PRACTICAL 7 STAINING STRATEGIES

Structure

7.1 INTRODUCTION

Practical 7 focuses on the different staining techniques used to visualize bacterial cell morphology. We have studied about the characteristics of different bacteria, yeast, fungi etc. in Unit 5 in the theory booklet. Here we will learn about the staining techniques which are used in microbiology to identify and study the bacterial cell morphology.

Objectives:

After undertaking this practical you will be able to:

- describe the different staining techniques, and
- identify the different bacteria using these staining techniques.

7.2 STAINING STRATEGIES: BASIC INTRODUCTION

Microorganisms are difficult to be seen in living state because of their minute, colourless and transparent nature. Moreover, there is limitation of insufficient contrast to bright field microscopy. Therefore, staining is of prime importance to visualize microbial cells and increase their contrast. Different stains can be used to see these tiny creatures. But first, what are *stains*?

Stains are *dyes used to increase contrast by their binding to certain parts of cells or cells themselves*. Chemically, stains are coloured organic compounds consisting of three portions as highlighted herewith and in Figure 7.1.

1. Benzene - colourless organic solvent
2. Chromophore - group with conjugated double bonds that impart colour to benzene or to dye
3. Auxochrome - gives property of ionization to the chromogen (Benzene + Chromophore)

Figure 7.1: Components of Stains

Dyes can bind with cells by ionic, covalent or hydrophobic bonding. Though ionic interaction is the most common means of attachment, dyes can also bind through covalent interactions or due to their solubility characteristic, e.g. Schiff's reagent in Feulgen procedure for DNA staining binds covalently to deoxyribose sugars after hydrochloric acid treatments. Similarly, Sudan III stains lipids because of its solubility in lipid. We shall learn about these dyes/ stains in this practical. Let us begin by studying about the types of stains.

7.3 TYPES OF STAINS

We studied above that dyes can bind with cells or cell components by ionic bonding. Ionizable dyes can be divided into two classes based on the nature of charged group. These include:

(1) *Acidic Stains (anionic)* – These are sodium, potassium, calcium or ammonium salts of coloured acids, which on ionization give negatively charged chromogen, as illustrated in Figure 7.2. These are best under acidic conditions. Most acidic dyes are unable to bind to the cell surface and also not able to penetrate into the cell because of repulsion by cell surface. These possess negatively charged groups, like, carboxyl (– COOH), phenolic, hydroxyl (–OH) so have a strong affinity to positively charged cell structure and macromolecules, like proteins.

Example: Eosin, Picric Acid, Rose Bengal, Acid Fuchsin etc.

Figure 7.2: Anionic chromogen

(2) *Basic Stains (Cationic)* – These are chloride or sulfate salts of coloured bases which on ionization give positively charged chromogen, as shown in Figure 7.3. As such, these have a strong affinity for the negative constituents of the cell (or these bind strongly to negatively charged component of the cell), e.g. nucleic acid. Surface of the bacterial cells is also negatively charged, so basic dyes are most often used in bacteriology. These work better at high pH (i.e. neutral / alkaline conditions). For this reason, basic dyes are made up in alkaline solution, e.g. Loeffler's methylene blue stain is prepared in KOH solution.

Figure 7.3: Cationic chromogen

The pH can affect the staining effectiveness by affecting the nature and degree of charge on cell components. Whereas, in neutral or alkaline environment, microbial cells are negatively charged, in acidic environment, these stain poorly with basic dyes. Different staining techniques can be used to visualize and study the different morphological characteristics of a cell. We shall learn about these staining techniques next.

7.4 TYPES OF STAINING TECHNIQUES

Staining can be positive or negative. Let us get to know about these two staining techniques.

Positive Staining – Here, a stain has a positively charged chromophore that gets attached to the negatively charged outer surface of the microbial cell and thus stains the cell, as shown in Figure 7.4.

Negative Staining – Here, a stain has a negatively charged chromophore that is repelled by the negative bacterial surface as shown in Figure 7.4. It results in indirect staining of microbial cell. It appears as clear area surrounded by coloured background. It is used for visualizing many cell structures like capsule. Negative stains also work better under neutral or alkaline conditions.

Figure 7.4: The staining principle

Microorganisms can also be stained either by simple staining or differential staining. What do we mean by simple or differential staining? Let's find out.

Simple Staining – Here single staining agent is used to determine the size, shape and arrangement of bacterial cells. It is simple and easy to perform. Dried suspension of microorganisms on slide is flooded with a dilute solution of a dye e.g. carbol fuchsin or crystal violet for 1-2 minutes. It is then rinsed several times in water to remove excess stain and is blotted dry.

Differential Staining – It divides bacteria into separate groups based on staining properties, like Gram's stain, Acid-fast stain etc. Here 2 or more contrasting stains are used. It is also used to visualize cell structures, like flagella stain, capsule stain, spore stain, etc. In differential staining at least 3 chemical reagents are used. First stain is called the *primary stain* and it imparts colour to all the cells. Second reagent is called *decolourizing agent* which may or may not remove the colour of the primary stain from the entire cell or part of the cell, depending upon the chemical composition of the cell. Third reagent is called the *counter stain* which has a contrasting colour to primary stain and does impart colour to the those cells or their parts which are decolourized by decolourizing agent. Cells which retain the primary stain cannot absorb the counter stain. In this way, cell types or their structures can be distinguished from each other on the basis of the stain that is retained. In some cases, another chemical reagent called *mordant* is also used to increase cell's affinity for a stain.

In the discussion above, we mentioned about gram staining and acid fast staining. A detailed discussion on these techniques follows.

7.4.1 Gram Staining

Gram staining is a differential staining procedure which divides bacteria into two groups: *Gram positive* and *Gram negative*, based on staining properties. The gram stain was developed in 1884 by the Danish Physician, *Dr. Christian Gram*. It is one of the most important and widely employed staining procedure in bacteriology. It is very useful and is almost essential in identifying an unknown bacteria.

The basis of gram staining is the difference in cell wall composition of two types of bacterial cells. Gram positives have thick cell wall, made up of multi-layered peptidoglycan with extensive cross-linking. On the other hand, gram negative cell wall has a very thin peptidoglycan layer surrounded by outer lipid rich outer membrane Fig. 7.6(b). There is no outer membrane in case of gram positives. This difference in cell wall leads to decolourization of gram negatives [as shown in Figure 7.5(a)] but not gram positives [as shown in Figure 7.5(b)] by decolourizing agent i.e., ethanol or acetone.

Figure 7.5: Gram staining

Gram stain uses four chemical reagents. There include

(i) *Primary Stain* – Crystal violet is the primary or first stain, which stains all the cells violet/purple as can be seen Figure 7.6 (a).

Figure 7.6 (a): Gram staining technique

(ii) *Mordant* – Gram's iodine serves as mordant. It binds with primary stain to form insoluble complex, crystal violet-iodine (CV-I) complex, that intensify the colour of the stain. At this point, all cells will appear purple – black.

(iii) *Decolourizing Agent* – 95% ethanol is used as a decolourizing agent. It has two functions –

(1) It acts as protein – dehydrating agent, and

(2) It is a lipid solvent.

The action of decolourizing agent depends on two factors –

(i) Concentration of lipids in cell wall.

(ii) Thickness of peptidoglycan layer.

In gram negatives, lipid content is high as compared to gram positives, so ethanol action increases the porosity of cell wall by dissolving the lipid layer in outer membrane. Peptidoglycan layer being thinner and less highly cross linked as compared to gram positives, is not able to retain the CV-I complex and cell become colourless. On the other hand, in gram positives, the pores become smaller due to dehydrating effect of the alcohol, so CV-I complex is retained by the thick highly cross linked peptidoglycan layer, and the cells remain purple as you may have seen in Figure 7.5(b). This step generates the differential aspect of the gram stain and is very critical. Look at Figure 7.5, it shows the staining colour for gram positive and gram negative.

Figure 7.6 (b): Gram positive and gram negative cell walls

Acetone can also be used as a decolourizing agent.

(iv) *Counter Stain* – Finally, the smear is counter stained with a simple basic dye different in colour from crystal violet. Safranin is the most commonly used counter stain. Since only gram negatives loses the crystal violet stain, these cells absorb safranin and appear pink to red as shown in Figure 7.5(a) and Figure 7.6(a). Gram positives remain purple in colour as can be seen in Figure 7.5(b) and 7.6(a) above.

7.4.2 Acid Fast Staining

Acid fast staining is a type of differential staining used for identification of certain bacteria, e.g. *Mycobacteria* which cannot be stained readily by simple staining technique or gram staining because of thick, waxy wall.

Ziehl-Neelsen staining is used for staining these bacteria. The technique was discovered first by *Robert Koch* during his pioneering investigations on tuberculosis and was the fore-runner of Ziehl-Neelsen stain used today for diagnosis of these organisms in laboratory.

Acid fast bacteria are called so because once these cells get stained with basic fuchsin, these resist decolourization with acid alcohol as can be seen in margin illustiation. This acid fastness red stain is due to high lipid content particularly of mycolic acid in their cell wall. Mycolic acid, as illustrated in Figure 7.7, is a group of branched chain hydroxyl lipids, which bind covalently to peptidoglycan of cell wall giving the cell surface a waxy, hydrophobic consistency.

Figure 7.7: Structure of mycolic acid and basic fuchsin

Three reagents are used in acid fast staining by Ziehl-Neelsen method. These are:

(1) *Primary Stain* - Ordinary aqueous stains like methylene blue, crystal violet, etc. are not able to stain most of the species of Mycobacteria. Carbol fuchsin, a red mixture of basic fuchsin and phenol can be used for staining these bacteria. Basic fuchsin, as illustrated in Figure 7.6, binds to carboxylic acid group of mycolic acid and phenol enhances penetration of basic fuchsin into lipid. Penetration is further enhanced by slow heating to steaming for 2-3 minutes. This drives the stain inside the cytoplasm. In modified Ziehl-Neelsen method, instead of heating, a wetting agent – Turgitol – can be added to the stain. This agent reduces surface tension between the cell wall of the mycobacteria and the stain. After staining, all cells become red as can be seen in Figure 7.7.

(2) *Decolourizing Agent* – Acid alcohol – a mixture of 95% ethanol and 3% HCI – is used for decolourization. Before decolourization, smear is allowed to cool to make waxy substances hardened. As primary stain is more soluble in cellular waxes than in decolourizing solution, acid-fast bacteria retain the primary stain. However, all non-acid fast bacteria loose primary stain because of the lack of cellular waxes as can be seen in Figure 7.8.

(3) *Counter Stain* – Methylene blue is used to stain previously decolourized cells. All non acid-fast bacteria de-stained by acid alcohol now absorbs counter stain and takes on its blue colour. All acid-fast bacteria remained red in colour due to primary stain as illustrated in Figure 7.8.

Genus *Mycobacterium* shows acid fastness. These are aerobic, slightly curved or straight rods that sometimes branch or form filaments. These filaments readily break down into rods and coccoid bodies, when disturbed. Some grow very slowly. Some are free-living saprophytes and some are pathogens, e.g. *M. bovis* could cause tuberculosis in cattles, *M. tuberculosis* causes tuberculosis in humans, *M leprae* causes leprosy in humans. Acid-fast stain has a diagnostic value in identifying these pathogenic mycobacteria.

Figure 7.8: Ziehl-Neelsen staining

7.4.3 Spore Staining (Schaeffer – Fulton Method)

What is an endospore? Look at the Figure 7.9, which illustrates an endospore. An endospore is a specialized, highly resistant, dormant structure formed within the vegetative cell of some bacteria e.g. *Bacillus* (rod), *Clostridium* (rod), *Sporosarcina* (cocci), *Desulfatomaculum* under adverse environmental conditions like scarcity of water and nutrients. It was discovered by German Botanist *Ferdinand Conn.* It is resistant to environment stresses and can survive extreme heat, lack of water and exposure to many toxic chemicals and radiations for years. This resistance is attributed to the presence of calcium dipicolinate, small acid soluble DNA binding proteins, dehydrated protoplast, presence of efficient DNA repair enzymes, spore coat and greater stability of cell proteins.Structurally, endospore has a complex structure. From outside, it is covered with exosporium – a thin delicate covering as you may have noticed in Figure 7.9. Beneath exosporium, a spore coat lies which consist of several layers of protein and under it is a cortex made up of peptidoglycan. Inside the cortex is a spore cell wall which surrounds the core (protoplast). Core is metabolically active and consists of ribosomes, nucleoid, etc. Endospores are very important clinically and also in food industry because of their resistance to heat and other lethal agents that normally kill vegetative cells. Further, growth of some endospore forming bacteria in foods is a problem as it may produce toxins and lead to diseases, as you have already studied in the Food Microbiology and Safety Course in Unit 5.

Figure 7.9: The endospore

One endospore is formed per bacterial cell. The process of formation of endospore is called *sporulation* that occurs in an organized manner over a period of several hours. Figure 7.10 illustrates the life cycle of spore forming bacteria. Sporulation is not a way of reproduction. No increase in number of cells occur by this process. The transformation of dormant spores into active vegetative cell is also a complex process (germination).

Figure 7.10: Life cycle of spore forming bacteria

Endospore cannot be stained by ordinary methods such as simple staining and gram staining because these dyes do not penetrate the wall of endospore. By treating with simple stain e.g. methylene blue, spores can be visible as colourless areas. The position of spores in mother cell varies with species. It may be located terminally, sub-terminally or centrally inside the vegetative cell, as illustrated in Figure 7.11. Also the diameter of the endospore may be the same or smaller or larger than the diameter of the vegetative cell.

Figure 7.11: Position of the endospore

Special stains are required to make spores *clearly visible*. Different techniques like *negative staining, dorner staining method, schaeffer fulton staining* method can be used. The most commonly used one is a schaeffer–fulton method. It is a differential staining technique using 2 different reagents – Malachite green and Safranin. Figure 7.25 graphically represents the staining process in Exercise 5.

Malachite green is a primary stain. Spores do not take the stain easily, so heating is applied by steaming for about 5 minutes. This makes the stain penetrate the endospore wall and make vegetative cell, as well as, endospores stain green as you can see in the margin illustration.

After primary stain, decolourization is done by tap water. Once the slide is cooled down, washing with tap water is done for about 30 seconds to one minute to remove the excess primary stain. As the stain does not have the strong affinity for vegetative cell, it is removed from the vegetative cell by tap water. So vegetative cells become colourless and spores remain green. Safranin is used as a counter stain. It is absorbed by the vegetative cells, which become colourless during decolourizing step. So after staining by this technique, spores will appear green and vegetative cells red. Figure 7.13 shows the staining as seen under the light microrcope.

Figure 7.13: Schaeffer – Fulton staining as seen under the microrcope

Next, let us learn about the capsule staining.

7.4.4 Capsule Staining (Anthony Staining Method)

A rigid, well-organized gelatinous layer called *capsule* is present outside the cell wall of many bacteria e.g. *Streptococus pneumoniae, Clostridium perfringens* and *Klebsiella pneumoniae*. It is mostly made up of polysaccharide (homo or hetero-polysaccharide) though in few it is proteinaceous in nature, e.g., in *Bacillus anthracis* it consists of poly-D-glutamic acid. The ability to form capsule is genetically determined. Capsule is not required for reproduction or growth of bacteria though it confers several advantages:

1. It protects the bacteria from phagocytosis by host phagocytes, so it contributes to the virulence of bacteria, e.g. *Streptococcus pneumoniae*.
2. It provides protection against dessication.
3. It helps the bacteria in attachment to host tissues e.g. in *Streptococcus mutans* capsule helps in attachment to the surface of the teeth and thus causing dental caries.
4. Capsule may be used as a source of nutrition by *S. mutans*.
5. Viscosity of a capsule may help in inhibiting the movement of nutrients out of the cell.
6. Capsule also exclude bacterial viruses and most hydrophobic toxic materials such as detergents.

Capsule staining is difficult because capsular material is soluble in water and may be dislodged or removed during vigorous washing. Negative staining can be used to visualize the capsule. Here presence of capsule is shown by mixing the bacteria in a loopful solution containing tiny colloidal suspension of coloured particles e.g. India Ink or nigrosin which cannot penetrate the cell or its capsule. After spreading the mixture in a thin film and air-drying, counter staining of cells is done with simple stains such as safranin, crystal violet etc. Capsule does not accept these dyes so appear as a halo surrounding coloured cells in a dark blue-black background as shown in Figure 7.14 (a) and (b).

Figure 7.14: Negative staining of capsule

Another method used for capsular staining is Anthony staining method, devised by *E.E. Anthony* in 1931. The method involves the use of a primary stain i.e., 1% aqueous crystal violet. Both the capsular material and the cell wall will take the colour of the stain and will appear dark blue. But the capsule being nonionic will not absorb the primary stain. It adheres to the capsule without binding to it. Because capsule is water soluble, therefore, copper sulphate is used both as a decolourizing agent and as a counter stain. It takes out the colour from the capsular material and counter stains the decolourized capsule which now appears as a light blue zone surrounding deep purple cells as shown in Figure 7.15.

Figure 7.15: Capsule staining using anthony staining method

In both *Klebsiella* and *Bacillus*, capsule can be seen surrounding the dark purple coloured cells. Capsule will appear as a clear zone in negative staining against dark background, whereas, it is light blue coloured in anthony's method.

Having learnt about the different staining techniques, next let us get to know about the composition of different stains, which are used in these staining techniques.

7.5 COMPOSITION OF DIFFERENT STAINS

In the earlier section you may have noticed that different stains are used for staining the specimen. The composition of these different staining reagents, used to stain bacterial cell or its parts, are given herewith. You will find this information useful in preparing these stains.

1. Methylene Blue

Methylene Blue	-	0.3 gm
Distilled Water	-	100 ml

2. Crystal Violet (Hucker's – for Gram Stain)

Solution A

Crystal Violet – (90% dye content) - 2 gm

Ethyl alcohol (95%)	-	20.0 ml

Solution B

Ammonium Oxalate	-	0.8 gm
Distilled Water	-	80.0 ml

Mix Solution A and Solution B

3. Crystal Violet (1% for capsule stain)

Crystal Violet (85% dye content)	-	1.0 gm
Distilled Water	-	100.0 ml

4. Carbol fuchsin (Ziehl's)

Solution A

Basic fuchsin (90% dye content)	-	0.3 gm
Ethyl alcohol (95%)	-	10.0 ml

Solution B

Phenol	-	5.0 gm
Distilled Water	-	95.0 ml

Mix Solutions A and B. Add two drops of triton x per 100 ml of stain for use in heatless method.

5. Gram's Iodine

Iodine	-	1 gm
Potassium Iodine	-	2.0 gm
Distilled Water	-	300.00 ml

6. Ethyl Alcohol (95%)

Ethyl Alcohol (100%)	-	95.0 ml
Distilled Water	-	5.0 ml

7. Acid Alcohol

Ethyl alcohol (95%)	-	97.0 ml
Hydrochloric acid	-	3.0 ml

8. Safranin

Safranin	-	0.25 ml
Ethyl alcohol (95%)	-	10.0 ml
Distilled Water	-	100.0 ml

9. Malachite Green

Malachite Green	-	5.0 gm
Distilled Water	-	100.0 ml

Different dyes or stains have different rates of staining, e.g. Methylene blue is one of the slowest stains while crystal violet is more reactive and may stain sufficiently in 15-30 seconds. Carbol fuchsin is even more powerful and requires a few seconds only for staining.

Before performing the staining process, preparation of bacterial smear and fixation of bacterial cells on glass slide is needed, otherwise these would be washed away during the staining procedure. What do we mean by fixation?

Fixation is the process of preserving internal and external structures of microorganisms. It leads to the killing of microbial cells and their firm attachment to the microscopic slides.

There are two ways of carrying out fixation. Let's get to know them.

(i) *Heat Fixation* – This is the usual method. Here, bacteria is fixed by gentle heating of air-dried bacterial film, which results in coagulation of bacterial proteins. It fixes the overall morphological features but not structures within the cell.

(ii) *Chemical Fixation* – Here chemical fixatives containing ethanol, acetic acid and formaldehyde etc. penetrates the cell and inactivates and immobilizes cellular components like proteins, lipids etc. It does less damage to the specimen than heat.

Fixation has following disadvantages also. These are highlighted herewith:

(i) It distorts the cell's appearance.

(ii) Motility can't be studied.

After fixation, stain is applied as per the procedure.

In our discussion so far, we have described the stains, their types and techniques. Along with this information, it is also important for us to know about the basic characteristics of some common bacteria, which can be observed by using the staining techniques we have discussed above.

You may recall studying about these basic characteristics of bacteria in Unit 5 in the Theory booklet. We suggest you look up the Unit once again now. To help you recapitulate, the basic characteristics of some important bacteria are also summarized herewith. So read them carefully, because you will find this information useful when you undertake the exercises given later in this practical.

7.6 CHARACTERISTICS OF SOME COMMON BACTERIA

We strongly recommend that you continue your study on this topic after reviewing with the information presented in Unit 5 in the Theory booklet. The basic characteristics of some common bacteria as presented here are also detailed in Unit 5.

(1) ***Escherichia*** – It belongs to order Enterobacteriales. It is the best-studied organism present in the colon of humans and other warm-blooded animals. It is commonly employed in research and used as indicator in analysis of water and food for faecal contamination. Figure 7.16 (a) and (b) shows *Escherichia* growth on media and as seen under the microscope.

Figure 7.16: Escherichia growth on media and as seen under the microscope

Escherichia is gram negative, non-sporulating straight rod, if motile peritrichously flagellated. These are facultative anaerobes, oxidase negative and have simple nutritional requirement. These carry out mixed acid fermentation. *Escherichia* sp. may play a nutritional role in intestine by synthesizing vitamins and also help in making large intestine anoxic. Usually *Escherichia* is not pathogenic, but some *Escherichia* sp. cause gastroenteritis, urinary tract diseases and also serious food borne diseases. Do you recall reading about *E. coli* O157:H7? This can cause serious form of illness.

(2) ***Klebsiella*** – It is gram negative, non-sporulating, non-motile and non-pigmented rod, which is commonly found in soil or water. Figure 7.17 (a) shows *Klebsiella* growth on media. It is the only enteric bacteria capable of nitrogen fixation. Some species are pathogenic, e.g. *Klebsiella pneumoniae*, cause a serious form of pneumonia in humans. Figure 7.17 (b) shows *Klebsiella* as seen under microscope.

Figure 7.17: Klebsiella growth on media and as seen under the microrcope

(3) ***Proteus*** – It is gram negative, non-sporulating rod, which is characterized by rapid motility (peritrichous flagella) and swarming type of growth on media. It is responsible for urinary tract infections and probably benefit in this regard from its ability to produce urease and degrade urea.

(4) ***Pseudomonas*** – *Pseudomonas* are aerobic, gram negative, straight or slightly curved rods as can be seen in Figure 7.18 (b) that are motile by polar flagella. Many species produces water- soluble pigments, which diffuses into the media e.g. *P. aeruginosa* produces soluble blue green pigments. Fluorescence pigment is produced by other species, which glows when illuminated by ultraviolet rays.

Figure 7.18: Pseudomonas growth on media and as seen under microscope

Pseudomonas is commonly present in soil and other natural environments and is responsible for causing diseases in plants and also in human and animals. It can cause infections of urinary tract, wounds, burn, blood and also abscesses and meningitis. *Pseudomonas* species are able to metabolize a wide variety of substrates. Many species are able to grow at refrigerated temperature. *Pseudomanas* sp. do not produce gas from glucose.

(5) ***Bacillus*** – It is gram positive [refer to Figure 7.19 (b)], endospore former, obligate aerobic or facultative aerobic rods belonging to order Bacilliales. Spores are spherical, oval or cylindrical and do not cause swelling of sporangia. It may be central or terminal in position.

Figure 7.19: Bacillus growth on media and as seen under microscope

Bacillus is commonly present in soil. Few are pathogenic, e.g., *B. anthracis* causes anthrax. It may be a possible agent of biological warfare. *B. cereus* is a cause of food poisoning. Some species e.g. *B. thuringiensis* are used as bioinsecticides. Several species produces antibiotics and extracellular hydrolytic enzymes.

(6) ***Staphylococcus*** – It is gram positive, nonsporulating facultative anaerobic cocci present in grape-like clusters as can be seen in Figure 7.20 (b). These have ability to tolerate drying and high salt in media. Many species are pigmented e.g. *Staphylococcus aureus* produces yellow pigmented colony. Number of toxins are produced by *Staphylococcus* species which contribute to their pathogenicity. *Staphylococus* is responsible for infection of surgical wounds, toxic shock syndromes, vomitting, nausea, food poisoning etc.

Figure 7.20: Staphylococcus growth on media and as seen under the microscope

Now that we have looked at the basic characteristics of some common bacteria, let us practice what we have learnt so far, by attempting the exercises 1-6 given in this practical. We will, however, first answer the question given in the next section to consolidate our understanding on staining techniques.

7.7 REVIEW QUESTIONS

1. What are stains?

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2. What is the difference between acidic and basic stains?

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3. Why basic stains are more often used for bacteriology?

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4. What is the need of fixation and how it is done?

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5. Define chromophore, mordant, simple staining and differential staining.

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6. Why the thick smear is not good for microscopic observation?

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7. Why is it essential that smear be air-dried?

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Now go ahead do the exercises.

EXERCISE 1

PREPARATION OF BACTERIAL SMEAR

Date : .....

Aim : To prepare bacterial smear.

Objective : After undertaking this exercise, you will be able to:

- prepare bacterial smear.

Requirements

Culture : 24 hours culture of *E. coli*

Reagent : Distilled water

Equipments : Glass slides, inoculating loop, bunsen burner, glassware marking pencil and glassware

Procedure:

Start the exercise by following the steps enumerated herewith.

(1) Take properly washed and dried glass slides for making bacterial smear. If slides are oily or greasy, wash them properly with soap and water followed by water rinse and a rinse with 95% alcohol. The slides are then dried.

(2) Now, label the slides properly by writing organism's name on either end of the slide by glassware marking pencil or permanent marker.

(3) Prepare the bacterial smear by using either liquid or solid culture medium as discussed herewith.

From Liquid Culture – Re-suspend the sedimented cells in broth culture by tapping the culture tube with finger. Spread one or two loopfuls of the culture with a sterile inoculating loop over the center of the slide. Allow smear to air dry.

Figure 7.21: Preparation of bacterial smear (and staining of cells for microscopic observation)

From Solid Culture – Place a loopful of water over the center of the slide. Transfer the culture to the drop with a sterile, cooled loop. Mix small amount of cells with this drop and spread in a circular motion of the loop. Allow smear to air dry completely. The smear should appear as semitransparent or translucent whitish film. Look up the graphical representation of the process given in Figure 7.21.

(4) Fix the bacterial smear on glass slide by heat fixation. This can be accomplished by rapid passage of air-dried smear over the Bunsen burner flame two-three times. So you have done this. The slide is ready for staining.

(5) Examine each slide for the confluent, whitish film or haze.

Precautions:

1. The slide should not be greasy.
2. Hold the slide by their edges only.
3. Label should not come in contact with staining reagents.
4. Smear should not be dense and thick.
5. Aseptic conditions should be maintained.

Observations and Results:

Record your observations in the format given herewith. Comment regarding the confluent, whitish film or haze appearing on the slide.

Observations	Results

Inference/Conclusion:

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Now submit your exercise for evaluation.

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Counsellor Signature

EXERCISE 2

SIMPLE STAINING OF BACTERIAL CULTURES

Date : .....

Aim : To perform simple staining of given bacterial culture(s).

Objectives : After undertaking this exercise, you will be able to:

- perform the different staining techniques, and
- stain given bacterial cultures.

Requirements

Culture : 24 hours bacterial culture of *E. coli* and *Bacillus* on nutrient agar or in broth.

Reagents : Methylene blue or crystal violet

Equipments and Glassware : Microscopic slides, inoculating loop, bunsen burner, staining tray, microscope, lens paper, oil, permanent glass marker, blotting paper.

Theory/Principle: In simple staining, a single staining agent preferably a basic dye is used. Commonly used basic stains are carbol fuchsin, crystal violet and methylene blue. Different exposure times are used for different dyes, e.g. for carbol fuchsin 15-30 seconds, crystal violet 20-60 seconds and methylene blue 1-2 minutes. Being positively charged, these binds strongly to bacterial cells, which have negative charge on their cell surface. Simple staining gives idea regarding size, shape and arrangement of cells. Its value lies in its simplicity and ease of use.

Procedure:

Now carry out the exercise following the steps given herewith and in Figure 7.22 herewith:

(1) Prepare thin bacterial smear on clean glass slide.

(2) Fix the bacterial smear on glass slide by heat fixation.

(3) Place the slide on the staining tray.

(4) Flood the smear with a staining reagent for appropriate exposure time.

(5) Remove the excess stain by gentle washing with tap water. The slide should be held parallel to the stream of water to avoid removal of microorganisms from slide.

(6) Blot dry the slide.

(7) Examine the dried slide under microscope in oil immersion (100x). First fix the slide at 10x and then move to 100x. (Oil immersion)

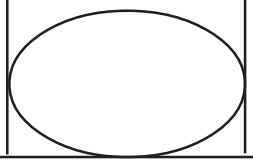
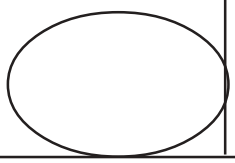
Figure 7.22: Graphic representation of procedure

Precautions:

1. Heat fix all the bacterial smears.
2. Use clean, grease free glass slides.
3. Wash the stained slide gently with tap water by holding it parallel to water stream to avoid loss of cells.
4. Use oil in very small quantity, otherwise it would spoil the lens.
5. Do not wipe the slide.
6. Maintain aseptic conditions.

Observations and Results:

Put down your observations of the practical in the format given herewith. Draw the morphology of bacterial cells as seen under the microscope in the space provided herewith.

Observations			**Results (Comment on the characteristic of the organism)**
Characteristics	**Organism**		
	E. coli	*Bacillus*	
Microscopic colour (Cell colour)			
Cell size			
Cell shape			
Cell Arrangement (solitary/chains/groups etc.) Representation of Microscopic field.			

Inference/Conclusion:

(Briefly summarize your findings here)

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Before you submit this exercise for evaluation, answer the questions given herewith under the section review questions.

Review Questions:

1. What is the advantage of simple staining?

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   .....

2. Why basic dyes are preferred over acidic dyes for staining?

   .....

   .....

   .....

3. What is the exposure time for different stains?

   .....

   .....

4. Can simple staining techniques be used to identify more than the morphological characteristics of microorganisms? Explain.

   .....

   .....

   .....

5. What would have happened if you won't fix the smear preparation?

   .....

   .....

Now submit the exercise for evaluation.

.....

Counsellor Signature

EXERCISE 3

GRAM STAINING OF BACTERIAL CULTURES

Date : .....

Aim : To perform gram staining of the given bacterial culture(s).

Objectives : After undertaking this exercise, you will be able to:

- perform gram staining of bacterial culture, and
- differentiate between gram positive and gram negative bacteria.

Requirements

Culture : 24 hrs. cultures of *E. coli, Bacillus, Pseudomonas, Staphylococcus* and a mixed culture.

Reagents : Crystal violet, Gram's iodine, 95% ethyl alcohol or acetone, Safranin, Immersion oil.

Equipments and Glassware : Bunsen burner, microscopic slides, inoculating loop, distilled water, staining tray, lens paper, filter paper.

Theory/Principle:

We have already studied about the principle of gram staining in section 7.4. Based on your understanding, briefly write the principle of gram staining here in the space provided. Write about the four chemical reagents used.

Basis of Gram Staining:

Four reagents:

Graphically illustrate the staining colour for gram positive and gram negative. Look up Figure 7.5 given earlier.

Gram Positive **Gram Negative**

Procedure:

Now carry out the exercise following the steps enumerated herewith. Figure 7.23 illustrates the procedure.

1. Prepare bacterial smear on a clean, non-greasy slide from the given culture(s). For this, a drop of water is placed on the slide, culture is transferred, mixed and spreaded by the circular motion of the sterile inoculating loop. Similarly, prepare a bacterial smear consisting of a mixture of organisms on one slide.

2. Now allow the bacterial smear to air dry and then heat fixed.

3. Put the slide on staining tray and flood the smear with crystal violet for one minute.

4. Rinse the slide gently under tap water.

5. Flood the smear with gram's iodine for one minute.

6. Wash gently with tap water.

7. Decolourize the slide with 95% ethanol or acetone by adding it drop-wise till the colour comes out.

8. Wash immediately with tap water.

9. Counter stain with safranin for 30-60 seconds.

10. Again wash gently with tap water.

11. Blot dry and examine in the microscope under oil immersion. A summary of the procedure and the process is highlighted herewith. Record your observations in the format given herewith:

Precautions:

1. Heat fix the smear, otherwise cells would be washed off.

2. Fresh 24 hrs old culture should be used. Gram variability can be seen in old culture. Since with age gram Positives lose their ability to retain primary stain, in gram variability some cells appear purple and others appear red.

3. Decolourization is a critical step. Decolourization is done till the colour of the primary stain comes out. Over-decolourization can make gram positives appear gram negatives and under decolourization can cause gram negatives to appear as gram positives.

4. Slide should be thoroughly washed after every step to remove excess reagent under running water or water applied with an eye dropper.

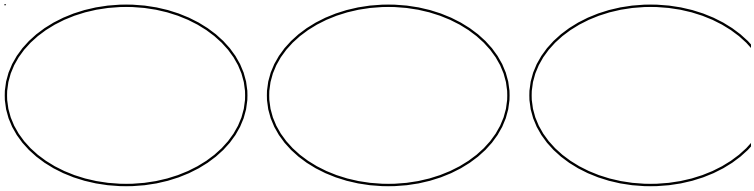
Observations and Results:

Record your observations and results in the given format:

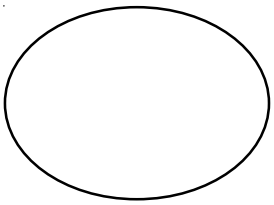
Characteristics						Results
Organisms (name)	**Culture 1**	**Culture 2**	**Culture 3**	**Culture 4**	**Mixture**	
Cell shape						
Cell arrangement						
Cell colour						
Gram reaction (+ve / –ve)						

Microscopic field

(draw the picture as you see under the microscope)



Culture 1 **Culture 2** **Culture 3** **Culture 4**



Mixed

Inference/Conclusion:

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Before you end this exercise, answer the review questions given herewith.

Review Questions:

1. What is the basis of gram staining?

   .....

   .....

2. Name the critical step in gram staining and why is it so critical?

   .....

   .....

   .....

3. Name two gram positive and two gram negative organisms.

   .....

   .....

4. What is the purpose of using Gram's iodine?

   .....

   .....

5. Why primary stain and counter stain have contrasting colours?

   .....

   .....

6. What are the advantages of differential staining procedures over the simple staining techniques?

   .....

   .....

7. Why gram staining is the differential staining? Name two other differential staining procedures.

   .....

   .....

   .....

8. State gram character of following organisms –

   (a) *E. coli*

   .....

   .....

(b) *Bacillus*

.....

.....

(c) *Staphylococcus*

.....

.....

(d) *Streptococcus*

.....

.....

(e) *Pseudomonas*

.....

.....

(f) *Proteus*

.....

.....

(g) *Klebsiella*

.....

.....

(h) *Micrococcus*

.....

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9. What would be the colour of a gram negative bacterium after gram staining by the conventional method?

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Now submit your exercise for evaluation.

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Counsellor Signature

EXERCISE 4

ACID FAST STAINING OF THE GIVEN CULTURE(S)

Date : .....

Aim : To perform acid fast staining (Ziehl – Neelsen Method) of the given culture(s).

Objective : After undertaking this exercise, you will be able to:

- conduct acid fast staining.

Requirements

Culture : Culture of *Mycobacterium smegmatis* and *Staphylococcus aureus*.

Reagents : Carbol fuchsin, acid alcohol, methylene blue, immersion oil.

Equipments and Glassware : Bunsen burner, inoculating loop, glass slide, microscope, permanent glass marker.

Theory/Principle:

We have already discussed the principle in sub-section 7.4.2. Recall the principle and the reagents used in acid-fast staining you learnt earlier and put them down in the space given herewith:

Basis of acid-fast staining:

Reagents used:

Procedure:

Carry out the exercise using the steps enumerated herewith. Figure 7.24, as illustrated in sub-section 7.4.2 earlier, graphically presents the procedure.

1. Label the clean, non-greasy slides and make smears of *M. smegmatis*, *S. aureus* and mixture of two on them.

2. Allow the smear to air dry and then fixed the smear by heating.
3. Cover the smear with carbol fuchsin and put the slide on a beaker of warm water. Allow the steam to pass through smear for five minutes. Replenish the stain, if needed.
4. Allow the slide to cool. Then wash the smear with tap water.
5. Decolourize the smear by adding acid alcohol drop wise till the clean alcohol with slight red tinge comes out.
6. Wash the slide with tap water.
7. Flood the smear with methylene blue for two minutes.
8. Wash the smear with tap water.
9. See under 100x after air drying.

Figure 7.24: Ziehl–Neelson method

Precautions:

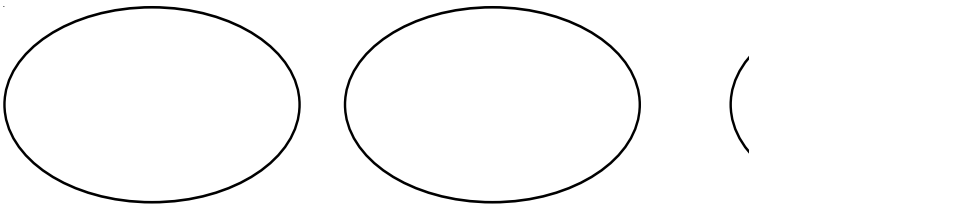
1. Do not allow carbol fuchsin to dry on heating.
2. Maintain aseptic conditions.
3. Decolourization should be done till no red colour comes out.

Observations and Results:

Record your observations and make the diagram of a microscopic field.

Characteristics				Results
	Culture 1	Culture 2	Mixture	
Organisms				
Cell colour				
Cell shape				
Cell arrangement				

Microscopic field



Culture 1 **Culture 2** **Culture 3**

Also answer the review questions given herewith.

Review Questions

1. Why acid-fast bacteria cannot be stained by simple or gram's stain?

   .....

   .....

   .....

2. Why acid fastness character is shown by some bacteria?

   .....

   .....

   .....

3. Why acid alcohol is used for decolourization rather than ethyl alcohol?

   .....

   .....

   .....

4. Why heating or surface active agent needed for primary staining?

.....

.....

5. Name two pathogenic bacteria showing acid fastness.

.....

.....

6. What properties does mycolic acid confer on cells that make it?

.....

.....

Now submit the exercise for evaluation.

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Counsellor Signature

EXERCISE 5

SPORE STAINING IN A GIVEN BACTERIAL CULTURE

Date : .....

Aim : To perform the spore staining in a given bacterial culture.

Objectives : After undertaking this exercise, you will be able to:

- perform spore staining, and
- differentiate between vegetative cells and spores.

Requirements

Culture : 24 hours culture of *Bacillus* sp. and *E. coli*.

Reagents : Malachite green, safranin, distilled water.

Equipments & Glassware : Glass slide, coverslip, beaker (100 ml), Bunsen burner, tripod stand, wire gauge, inoculating loop, staining tray, blotting paper, microscope.

Theory/Principle:

We have already discussed the principle of spore staining in sub-section 7.4.3. Read the section once again and write the principle and the spore forming technique in the space provided herewith.

Procedure

Carry out the exercise following the steps enumerated herewith. Figure 7.25 graphically presents the procedure.

1. Take a clean, non-greasy slide. Prepare bacterial smear by taking a small amount of culture and mixing it with a drop of water on a slide. Spread it in a rounded manner.
2. Air dry and heat fix the smear.
3. Keep the slide on a beaker having boiling water. Flood the smear with malachite green and allow the steam to pass through it for 4-5 minutes. Replenish the stain if it starts evaporating and drying.
4. Remove the slide from the beaker and allow it to cool.
5. Decolourize the smear by washing slowly under running tap water.
6. Counter stain the smear with safranin for 30 seconds.
7. Wash the slide again with tap water.
8. Blot dry the slide with blotting paper and observe under oil immersion.

Precautions:

1. Do not allow malachite green to dry on slide.
2. Decolourization should be done only after cooling the slide.
3. Use aseptic conditions.

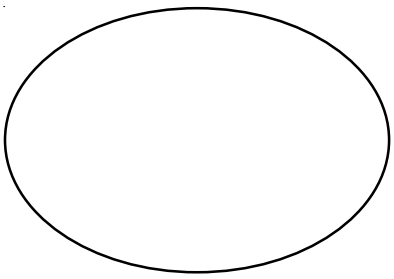
Figure 7.25: Spore staining

Observations and Results:

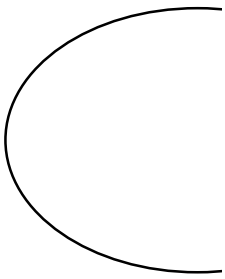
Based on your observations (as you see the slide under the microscope), make the diagram and comment on the position of endospore, in the format given herewith.

Observations			Results
Characteristics	***Bacillus***	***E. coli***	
Colour of the Spore			
Size of the Spore (small/large)			
Location of the Spore (central/ terminal/sub-terminal			
Colour of the Vegetative Cell			
Shape of the Vegetative Cell			
Shape of the Spore			

Representative Microscopic Field (draw the Figure/diagram of the cells as you see under the microscope



***Bacillus* species**



E. coli

Inference / Conclusion:

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Also answer the review questions given herewith.

Review Questions

1. Name one rod shape and one coci endospore former.

   .....

   .....

2. What is the importance of endospore formers in food industry?

   .....

   .....

3. Name the bacteria having terminal, sub-terminal and centrally located endospores.

   .....

   .....

4. Why is steaming necessary in spore staining?

   .....

   .....

5. What is the function of water in spore staining?

   .....

   .....

6. What is the colour of endospore and vegetative cell in the staining?

   .....

   .....

Submit the exercise for evaluation.

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Counsellor Signature

EXERCISE 6

CAPSULE STAINING IN A GIVEN CULTURE

Date : .....

Aim : To perform the capsule staining of a given culture(s).

Objectives : After undertaking this exercise, you will be able to:

- carry out capsule staining using Anthony Staining method.

Requirements

Culture : 24 hours culture of *Klebsiella* sp. and *Bacillus* species.

Reagents : Crystal violet aqueous (1% w/v), 20% copper sulphate, nigrosin dye or india ink.

Equipments and Glassware : Inoculating loop, bunsen burner, glass slide, blotting sheet.

Theory/Principle:

Look up sub-section 7.4.4 for the purpose / theory of capsule staining. Write the basic concept of capsule staining and the principle behind Negative and Anthony Staining Method in the space provided herewith.

Basic Principle of Capsule Staining

Basic Principle of Negative and Anthony Staining Method:

Procedure:

We have learnt that Capsule staining can be undertaken either by negative staining or Authony method. Now carry out the exercise by following the steps enumerated herewith and also illustrated in Figure 7.26 (a) and (b) for the two methods.

For Negative Staining

1. Label the clean non-greasy slide.

2. Put a drop of nigrosin/India Ink dye at one end of the slide and mix it evenly with a loopful culture of given bacterial sample.

3. Spread the mixture over a slide as a thin film with the help of another slide held at 30^{o} angle as illustrated in the figure in margin and pushed away to the other end of the slide.

4. Air dry the smear.

5. Stain the smear with Crystal violet/Safranin for 1 minute and wash gently with water.

6. Finally press the slide with the help of a filter paper and observe under oil-immersion objective of the microscope.

For Anthony Staining Method

1. Take the clean non-greasy slide and prepare a bacterial smear.

2. Air dry the smear and flood the slide with crystal violet for 1-2 minutes.

3. Wash the slide with 20% copper sulphate as shown in the Figure 7.26(b) and gently blot dry it.

4. Observe under oil immersion.

Precautions:

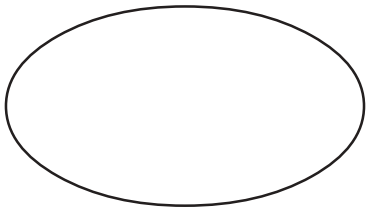
1. Never heat fix the smear. This is because by heating shrinkage can occur which may create a clear zone around the cell. It is an artifact and may be mistaken for a capsule.

2. Washing off the smear with water should be avoided because capsule may be removed with vigorous washing.

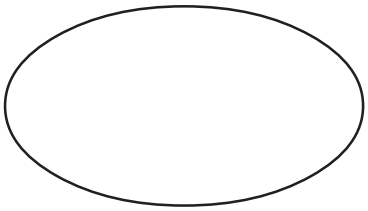
Observations and Results:

Record the exercise step-by-step, giving the observations (as highlighted in the format given herewith), and results.

Characteristics	Observations		Results
	Organisms		
	Klebsiella	*Bacillus*	
Capsule Size			
Colour of the Cell			
Colour of Capsule			



Klebsiella sp.



Bacillus sp.

Drawing of a representative microscopic field

Inference / Conclusion:

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Before you finish this exercise, answer the review questions given herewith to recapitulate what you have learnt in this exercise.

Review Questions

1. What is a capsule? Write its significance for the bacteria.

   .....

   .....

2. What is the function of India Ink or nigrosin in negative staining of a capsule?

   .....

   .....

3. Mention the function of copper sulphate in Anthony's method.

.....

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4. Why heat fixing should be avoided in capsule staining?

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Now submit the exercise for evaluation.

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Counsellor Signature