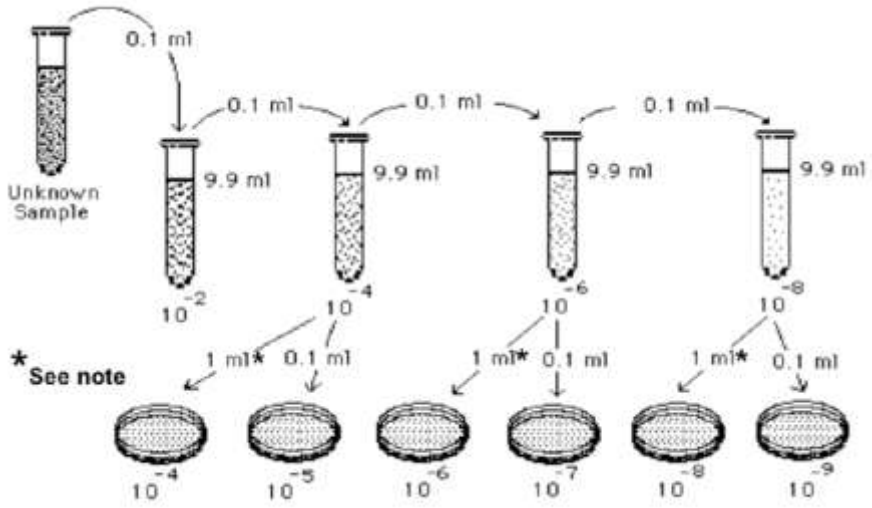
Materials Required

1. Bacterial culture
2. Test tubes
3. Petri plates
4. Pipette
5. Marker pen

Procedure

1. Label four 9.9 ml saline tubes 10-2, 10-4, 10-6, and 10-8, respectively. Label six BHI plates 10-4 to 10-9.
2. Vortex the unknown sample to ensure an even distribution of bacteria. Aseptically remove 0.1 ml of sample with a sterile pipette and transfer it to the 10-2 dilution tube (see diagram).
3. Vortex the 10-2 tube and transfer 0.1 ml to the 10-4 tube.

4. Again vortex the 10-4 dilution tube and transfer 0.1 ml to the 10-6 tube. Vortex this last tube well.
5. Vortex the 10-6 tube, transfer 0.1 ml to 10-8 tube and vortex again.
6. Using a new sterile pipette, aseptically transfer 1.0 ml from the 10-4 dilution tube to the plate labeled 10-4 and 0.1 ml to the plate labeled 10-5. Spread the inoculum on the surface of the agar in each plate using an alcohol-dipped, flamed, metal spreader. Dip the spreader into the alcohol jar and quickly take it through the flame and let the alcohol burn off after each spreading. Do not allow the spreader to get too hot. Never hold the spreader in the flame for more than a second.
7. Repeat the above to transfer 1.0 and 0.1 ml from the 10-6 dilution tube to the 10-6 and 10-7 plates, respectively. In the same manner establish the 10-8 and 10-9 plates. Do not discard your dilution tubes. (See note above)
8. Allow the surface of the agar to dry before you move or invert the plates. Incubate the plates at 37°C for 2 days.



Result

The generation time of the given bacterial culture by viable count method was found to be 30 minutes.

MEASUREMENT OF MICROBIAL GROWTH - DIRECT COUNT

Aim

To enumerate bacterial cells by direct microscopic count using haemocytometer

Principle:

Direct microscopic counts are performed by spreading a measured volume of sample over a known area of a slide, counting representative microscopic fields, and relating the averages back to the appropriate volume-area factors. Specially constructed counting chambers, such as the Petroff-Hauser and Levy counting chambers, simplify the direct counting procedure because they are made with depressions in which a known volume overlies an area that is ruled into squares. The ability to count a defined area and convert the numbers observed directly to volume makes the direct enumeration procedure relatively easy.

Direct counting procedures are rapid but have the disadvantage that they do not discriminate between living and dead cells. This method is used to assess the sanitation level of a food product and in performing blood cell counts in hematology. The differential white blood cell count, which is used as an indication of the nature of a microbial infection, involves direct counting of blood cells that have been stained to differentiate different types of white blood cells.

If there is an average of 24 cells/square than the number of bacteria/mm^2 = 24 X 25 squares

= 600 cells/mm^2

To convert the number of cells/mm^2. It is again multiplied by the depth of the chamber

= 600 X 10

= 6000 cells/mm^2

To calculate the number of cells/ml of the sample; there are

6000 cells/mm^3 = 10^{-3} cm^3 = 0.01

No. of cells = 6000 X 10^3

= 60.00000 cells/ml [cm^3]

= 6 X 10^6 cells/ml

Materials Required

1. Bacterial culture
2. Heamocytometer
3. Coverslip
4. Microscope
5. Marker pen

Procedure

1. Clean a counting chamber with methanol and lens paper and then place it on the microscope stage.
2. Using the 4X objective find the ruled area on one side of the chamber and not the size and arrangements of larger squares and their small square subdivisions.
3. Shake the yeast suspension to distribute the cells evenly. Take out the counting chamber without changing the focus on the 4X objective. Place a coverslip over the calibrated surface of the counting chamber.
4. Using a transfer pipette, transfer some of the yeast suspension to the groove of the counting chamber to fill the chamber by capillary action.
5. Carefully place the counting chamber back onto the microscopic stage and observe the cells under 4X. You may need to reduce the amount of light by closing the diaphragm of the condenser to be able see the cells
6. Switch to the high-dry objective (40 X) and count the number of yeast cells in at least 50 of the small squares. If cells fall on a line, include in your count those on the top

and left-hand lines and exclude those on the bottom and right-hand lines. (If the yeast cells are too dense to count, dilute your sample and start again.)

7. Calculate the average number of yeast cells per Small Square. Then calculate the number of yeasts per ml by dividing the average number of yeasts per Small Square by the volume of each small square which is 0.00025 µl. If you diluted the sample you must also multiply your results by the dilution factor to determine theconcentration of yeast cells in the original sample. Record your calculations and results.

Result

The number of bacterial cells present min the given sample by direct count method after 5 hours was found to be $4X10^6$ cells/ml.

MEASUREMENT OF MICROBIAL GROWTH - TURBIDITY METHOD

Aim

To measure the growth of bacteria by turbidity method

Principle:

The bacteria growing in a liquid medium, the culture appears **turbid**. This is because a bacterial culture acts as a colloidal suspension that blocks and reflects light passing through the culture. Within limits, the light absorbed by the bacterial suspension will be directly proportional to the concentration of cells in the culture. By measuring the amount of light absorbed by a bacterial suspension, one can estimate and compare the number of bacteria present.

The instrument used to measure turbidity is a **spectrophotometer.** It consists of a light source, a filter which allows only a single wavelength of light to pass through, the sample tube containing the bacterial suspension, and a photocell that compares the amount of light coming through the tube with the total light entering the tube.

The ability of the culture to block the light can be expressed as either percent of light transmitted through the tube or the amount of light absorbed in the tube. The**percent of light transmitted** is inversely proportional to the bacterial concentration. (The greater the number of bacteria, the lower the percent light transmitted.) The **absorbance**, or optical density, is directly proportional to the cell concentration. (The greater the number of bacteria, the higher the absorbance.)

Turbidimetric measurement is often correlated with some other method of cell count, such as the direct microscopic method or the plate count. In this way, turbidity can be used as an indirect measurement of the cell count. For example:

1. Several dilutions can be made of a bacterial stock.
2. A Petroff-Hausser counter can then be used to perform a direct microscopic count on each dilution.
3. Then a spectrophotometer can be used to measure the absorbance of each dilution tube.
4. A standard curve comparing absorbance to the number of bacteria can be made by plotting absorbance versus the number of bacteria per cc.
5. Once the standard curve is completed, any dilution tube of that organism can be placed in a spectrophotometer and its absorbance read. Once the absorbance is determined, the standard curve can be used to determine the corresponding number of bacteria per cc.

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 10^7 cells or greater.

Materials Required

1. Bacterial culture *E.coli*
2. Heamocytometer
3. Cuvette
4. Sterile Nutrient Broth
5. Test tubes
6. Test tube racks
7. Spectrophotometer

8. Marker pen

Procedure

1. Put the **ORIGINAL tube of *E. coli*** and four tubes of the sterile Nutrient Broth 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.***
    a. The wavelength is preset somewhere between 550- 600nm. DO NOT change it! (Instructor will set the wavelength)
    b. Standardize the spectrophotometer as directed.
    c. Obtain the 6 micro-cuvettes. The cuvettes will look like either of the 2 shown to the right. The **lined or etched sides of the cuvettes face you**, with the clear sides facing the light source.
    d. The microcuvette must contain1ml for the spectrophotometer to read the fluid, but you can guesstimate the amount by eyesight. Notice the arrow on picture above showing where level of fluid must be.
    e. The BLANK used to standardize the machine is sterile nutrient broth: it is called the BLANK because it has a sample concentration equal to zero. Pipette 1ml of the sterile NB into one of the micro-cuvettes. Place into the black cuvette holder (**red line** towards you), close the cover and read. Save BLANK to re-standardize the machine to infinity absorbance and zero absorbance before each reading because the settings tend to drift.
    f. Pipette 1ml of the original bacterial specimen into a second micro-cuvette. Place in cuvette holder and read. When read, discard micro-cuvette into bleach container on your table. Next pipette the 1/2 dilution into the third cuvette and read it. Repeat this with the 1/4, 1/8, and 1/16 dilutions.

Result

The generation time of the given bacteria by turbidity method was found to be 30 minutes.

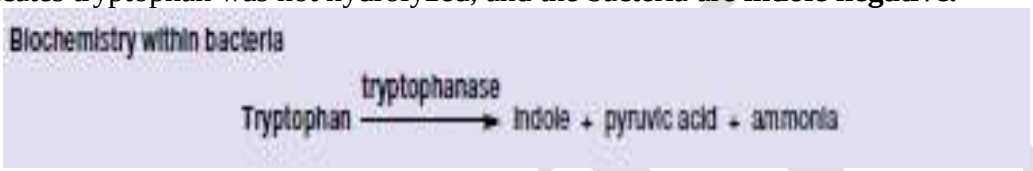
BIOCHEMICAL CHARACTERIZATION - INDOLE TEST

Aim

To check the ability of the microorganism for the production of indole from tryptophan

Principles

The amino acid **tryptophan** is found in nearly all proteins. Bacteria that contain the enzyme **tryptophanase** can hydrolyze tryptophan to its metabolic products, namely, indole, pyruvic acid, and ammonia. The bacteria use the pyruvic acid and ammonia to satisfy nutritional needs; indole is not used and accumulates in the medium. The presence of indole can be detected by the addition of **Kovacs' reagent.** Kovacs' reagent reacts with the indole, producing a bright red compound on the surface of the medium. Bacteria producing a red layer following addition of Kovacs' reagent are **indole positive;** the absence of a red color indicates tryptophan was not hydrolyzed, and the bacteria are **indole negative.**



Materials per Student

1. 24- to 48-hour tryptic soy broth cultures of *Escherichia coli, Proteus vulgaris, Klebsiella.*
2. Peptone broth tubes each containing 5 ml of medium
1. Kovacs' reagent
2. Bunsen burner
3. Inoculating needle
4. 4-ml pipettes with pipettor
5. Marker pen
6. disposable gloves
7. Test-tube rack

Procedure

First Period

1. Label each of the SIM deep tubes with the name of the bacterium to be inoculated (*E. coli*, *P. vulgaris,* and *E. aerogenes*), your name, and date.
2. Using aseptic technique, inoculate each tube by a stab inoculation or with a loopful of culture.
3. Incubate the tubes for about 24 hours at 35°C.

Second Period

1. Remove the tubes from the incubator and while wearing disposable gloves, add 0.5 ml (about 10 drops) of Kovacs' reagent to each tube, and shake the tube gently. A deep red develops in the presence of indole. Negative reactions remain colorless or light yellow.
2. Based on your observations, determine and record in the report for exercise 25 whether or not each bacterium was capable of hydrolyzing the tryptophan.

Result

After addition of Kovac's reagent into the tubes containing peptone broth inoculated with *E.coli* a cherry red colour was formed at the top of the broth indicating a positive reaction tubes containing culture of *Klebsiella* showed no change in colour thus indicating a negative reaction.

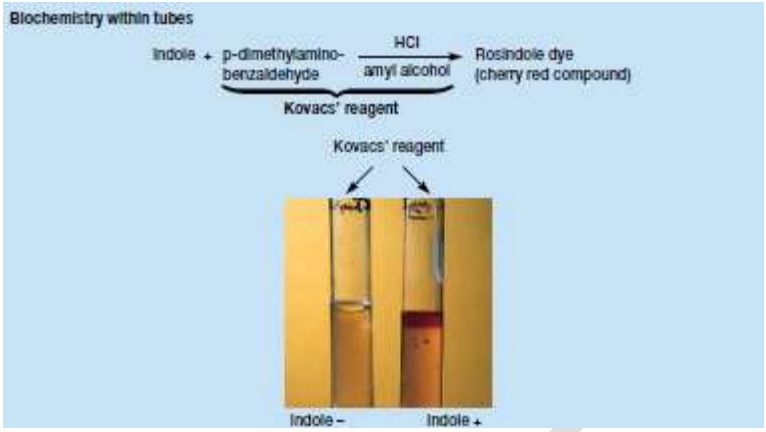


Fig: Indole Test. The tube on the left is indole negative and the tube on the right is indole positive.

BIOCHEMICAL CHARACTERIZATION - METHYL RED TEST

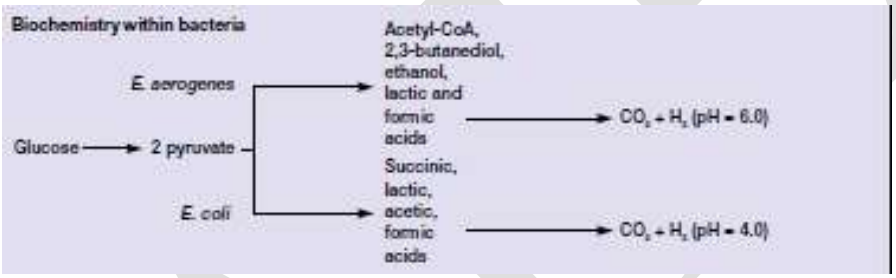
Aim

Determine the ability of some bacteria to oxidize glucose with the production of acid end products

Principles

All enteric bacteria catabolize glucose for their energy needs; however, the end products vary depending on the enzyme pathways present in the bacteria. The Ph indicator **methyl red** detects a pH change to the acid range as a result of acidic end products such as lactic, acetic, and formic acids. This test is of value in distinguishing between *E. coli* (a mixed acid fermenter) and *E. aerogenes* (a butanediol fermenter).

Mixed acid fermenters such as *E. coli* produce a mixture of fermentation acids and thus acidify the medium. **Butanediol fermenters** such as *E. aerogenes* form butanediol, acetoin, and fewer organic acids. The pH of the medium does not fall as low as during mixed acid fermentation. As illustrated in figure 25.3, at a pH of 4, the methyl red indicator turns red—a **positive methyl red test.** At a pH of 6, the indicator turns yellow—a **negative methyl red test.**



Materials per Student

3. 24- to 48-hour tryptic soy broth cultures of *Escherichia coli, Proteus vulgaris, Klebsiella.*
4. Peptone broth tubes each containing 5 ml of medium
8. Methyl red indicator
9. Bunsen burner
10. Inoculating needle
11. 4-ml pipettes with pipettor
12. Marker pen
13. disposable gloves
14. Test-tube rack

Procedure

First Period

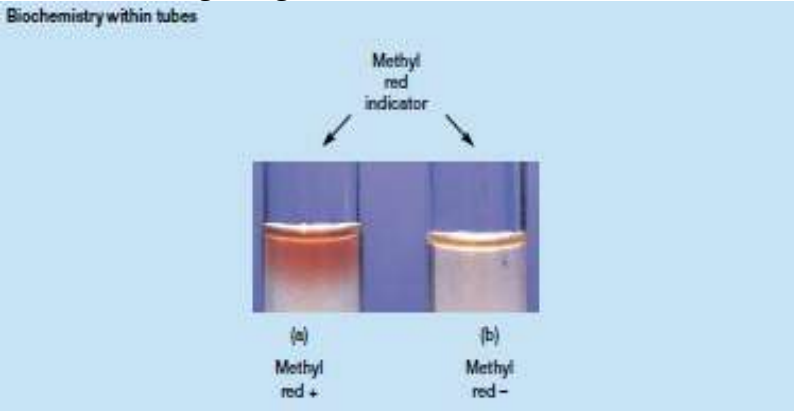
1. Label each of the MR-VP broth media tubes with the name of the bacterium (*E. coli* and *Klebsiella*) to be inoculated, your name, and date.
2. Using aseptic technique, inoculate each tube with the appropriate bacterium by means of a loop inoculation.
3. Incubate all tubes at 35°C for 24 to 48 hours. For slow fermenters, it may take four to five days.

Second Period

1. To the culture in each tube, add 0.2 ml (about 4 to 5 drops) of methyl red indicator. Carefully note any color change (a red color is positive).
2. Based on your observations, determine and record in the report for exercise 25 whether or not each bacterium was capable of fermenting glucose, lowering the pH of the medium.

Result

On addition of methyl red indicator in the test tube containing the culture of *E.coli* showed bright red colour indicating a positive reaction. The test tubes containing culture of *Klebsiella* showed yellow colour indicating a negative reaction.



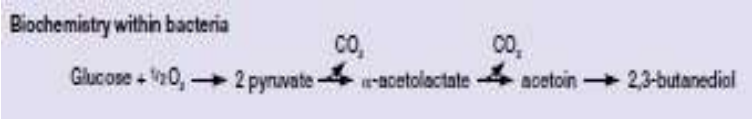
BIOCHEMICAL CHARACTERIZATION - VOGES-PROSKAUER TEST

Aim

To check the ability of the microorganism for the production of acetyl methyl carbinol from glucose

Principles

The **Voges-Proskauer test** (named after Daniel Voges, German physician, and Bernhard Proskauer, German bacteria that ferment glucose, leading to **2, 3-butanediol** accumulation in the medium. The addition of 40% KOH and a 5% solution of alpha-naphthol in absolute ethanol (Barritt's reagent) will detect the presence of **acetoin**—a precursor in the synthesis of 2,3-butanediol. In the presence of the reagents and acetoin, a cherry-red color develops. Development of a red color in the culture medium 15 minutes following the addition of Barritt's reagent represents a **positive VP test;** absence of a red color is a **negative VP test**



Materials per Student

9. 24- to 48-hour tryptic soy broth cultures of *Escherichia coli, Proteus vulgaris, Klebsiella.*
10. Peptone broth tubes each containing 5 ml of medium
11. Barritt's reagent (solutions A and B)
12. Bunsen burner
13. Inoculating needle
14. 4-ml pipettes with pipettor
15. Marker pen
16. disposable gloves
17. Test-tube rack

Procedure

First Period

4. Label each of the MR-VP broth media tubes with the name of the bacterium (*E. coli* and *Klebsiella*) to be inoculated, your name, and date.
5. Using aseptic technique, inoculate each tube with the appropriate bacterium by means of a loop inoculation.
6. Incubate all tubes at 35°C for 24 to 48 hours. For slow fermenters, it may take four to five days.

Second Period

1. Add 0.6 ml of Barritt's solution A and 0.2 ml of solution B to each culture, and shake vigorously to aerate. Positive reactions occur at once or within 20 minutes and are indicated by the presence of a red color.
2. Based on your observations, determine and record in the report whether or not each bacterium was capable of fermenting glucose, with the production of acetylmethylcarbinol.

Result

On addition of Barrits reagent A and B *Klebsiella* showed pinkish to crimson red colour indicating a negative reaction. *E.coli* showed no colour change indicating a negative reaction.

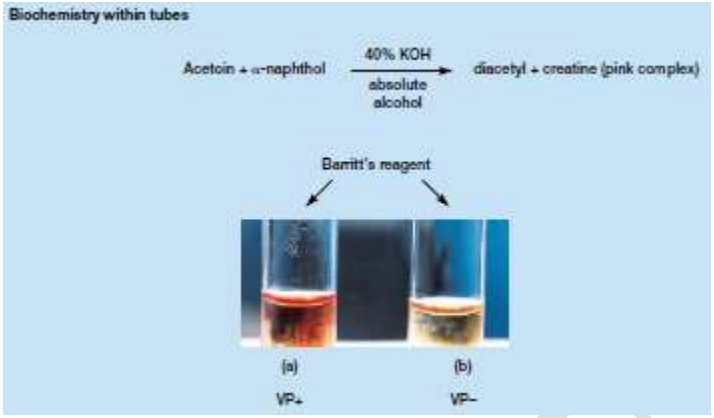


Fig: Voges-Proskauer Test. (a) *Enterobacter aerogenes*, VP+. (b) *Escherichia coli*, VP–.

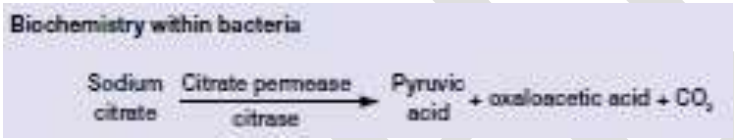
BIOCHEMICAL CHARACTERIZATION - CITRATE UTILIZATION TEST

Aim

To detect the ability of the microorganism to utilize citrate as the sole carbon source in the presence of translocating enzyme

Principles

The **citrate utilization test** determines the ability of bacteria to use **citrate** as a sole carbon source for their energy needs. This ability depends on the presence of a **citrate permease** that facilitates transport of citrate into the bacterium. Once inside the bacterium, citrate is converted to pyruvic acid and CO_2. Simmons citrate agar slants contain sodium citrate as the carbon source, NH_4 + as a nitrogen source, and the pH indicator bromothymol blue. This test is done on slants since O_2 is necessary for citrate utilization. When bacteria oxidize citrate, they remove it from the medium and liberate CO_2. CO_2 combines with sodium (supplied by sodium citrate) and water to form sodium carbonate—an alkaline product. This raises the pH, turns the pH indicator to a blue color, and represents a **positive citrate test;** absence of a color change is a **negative citrate test**. Citrate-negative cultures will also show no growth in the medium.



Materials per Student

18. 24- to 48-hour tryptic soy broth cultures of *Escherichia coli, Enterobacter aerogenes, Klebsiella.*
19. Simmons citrate agar slants
20. Bunsen burner
21. Inoculating needle
22. Marker pen
23. disposable gloves
24. Test-tube rack

Procedure

First Period

1. Label each of the Simmons citrate agar slants with the name of the bacterium (*E. coli, Enterobacter aerogenes* and *Klebsiella*) to be inoculated, your name, and date.
2. Using aseptic technique, inoculate each bacterium into its proper tube by means of a stab-and-streak inoculation.
3. Incubate these cultures for 24 to 48 hours at 35°C.

Second Period

1. Examine the slant cultures for the presence or absence of growth and for any change in color from green to blue.
2. Based on your observations, determine and record in the report for exercise 25 whether or not each bacterium was capable of using citrate as an energy source. The development of a deep blue color is a positive test.

Result

The slant inoculated with the culture of *Klebsiella* sp turned from green to Prussian blur colour, which suggested that the organism has sterilised citrate as sole source of carbon. The tube was inoculated with *E.coli* showed no change in colour indicating a negative result.

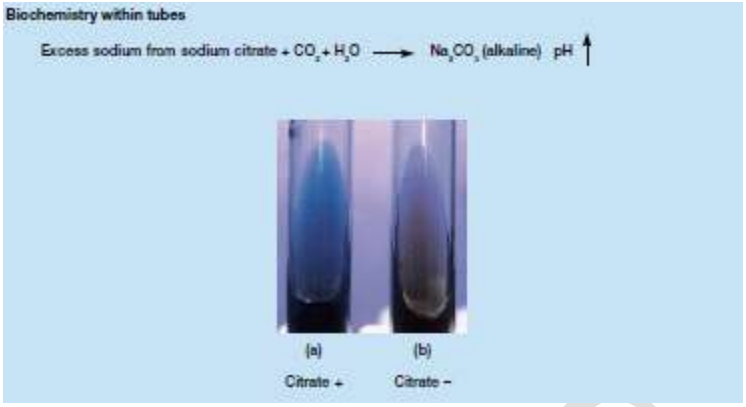


Fig: Citrate Test. (a) *Enterobacter aerogenes;* blue color is positive. (b) *Escherichia coli;* green color is negative.

BIOCHEMICAL CHARACTERIZATION - TRIPLE SUGAR IRON AGAR TEST

Aim

To differentiate the different genera of the enterobacteriaceae based on carbohydrate (glucose, lactose, sucrose) fermentation and H_2S production.

Principles

As originally described in 1911 by F. F. Russell, the **triple sugar iron (TSI) agar test** is generally used for the identification of enteric bacteria (*Enterobacteriaceae*). It is also used to distinguish the *Enterobacteriaceae* from other gram-negative intestinal bacilli by their ability to catabolize glucose, lactose, or sucrose, and to liberate sulfides from ferrous ammonium sulfate or sodium thiosulfate.TSI agar slants contain a 1% concentration of lactose and sucrose, and a 0.1% glucose concentration. The pH indicator, phenol red, is also incorporated into the medium to detect acid production from carbohydrate fermentation. Often Kligler Iron Agar (named after I. J. Kligler in 1917), a differential medium similar to TSI, is used to obtain approximately the same information. TSI slants are inoculated by streaking the slant surface using a zig-zag streak pattern and then stabbing the agar deep with a straight inoculating needle. Incubation is for 18 to 24 hours in order to detect the presence of sugar fermentation, gas production, and H2S production. The following reactions may occur in the TSI tube:

1. **Yellow butt (A) and red slant (A)** due to the fermentation of glucose (phenol red indicator turns yellow due to the persisting acid formation in the butt). The **slant remains red (alkaline) (K)** because of the limited glucose in the medium and, therefore, limited acid formation, which does not persist.
2. A**yellow butt (A) and slant (A)** due to the fermentation of lactose and/or sucrose (yellow slant and butt due to the high concentration of these sugars) leading to excessive acid formation in the entire medium.
3. **Gas formation** noted by splitting of the agar.
4. **Gas formation (H2S)** seen by blackening of the agar.
5. **Red butt (K) and slant (K)** indicates that none of the sugars were fermented and neither gas nor H2S were produced.

Materials per Student

1. 24- to 48-hour tryptic soy broth cultures of *Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa*, and *Shigella.*
2. 5 triple sugar iron agar slants
3. Bunsen burner
4. Inoculating needle
5. Incubator set at 35°c
6. Test-tube rack

Procedure

First Period

1. Label each of the TSI agar slants with the name of the bacterium to be inoculated. Use one of the tubes as a control. Place your name and date on each tube.
2. Using aseptic technique, streak the slant with the appropriate bacterium and then stab the butt. Screw the caps on the tubes but do not tighten!
3. Incubate for only 18 to 24 hours at 35°C for changes in the butt and on the slant. Tubes should be incubated and checked daily for up to seven days in order to observe blackening.

Second Period

1. Examine all slant cultures for the color of the slant and butt, and for the presence or absence of blackening within the medium.
2. Record your results

The Possible Reactions and Results in TSI Agar for the Various Bacteria Used in This Experiment.

No carbohydrate fermentation or hydrogen sulfide production

Example: *Alcaligenes faecalis*

glucose, lactose, sucrose → glucose, lactose, sucrose
(red slant/red butt) **(K; red slant/red butt)**

cysteine → cysteine
(no black color)

Glucose fermentation only

Example: *Shigella flexneri*

glucose → decrease in pH due to acid
(red butt) **(A; yellow butt)**

(continued)

lactose, sucrose → lactose, sucrose
(red slant) **(K; red slant)**

cysteine → cysteine
(no black color)

Glucose fermentation only with hydrogen sulfide production

Example: *Pseudomonas aeruginosa*

glucose → decrease in pH due to acid
(red butt) **(A; yellow butt)**

lactose, sucrose → lactose, sucrose
(red slant) **(K; red slant)**

cysteine → H_2S production

$H_2S + FeSO_4 \rightarrow FeS$
(black color in media)

Lactose and/or sucrose and glucose fermentation

Example: *Escherichia coli*

glucose → decrease in pH due to acid
(red butt) **(A; yellow butt)**

lactose and/or sucrose → decrease in pH due to acid
(red butt) **(A; yellow slant)**

cysteine → cysteine
(no black color in media)

Lactose and/or sucrose and glucose fermentation with hydrogen sulfide (H_2S) production

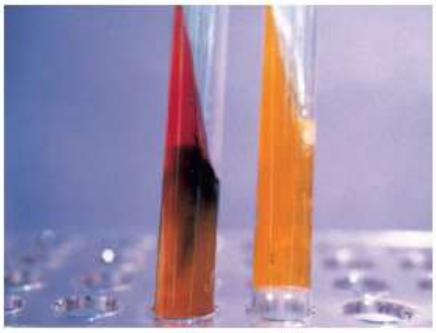
Example: *Proteus vulgaris*

glucose → decrease in pH due to acid
(red butt) **(A; yellow butt)**

lactose and/or sucrose → decrease in pH due to acid
(red slant) **(A; yellow butt)**

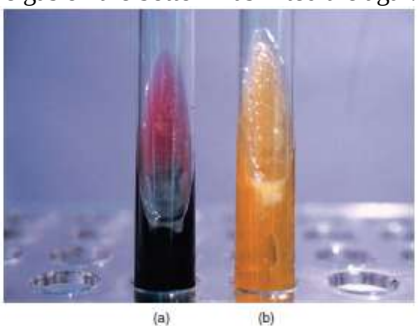
cysteine → H_2S production

$H_2S + FeSO_4 \rightarrow FeS$ production
(black color in media)



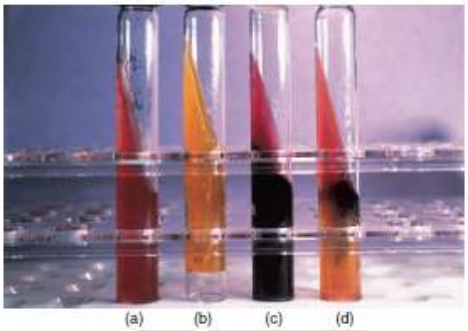
	Tube a	Tube b
Slant	K	A
Butt	A	A
Gas	-	+
H_2S	+	-

Fig: Triple Sugar Iron Reactions (TSI-1) and Their Interpretation. (**a**) The tube on the left has a yellow butt (acid), red slant (alkaline), H2S production as indicated by blackening of the agar, and no gas production. (**b**) The tube on the right shows no H2S formation, a yellow slant (acid), gas production, and an acid butt. Note that the gas on the bottom has lifted the agar.



	Tube a	Tube b
Slant	K	A
Butt	K	A
Gas	-	-
H_2S	-	-

Fig: Triple Sugar Iron Reactions (TSI-2) and Their Interpretation. (**a**) The tube on the left has a red butt (alkaline), red slant (alkaline), and no acid or H2S production. (**b**) The tube on the right has a yellow slant (acid), yellow butt (acid), and no gas or H2S production.



	Tube a	Tube b	Tube c	Tube d
Slant	-	A	K	K
Butt	-	A	K	A
Gas	-	+	-	-
H_2S	-	-	+	+

Figure 21.3 Triple Sugar Iron Reactions (TSI-3) and Their Interpretation. (**a**) The tube on the left is an uninoculated control. Notice the red color. (**b**) The second tube from the left has a yellow slant (acid), yellow butt (acid), gas production at the bottom of the tube, and no H2S production. This would indicate a weak lactose fermenter. (**c**) The third tube from the left has a red slant (alkaline), red butt (alkaline), and the black indicates H2S production, but no gas. (**d**) The tube on the right has a red slant (alkaline), yellow butt (acid), H2S production, but no gas production. This would indicate a nonlactose fermenter.

Result

The given organisms were inoculated in the TSI agar tubes and the results were tabulated.

Results of TSI Reaction				
Bacterium	**TSI Reaction**			
	Butt	**Slant**	**H2S**	**Gas**
Enterobacter	A	A	-	+
Escherichia	A	A or K	-	+
Klebsiella	A	A	-	+
Citrobacter	A	K or A	V	+
Proteus vulgaris	A	A or K	+	+
Serratia	A	K or A	-	V
Shigella	A	K	-	-
Salmonella typhi	A	K	+	-

A = acid, K = alkaline, V = varies between species

BIOCHEMICAL CHARACTERIZATION - CATALASE TEST

Aim

1. Understand the biochemical process of hydrogen peroxide detoxification by aerobic bacteria through the production of the enzyme catalase.
2. Describe how catalase production can be determined.
3. Perform a catalase test**.**

Principles

Some bacteria contain flavoproteins that reduce O_2, resulting in the production of **hydrogen peroxide** (H_2O_2) or **superoxide** (O_2 –). These are extremely toxic because they are powerful oxidizing agents and destroy cellular constituents very rapidly. A bacterium must be able to protect itself against such O2 products or it will be killed.

Many bacteria possess enzymes that afford protection against toxic O_2 products. Obligate aerobes and facultative anaerobes usually contain the enzymes **superoxide dismutase,** which catalyzes the destruction of superoxide, and either **catalase** or **peroxidase,** which catalyze the destruction of hydrogen peroxide as follows:

$$2O_2 + 2H^+ \xrightarrow[\text{dismutase}]{\text{superoxide}} \underset{\text{Oxygen}}{O_2} + \underset{\text{Hydrogen peroxide}}{H_2O_2}$$

$$2H_2O_2 \xrightarrow[\text{peroxidase}]{\text{catalase or}} \underset{\text{Water}}{2H_2O} + \underset{\text{Free oxygen}}{O_2}$$

Most strict anaerobes lack both enzymes and therefore cannot tolerate O_2. Catalase production and activity can be detected by adding the substrate H_2O_2 to an appropriately incubated (18- to 24-hour) tryptic soy agar slant culture. If catalase was produced by the bacteria, the above chemical reaction will liberate free O_2 gas. Bubbles of O_2 represent a positive catalase test; the absence of bubble formation is a negative catalase test. Catalase activity is very useful in differentiating between groups of bacteria. For example, the morphologically similar *Enterococcus* (catalase negative) and *Staphylococcus* (catalase positive) can be differentiated using the catalase test

Materials Required

1. 18- to 24-hour tryptic soy broth cultures of *Staphylococcus aureus*
2. Nutrient agar slants
3. 3% hydrogen peroxide (H2O2)(caustic) or Difco's
4. Bunsen burner
5. Inoculating loop
6. Pasteur pipette with pipettor
7. Incubator set at 35°C
8. Test-tube rack
9. Wax pencil
10. Clean glass slides
11. Wooden applicator stick (or Nichrome wire loop)

Procedure

First Period

1. Label each of the tryptic soy agar slants with the name of the bacterium to be inoculated, your name, and date.
2. Using aseptic technique, heavily inoculate each experimental bacterium into its appropriately labeled tube by means of a streak inoculation.
3. Incubate the slants at 35°C for 18 to 24 hours.

Second Period

1. To test for catalase, set the slant in an inclined position and pipette several drops of a 3% solution of H_2O_2 over the growth on the slant.
2. The appearance of gas bubbles indicates a positive test; the absence of gas bubbles is a negative test.
3. Based on your observations, determine and record in the report whether or not each bacterium was capable of catalase activity.

Result:

The given bacterial sample has the capability of producing catalase. It was identified by the gas bubble formation when adding hydrogen peroxide in the bacterial sample.

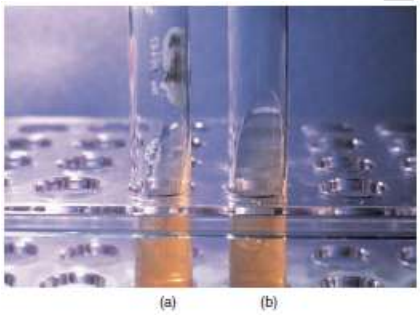


Fig: Catalase Test on Slants. (**a**) *Staphylococcus aureus,* catalase positive. Notice the bubbles of oxygen (tube on the left). (**b**) *Enterococcus faecalis,* catalase negative; note the absence of bubbles (tube on the right).

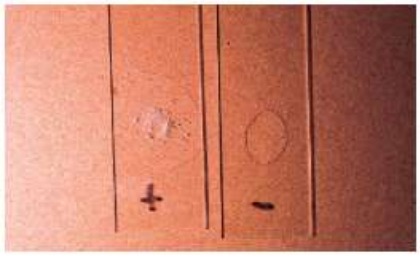


Fig: Catalase Test on Slides. A positive catalase reaction (left slide) shows gas bubbles; a negative catalase reaction reveals an absence of gas bubbles (right slide).

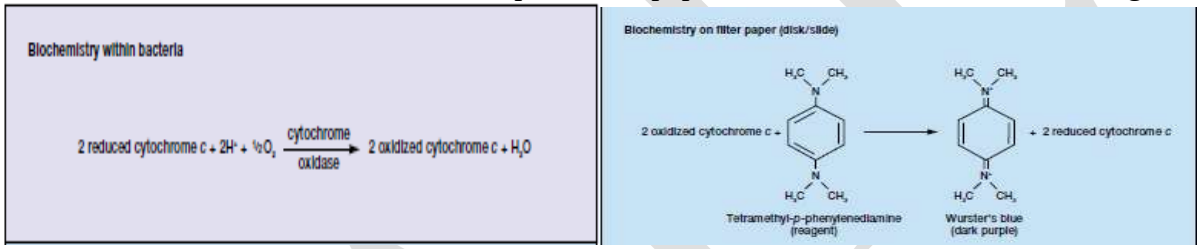
BIOCHEMICAL CHARACTERIZATION - OXIDASE TEST

Aim

1. Understand the biochemistry underlying oxidase enzymes
2. Describe the experimental procedure that enables one to distinguish between groups of bacteria based on cytochrome oxidase activity
3. Give examples of oxidase-positive and oxidase negative bacteria
4. Perform an oxidase test

Principles

Oxidase enzymes play an important role in the operation of the electron transport system during aerobic respiration. **Cytochrome oxidase** (*aa*3 type) uses O_2 as an electron acceptor during the oxidation of reduced cytochrome *c* to form water and oxidized cytochrome *c*. The ability of bacteria to produce cytochrome oxidase can be determined by the addition of the oxidase test reagent or test strip (tetramethyl-*p*-phenylenediamine dihydrochloride or an Oxidase Disk, p-aminodimethylaniline) to colonies that have grown on a plate medium. Or, using a wooden applicator stick, a bacterial sample can either be rubbed on a Dry Slide Oxidase reaction area, on a KEY test strip, or filter paper moistened with the oxidase reagent.



The light pink oxidase test reagent (Disk, strip, or Slide) serves as an artificial substrate, donating electrons to cytochrome oxidase and in the process becoming oxidized to a purple and then dark purple compound in the presence of free O2 and the oxidase. The presence of this dark purple coloration represents a positive test. No color change or a light pink coloration on the colonies indicates the absence of oxidase and is a negative test.

Materials Required

1. Young 24-hour culture of *Escherichia coli* and *Pseudomonas aeruginosa*
2. Nutrient agar plates
3. Bunsen burner
4. Inoculation loops
5. Marker pens
6. Pasteur pipette with pipettor
7. Oxidase Disks with oxidase reagent (tetramethyl-p-phenylenediamine dihydrochloride)
8. Wooden applicator sticks

Procedure

First Period

1. With a wax pencil, divide the bottom of a tryptic soy agar plate into three sections and label each with the name of the bacterium to be inoculated, your name, and date.
2. Using aseptic technique, make a single streak-line inoculation on the agar surface with the appropriate bacterium.
3. Incubate the plate in an inverted position for 24 to 47 hours at 35°C.

Second Period

1. Using a wooden applicator stick transfer the bacterial sample to the oxidase disc.

2. Observe the colony or sample for the presence or absence of a color change from pink to purple, and finally to dark purple. This color change will occur within 20 to 30 seconds. Color changes after 20 to 30 seconds are usually disregarded since the reagent begins to change color with time due to auto-oxidation. Oxidase-negative bacteria will not produce a color change or will produce a light pink color.
3. Based on your observations, determine and record in the report for exercise 30 whether or not each bacterium was capable of producing oxidase.

Result:

After 5-10 seconds on emulsifying the culture of pseudomonas, the oxidase disc turned into deep purple colour indicating a positive reaction. The oxidase disc remains unchanged in colour even after one minute for culture of *E.coli* which indicates a negative reaction.

Fig: Oxidase Test. Note the purple to dark purple color after the colonies have been added to oxidase disc with oxidase reagent.

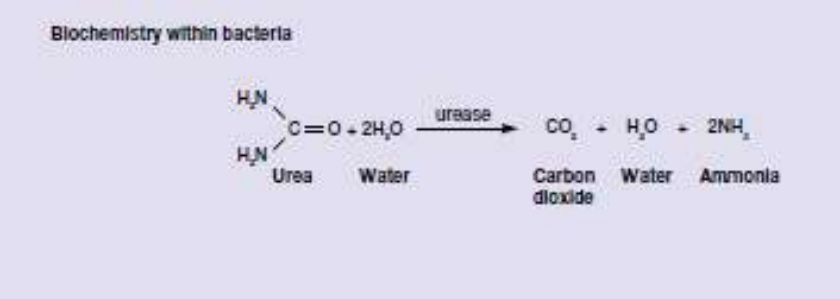
BIOCHEMICAL CHARACTERIZATION - UREASE TEST

Aim

1. Understand the biochemical process of urea hydrolysis
2. Determine the ability of bacteria to degrade urea by means of the enzyme urease
3. Perform a urease test

Principles

Some bacteria are able to produce an enzyme called **urease** that attacks the nitrogen and carbon bond in amide compounds such as urea, forming the end products ammonia, CO_2, and water.



Urease activity (the **urease test**) is detected by growing bacteria in a medium containing urea and usinga pH indicator such as phenol red. When urea is hydrolyzed, ammonia accumulates in the medium and makes it alkaline. This increase in Ph causes the indicator to change from orange-red to deep pink or purplish red (cerise) and is a positive test for urea hydrolysis. Failure of a deep pink color to develop is a negative test.

Materials Required

1. 24- to 48-hour nutrient agar slants of *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, and *Salmonella.*
2. 5 Christensen's urea agar tubes
3. Bunsen burner
4. Test-tube rack
5. Inoculating loop
6. Incubator set at 35°C
7. Marker pen

Procedure

First Period

1. Label each of the urea broth tubes with the name of the bacterium to be inoculated, your name, and date.
2. Using aseptic technique, inoculate each tube with the appropriate bacterium by means of a loop inoculation.
3. Incubate the tubes for 24 to 48 hours at 35°C.

Second Period

1. Examine all of the urea broth cultures and urea disk or urease tablet tubes to determine their color
2. Based on your observations, determine and record in the report for exercise 31 whether each bacterium was capable of hydrolyzing urea.

Result:

Christensen's urea agar slants inoculated with the culture of *Klebsiella* turned pink in colour which indicated a negative reaction for urease where as the slant inoculated with the culture of *E.coli* have no change in colour by indicating a negative reaction.

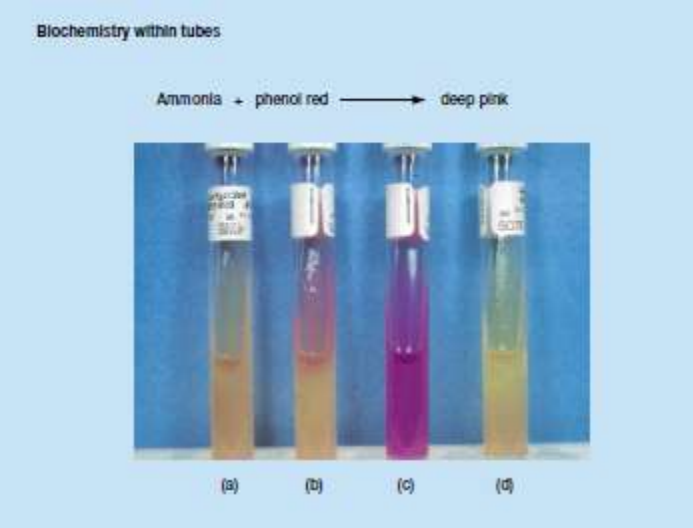


Fig: Urea Hydrolysis. (**a**) Uninoculated control. (**b**) Weakly positive reaction (delayed positive). (**c**) Very rapid positive reaction. (**d**) Negative reaction.

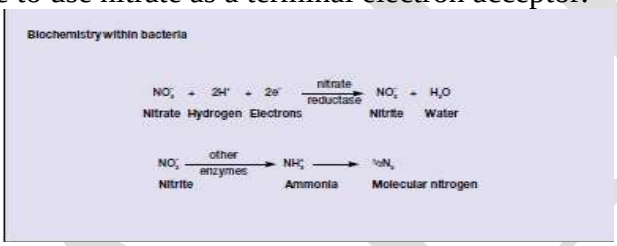
BIOCHEMICAL CHARACTERIZATION - NITRATE REDUCTION TEST

Aim

1. Understand the biochemical process of nitrate reduction by bacteria
2. Describe how nitrate reduction can be determined from bacterial cultures
3. Perform a nitrate reduction test

Principles

Chemolithoautotrophic bacteria (bacteria that obtain energy through chemical oxidation; they use inorganic compounds as electron donors and CO2 as their primary source) and many chemoorganoheterotrophs (bacteria that require organic compounds for growth; the organic compounds serve as sources of carbon and energy) can use nitrate (NO^-_3) as a terminal electron acceptor during anaerobic respiration. In this process, nitrate is reduced to nitrite (NO^-_2) by **nitrate reductase**. Some of these bacteria possess the enzymes to further reduce the nitrite to either the ammonium ion or molecular nitrogen. The ability of some bacteria to reduce nitrate can be used in their identification and isolation. For example, *E. coli* can reduce nitrate only to nitrite, *P. aeruginosa* reduces it completely to molecular nitrogen, and *S. epidermidis* is unable to use nitrate as a terminal electron acceptor.



The **nitrate reduction test** is performed by growing bacteria in a culture tube with a nitrate broth medium containing 0.5% potassium nitrate (KNO_3). After incubation, the culture is examined for the presence of gas and nitrite ions in the medium. The gas (a mixture of CO2 and N2) is released from the reduction of nitrate (NO3) and from the citric acid cycle (CO2). The nitrite ions are detected by the addition of sulfanilic acid and N, N-dimethyl-1-naphthylamine to the culture. Any nitrite in the medium will react with these reagents to produce a pink or red color. If a culture does not produce a color change, several possibilities exist: (1) the bacteria possess nitrate reductase and also reduce nitrite further to ammonia or molecular nitrogen; (2) they possess other enzymes that reduce nitrite to ammonia; or (3) nitrates were not reduced by the bacteria. To determine if nitrates were reduced past nitrite, a small amount of zinc powder or 5 to 10 drops of SpotTest nitrate reagent C is added to the culture containing the reagents. Since zinc reduces nitrates to nitrites, a pink or red color will appear and verifies the fact that nitrates were not reduced to nitrites by the bacteria. If a red color does not appear, the nitrates in the medium were reduced past the nitrite stage to either ammonia or nitrogen gas.

Materials Required

1. 24- to 48-hour broth cultures of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus epidermidis*
2. Garden soil
3. Bunsen burner
4. Inoculating loop
5. 1-ml pipette with pipettor
6. Nitrate broth tubes or nitrate agar slants
7. Nitrite test reagent A
8. Nitrite test reagent B

9. Test-tube rack
10. Incubator set at 35°C
11. 5 sterile test tubes
12. Marker pens
13. Disposable gloves

Procedure

First Period

1. Label three tubes of nitrate broth or nitrate agar slants with the three respective bacteria (*E. coli*, *P. fluorescens,* and *S. epidermidis*); label the fourth tube "garden soil" and the fifth tube "control." Add your name and date to each tube. The control tube serves two purposes:
   (1) To determine if the medium is sterile and
   (2) To determine if any O_2 comes out of the medium instead of out of the gas produced by the bacteria.
2. Using aseptic technique, inoculate three tubes with the respective bacteria, and the fourth with about a gram of garden soil.
3. Incubate all five tubes for 24 to 48 hours at 35°C.

Second Period

1. Observe the tubes for the presence of growth, and the absence of growth in the control tube.
2. With a pipette and pipettor, while wearing disposable gloves, add 0.5 ml of nitrate test reagent A and 0.5 ml of test reagent B to each of the culture tubes and mix. A distinct pink or red color indicates a positive test, provided the uninoculated control medium is negative.
3. Negative tests should be confirmed by adding several grains of zinc powder or 5 to 10 drops of Difco's nitrate reagent C and gently shaking the tube. If nitrate is present in the medium, it will turn red within 5 to 10 minutes; if it is absent, there will be no color change.
4. Record your results in the report

Result:

Upon addition of solution a and B the medium with *E.coli* culture; there was no colour change in the medium or further addition of Zinc the medium remained unchanged, which suggested that nitrates were reduced beyond nitrite ie., to ammonia to molecular nitrogen upon the addition of Solution A, B to the medium with *Pseudomonas* culture, there was no change in the colour of medium but on the addition of the medium changed to red colour, which indicated that nitrate not reduces to nitrite.

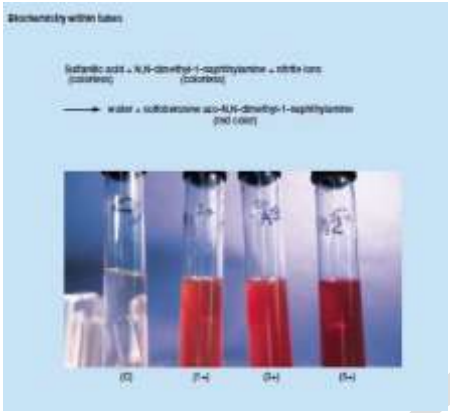


Fig: Nitrate Reduction. After 24 to 48 hours of incubation, nitrate reagents are added to the culture tubes. The tube on the left (C) is a negative broth control. The second tube (1+) is weakly positive, the third tube (3+) is more positive, and the tube on the right (5+) is very positive for nitrate reduction to nitrite as indicated by the deep red color.

BIOCHEMICAL CHARACTERIZATION - CARBOHYDRATE FERMENTATION TEST

Aim

1. To find the ability of microorganisms to ferment the given Carbohydrate.
2. To determine the ability to degrade amino acids.
3. To determine the ability of microorganism to produce gaseous end products in fermentation.

Principles

A metabolic process performed by almost all types of bacteria is known as fermentation. This will result in the production of ATP, the ultimate energy source of the organism. This will happen either in the presence or absence of atmospheric oxygen. Bacteria utilize the nutrients in their environment to produce ATP for their biological processes such as growth and reproduction. The enzyme systems in bacteria allow them to oxidize environmental nutrient sources. Bacteria will use different energy sources in the medium depends on the specific enzymes of each bacteria.

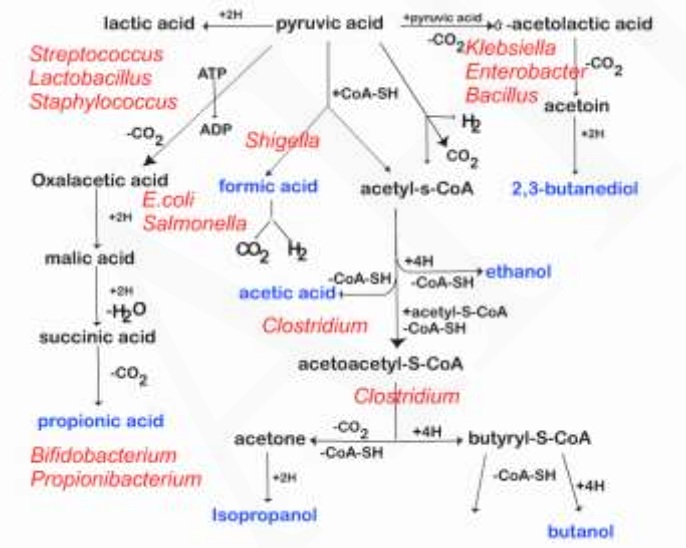


Fig: The sugar fermentation pattern may be unique to a particular species or strain. Sugar fermentation pattern of different bacteria

Many bacteria possess the enzymes system required for the oxidation and utilization of the simple sugar, glucose. Some bacteria have the ability to degrade complex carbohydrates like lactose, sucrose or even polysaccharides. Such bacterium should possess the enzymes that should cleave the glycosidic bonds between the sugar units and the resulting simple carbohydrate can be transported into the cell. Lactose is a disaccharide consisting of the glucose and galactose connected by glycosidic bond. The bacteria which produce the enzyme lactase will break this bond and thus release free glucose that can be easily utilized by the organism. The characteristics feature of the enzyme production in the bacteria enables them to use diverse carbohydrates and this will aid in the identification of unknown bacteria.

Phenol Red Carbohydrate Fermentation Broth:

Phenol red broth is a general purpose fermentation media comprising of trypticase, sodium chloride, phenol red and a carbohydrate. The trypticase provides amino acids, vitamins, minerals and other nitrogenous substances making it a nutritious medium for a variety of organisms. Sodium chloride helps in maintaining the osmotic balance and provides the

essential electrolytes for the transport into the cell while the carbohydrate acts as the energy source. The phenol red is the pH indicator and is initially neutral (pH 7). It supports the growth of most organisms whether they are able to ferment sugar or not. When the bacterium is inoculated into the tube, the bacterium which ferments the sugar will result in the production of acid that will change the color of phenol red. Fermentation reactions often begin with glycolysis. Glucose acts as an electron donor in the fermentation reaction, pyruvate, and metabolic product of glucose act as an electron acceptor. The other disaccharides and polysaccharides are hydrolyzed into glucose or converted into glucose and then the fermentation reaction will occur. Finally the reaction will result in the end products such as acid, ethanol, Hydrogen and Carbon dioxide and other compounds. This depends on the species of bacteria. Phenol red broth is a test is differential for gram negative bacteria.

When the organism ferments carbohydrates, acidic organic by products (Lactic acid, formic acid or acetic acid) is accumulated which turns the medium into yellow color with reduction in the pH (acidic). The inverted Durham tubes will detect the presence of gas. The degradation of peptones in the broth may result in the production of alkaline end products, which will change the broth color to pink often at the top of the tube.

Materials Required

1. Phenol Red Carbohydrate Fermentation Broth.
2. Bacterial culture.
3. Inoculation loop.
4. Incubator (37^0 C).

Procedure

I. Preparation of Carbohydrate Fermentation Broth

Weigh and dissolve trypticase, Sodium chloride, and Phenol red in 100 ml distilled water and transfer into conical flasks.

1. Add 0.5% to 1% of desired carbohydrate into all flasks.
2. Insert inverted Durham tubes into all tubes, the Durham tubes should be fully filled with broth.
3. Sterilize at 115^0 C for 15 minutes.
4. **Important**: Do not overheat the Phenol red Carbohydrate fermentation broth. The overheating will result in breaking down of the molecules and form compounds with a characteristic color and flavour. The process is known as caramelization of sugar (the browning of sugar).
5. Transfer the sugar into screw capped tubes or fermentation tubes and label properly.

Ingredients of the Fermentation Broth:

Trypticase: 1g

1. Carbohydrate: 0.5g
2. Sodium Chloride: 0.5g
3. Phenol red : 0.0189mg

*Autoclave at 115^o C for 15 minutes

II. Inoculation of Bacterial Culture into the Phenol Red Carbohydrate Broth

1. Aseptically inoculate each labeled carbohydrate broth with bacterial culture.(keep uninoculated tubes as control tubes).
2. Incubate the tubes at 18-24 hours at 37^oC.
3. Observe the reaction.

Precautions:

1. After inoculation into a particular sugar, sterilize the loop in order to avoid cross contamination of the tube with other sugars.

2. Keep uninoculated sugar tubes as control tubes.
3. Do not use the tubes with Durham tubes that are partially filled or with bubbles.
4. Over incubation will help the bacteria to degrade proteins and will result give false positive results.

Result:

1. **Acid production**: Changes the medium into yellow color- organism ferments the given carbohydrate and produce organic acids there by reducing the ph of the medium into acidic.
2. **Acid and Gas production**: Changes the medium into yellow color-organism ferments the given Carbohydrate and produce organic acids and gas. Gas production can be etected by the presence of small bubbles in the inverted durham tubes.
3. **Absence of fermentation**: The broth retains the red color. The organism cannot utilize the carbohydrate but the organism continues to grow in the medium using other energy sources in the medium.

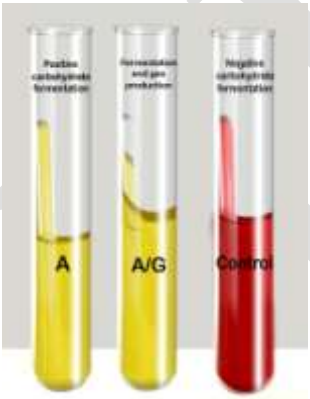


Fig: Results of carbohydrate fermentation test

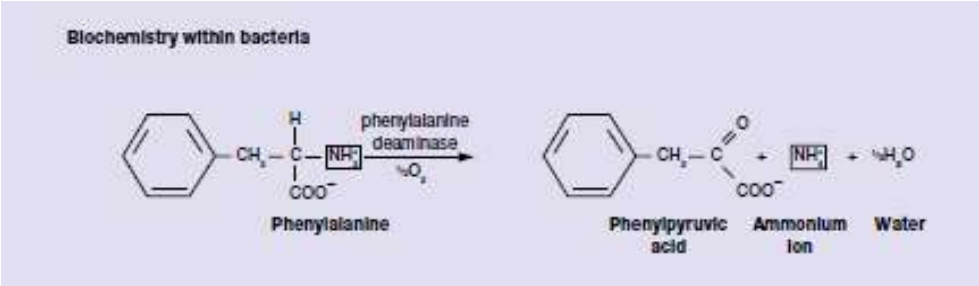
BIOCHEMICAL CHARACTERIZATION - AMINO ACID UTILIZATION TEST

Aim

To test the ability of bacteria to utilize amino acid

Principles

Phenylalanine deaminase catalyzes the removal of the amino group (NH_3^+) from phenylalanine. The resulting products include organic acids, water, and ammonia. Certain enteric bacteria (e.g., *Proteus*, *Morganella*, and *Providencia*) can use the organic acids in biosynthesis reactions. In addition, the deamination detoxifies inhibitory amines.



The **phenylalanine deaminase test** can be used to differentiate among enteric bacteria such as *E. coli* and *P. vulgaris*. *P. vulgaris* produces the enzyme phenylalanine deaminase, which deaminates phenylalanine, producing phenylpyruvic acid. When ferric chloride is added to the medium, it reacts with phenylpyruvic acid, forming a green compound. Since *E. coli* does not produce the enzyme, it cannot deaminate phenylalanine. When ferric chloride is added to an *E. coli* culture, there is no color change.

Materials Required

1. 24- to 48-hour broth cultures of *Escherichia coli* and *Proteus vulgaris*
2. 3 phenylalanine deaminase agar slants
3. 10% aqueous ferric chloride solution (or 10% FeCl3 in 50% HCl)
4. inoculating loop
5. Pasteur pipette with pipettor
6. test-tube rack
7. incubator set at 35°C
8. Marker pen

Procedure

First Period

1. Label two slants of phenylalanine deaminase agar with the name of the bacterium to be tested. Use another slant as a control. Add your name and date to each slant.
2. Using aseptic technique, inoculate each of the slants with the respective bacteria.
3. Incubate aerobically at 35°C for 18 to 24 hours.

Second Period

1. With the Pasteur pipette, add a few drops of the 10% FeCl3 to the growth on the slant. Rotate each tube between your palms to wet and loosen the bacterial growth. The presence of phenylpyruvic acid is indicated by the development of a green color within 5 minutes and indicates a positive test for phenylalanine deamination. If there is no color change after adding the reagent, the test is negative, and no deamination has occurred.

2. Based on your observations, determine and record in the report for exercise 33 which of the bacteria were able to deaminate phenylalanine.

Result:
The appearance of green color indicated the presence of phenyl pyruvic acid and said to be positive test.

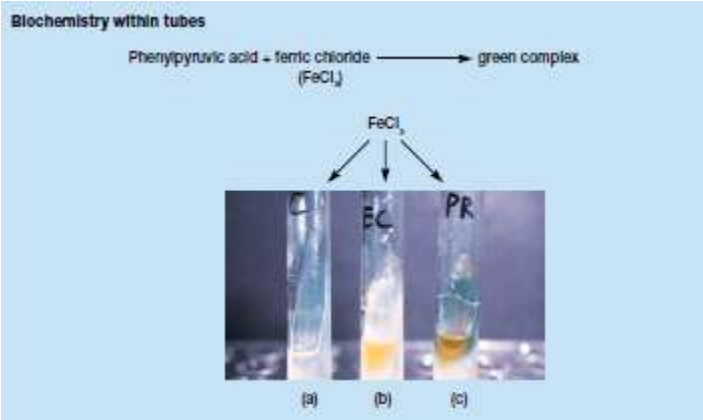


Fig: Phenylalanine Deamination. (**a**) Uninoculated control. (**b**) Phenylalanine negative. (**c**) Phenylalanine positive.

BIOCHEMICAL CHARACTERIZATION - HYDROLYSIS OF POLYMERS – STARCH HYDROLYSIS

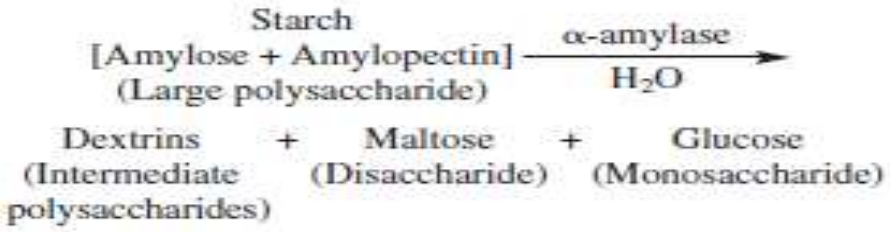
Aim

To demonstrate starch hydrolysis and to isolate starch degrading bacteria from soil

Principles

Many bacteria produce enzymes called **hydrolases.** Hydrolases catalyze the splitting of organic molecules into smaller molecules in the presence of water. This exercise will present the hydrolysis of the carbohydrate starch.

The starch molecule consists of two constituents: **amylose,** an unbranched glucose polymer (200 to 300 units) and **amylopectin,** a large branched polymer. Both amylopectin and amylose are rapidly hydrolyzed by certain bacteria, using their α-**amylases,** to yield dextrins, maltose, and glucose, as follows:

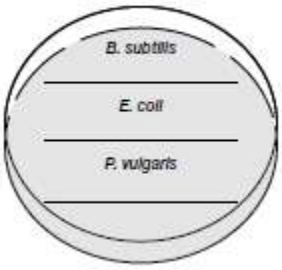


Gram's iodine can be used to indicate the presence of starch. When it contacts starch, it forms a blue to brown complex. Hydrolyzed starch does not produce a color change. If a clear area appears after adding Gram's iodine to a medium containing starchand bacterial growth, α-**amylase** has been produced by the bacteria. If there is no clearing, starch has not been hydrolyzed.

Procedure

First Period: Starch Hydrolysis Test

1. With a wax pencil, divide a starch agar plate into three straight sections as indicated. Label each with the bacterium to be inoculated. Add your name and date to the plate.
3. Using aseptic technique, streak the respective bacteria onto the plate in a **straight line** within the section.
4. Incubate the plate for 24 to 48 hours at 35°C.



Second Period: Starch Hydrolysis Test

1. Place several drops of Gram's iodine on each of the line streaks on the starch agar plate. If the area around the line of growth is clear, starch has been hydrolyzed, and the test is positive; if it is not clear or the entire medium turns blue, starch has not been hydrolyzed, and the test is negative.
2. If the results are difficult to read, an alternative procedure is to invert the plate (after removing the lid) over a beaker containing iodine crystals. The rising vapor will react

with the starch without the interference of the red-brown color of the unreacted iodine.

3. Record your results and report.

Result:

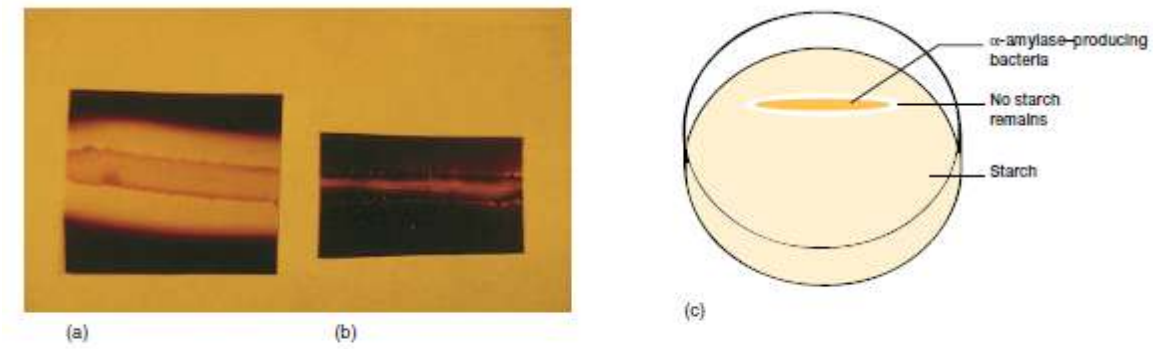


Fig: Test for Starch Hydrolysis after Adding Gram's Iodine. (**a, c**) Positive hydrolysis. The complete breakdown of all starch is shown by the clear (white) halo. (**b**) Negative hydrolysis. Starch remains intact—no color change as indicated by the purple to brown color around the streak.

BIOCHEMICAL CHARACTERIZATION - HYDROLYSIS OF POLYMERS – LIPID HYDROLYSIS

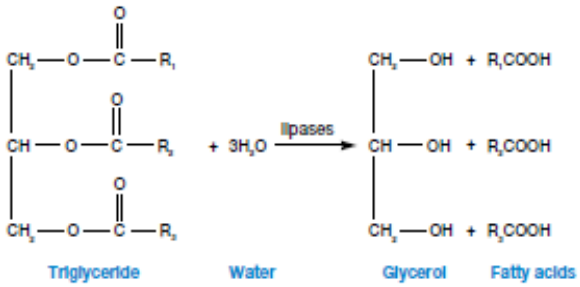
Aim

To demonstrate the lipid hydrolysis and to isolate lipid hydrolising bacteria from soil

Principles

Lipids are high molecular weight compounds possessing large amounts of stored energy. The two common lipids catabolized by bacteria are the **triglycerides (triacylglycerols)** and **phospholipids.** Triglycerides are hydrolyzed by the enzymes called **lipases** into glycerol and free fatty acid molecules as indicated in the following diagram. Glycerol and free fatty acid molecules can then be taken up by the bacterial cell and further metabolized through reactions of glycolysis, _-oxidation pathway, and the citric acid cycle. These lipids can also enter other metabolic pathways where they are used for the synthesis of cell membrane phospholipids. Since phospholipids are functional components of all cells, the ability of bacteria to hydrolyze host-cell phospholipids is an important factor in the spread of pathogenic bacteria.

In addition, when lipase-producing bacteria contaminate food products, the lipolytic bacteria hydrolyze the lipids, causing spoilage termed **rancidity.**



When these same lipids are added to an agar solidified culture medium and are cultured with lipolytic bacteria, the surrounding medium becomes acidic due to the release of fatty acids. By adding a pH indicator to the culture medium, it is possible to detect the hydrolysis of lipids by a color change. For example, spirit blue agar with Bacto lipase reagent has a lavender color. It turns royal blue around lipolytic bacterial colonies due to the acid pH.

Procedure

First Period

1. With a wax pencil, divide the bottom of a spirit blue agar plate in half and label half the plate *P. mirabilis* and the other half *S. epidermidis.* Place your name and date on the plate.
2. Spot-inoculate (figure 23.1*a*) the spirit blue agar plates with the respective bacteria.
3. Incubate the plate in an inverted position for 24 to 48 hours at 35°C.

Second Period

1. Examine the plate for evidence of lipid hydrolysis. Hydrolysis is evidenced by a blue zone around the bacterial growth. If no lipid hydrolysis has taken place, the zone around the colony will remain lavender.
2. Measure the zone of hydrolysis and record your results.

Result:

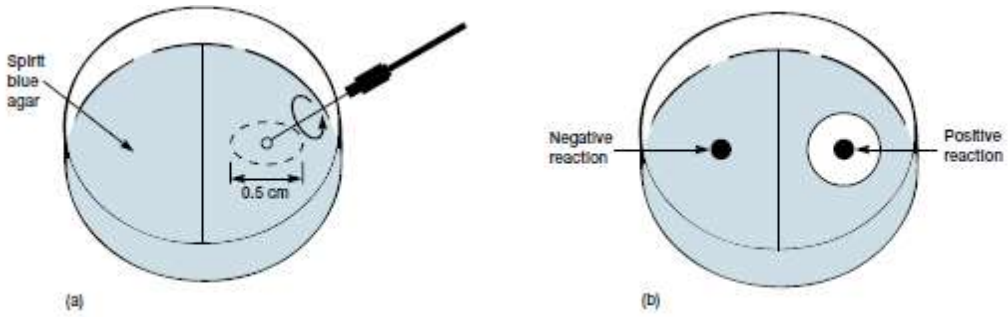


Fig: Lipid Hydrolysis. (**a**) Procedure for spot inoculating a spirit blue agar plate. (**b**) Positive and negative reactions.

BIOCHEMICAL CHARACTERIZATION - HYDROLYSIS OF POLYMERS – CASEIN HYDROLYSIS

Aim

To demonstrate the casein hydrolysis and to isolate casein hydrolising bacteria from soil

Principles

Casein is a large milk protein incapable of permeating the plasma membrane of bacteria. (Its presence is the reason milk is white.) Therefore, before casein can be used by some bacteria as their source of carbon and energy, it must be degraded into amino acids. Bacteria accomplish this by secreting **proteolytic enzymes** that catalyze the hydrolysis of casein to yield amino acids, which are then transported into the cell and catabolized.

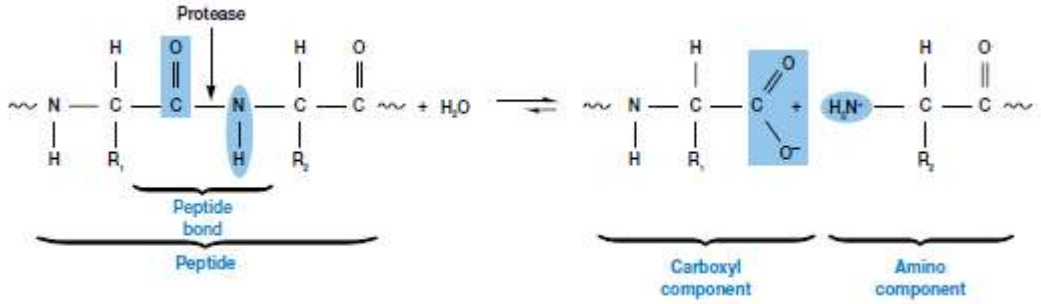


Fig: Proteolytic Hydrolysis.

When milk is mixed with plate count agar, the casein in the milk makes the agar cloudy. Following inoculation of the plate count agar, bacteria that liberate proteases (e.g., caseinase) will produce a **zone of proteolysis** (a clear area surrounding the colony). Clearing of the cloudy agar (a positive reaction) is the result of a hydrolytic reaction that yields soluble amino acids (figure 26.2*b*). In a negative reaction, there is no protease activity, and the medium surrounding the bacterial colony remains opaque.

Materials Required

1. 24- to 48-hour tryptic soy broth cultures of *Escherichia coli*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* tubes of plate count agar (tryptone glucose yeast agar)
2. Inoculating loop
3. Boiling water bath
4. Sterile petri plates
5. Water bath set at 48° to 50°c
6. Water bath set at 35°c
7. Bunsen burner
8. Test-tube rack
9. Marker pen
10. Sterile skim milk
11. 5-ml pipette with pipettor

Procedure

First Period

1. Melt the tubes of plate count agar by placing them in the boiling water bath. After melting, place the tubes in the 48° to 50°C water bath for 10 minutes.
2. With a wax pencil, mark the bottom of a petri plate into three sections: label one *E. coli*, the second, *B. subtilis*, and the third, *P. aeruginosa.* Add your name and date to the plate.

3. Pipette 2 ml of warm (48° to 50°C) sterile skim milk into the petri plate. Add the melted agar and mix thoroughly by moving the plate in a circular motion. Allow this medium to gel on a cool, level surface.
4. As shown in figure 14.3, aseptically spotinoculate each third of the petri plate with the appropriate bacterium as per the label. Place a loopful of culture on the center of each section and spread it in a circular fashion to cover an area about the size of a dime or less (5 to 8 mm in diameter).
5. Incubate the plate in an inverted position at 35°C for 24 to 48 hours.

Second Period

1. Examine the plate count agar for the presence or absence of a clear zone (zone of proteolysis) surrounding the growth of each of the bacterial test organisms. You can see the clear zones best against a black background.
2. Based on your observations, determine and record in the report for exercise 26 which of the bacteria were capable of hydrolyzing the casein. Also, measure the zone of hydrolysis for each colony.

Result

The clear zone was observed around few colonies indicating the milk protein casein was degraded by bacteria preferably by the production of enzyme caseinase or proteinase selected colonies may be streaked as a single line at the centre of a fresh skim milk agar plate to demonstrate the extend of hydrolysis of the organism.

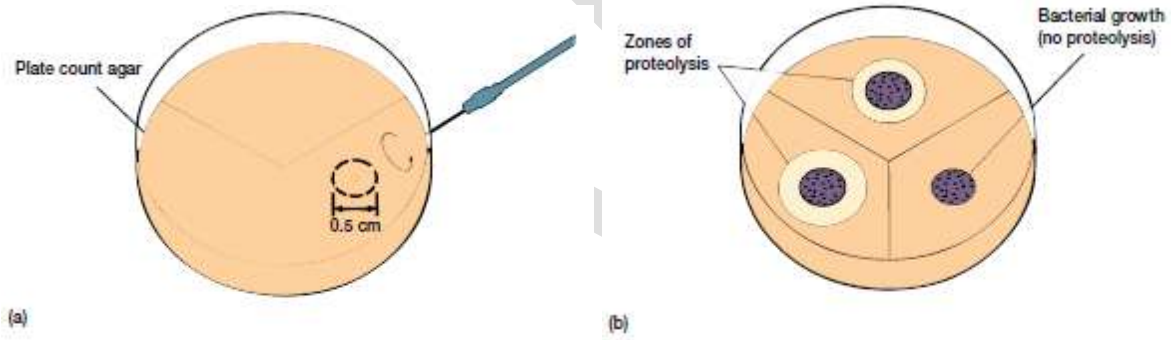


Fig: Procedure for Determining Casein Hydrolysis. (**a**) Spot inoculation of a plate count agar with milk plate. (**b**) Plate exhibiting two zones of proteolysis.

BIOCHEMICAL CHARACTERIZATION - HYDROLYSIS OF POLYMERS – GELATIN HYDROLYSIS

Aim

To demonstrate the gelatin is derived from hydrolyzing the collective tissue collagen.

Principles

When boiled in water, the connective tissue collagen (which is stringy, insoluble, and indigestible) changes into **gelatin,** a soluble mixture of polypeptides. Certain bacteria are able to hydrolyze gelatin by secreting a proteolytic enzyme called **gelatinase.** The resulting amino acids can then be used as nutrients by the bacteria. Since hydrolyzed gelatin is no longer able to gel, it is a liquid. The ability of some bacteria to digest gelatin is an important characteristic in their differentiation. For example, when grown on a gelatin medium (Thiogel), *Clostridium perfringens* causes liquefaction, whereas *Bacteroides fragilis* does not. Gelatin hydrolysis can also be used to assess the pathogenicity of certain bacteria.

The production of gelatinase can often be correlated with the ability of a bacterium to break down tissue collagen and spread throughout the body of a host. Gelatin liquefaction (the formation of a liquid) can be tested for by stabbing nutrient gelatin deep tubes. Following incubation, the cultures are placed in a refrigerator or ice bath at 4°C until the bottom resolidifies. If gelatin has been hydrolyzed, the medium will remain liquid after refrigeration. If gelatin has not been hydrolyzed, the medium will resolidify during the time it is in the refrigerator. Nutrient gelatin may require up to a 14-day incubation period for positive results.

Materials Required

1. 24- to 48-hour tryptic soy broth cultures of *Escherichia coli* and *Proteus vulgaris*
2. 4 nutrient gelatin deep tubes
3. Bunsen burner
4. Inoculating loop
5. 1-ml pipettes with pipettor
6. Refrigerator or ice-water bath
7. Test-tube rack
8. Incubator set at 35°c
9. 3 sterile test tubes
10. Marker pen

Procedure

First Period

1. Label three nutrient gelatin deeps with your name, date, and the bacterium to be inoculated. Label the fourth tube "control."
2. Using aseptic technique, inoculate three of the deeps with the appropriate bacterium by stabbing the medium f of the way to the bottom of the tube.
3. Incubate the four tubes for 24 to 48 hours or longer at 35°C. The incubation time depends on the species of bacteria; some may require incubation for up to 2 weeks. If the latter is the case, observe on days 7 and 14.

Second Period

1. Remove the nutrient gelatin deep tubes from the incubator and place them in the refrigerator at
2. 4°C for 30 minutes or in an ice bath for 3 to 5 minutes.
3. When the bottom resolidifies, remove the tubes and gently slant them. Notice whether or not the surface of the medium is fluid or liquid. If the nutrient gelatin is liquid, this

indicates that gelatine has been hydrolyzed by the bacterium. If no hydrolysis occurred, the medium will remain a gel. The uninoculated control should also be negative.

Result

Nutrient gelatin tubes inoculated with Bacillus sp showed gelatin liquefaction even after incubation at 4°C for 30 minutes which was a positive reaction for gelatinase a protease compound.