Laboratory II: Microbiology

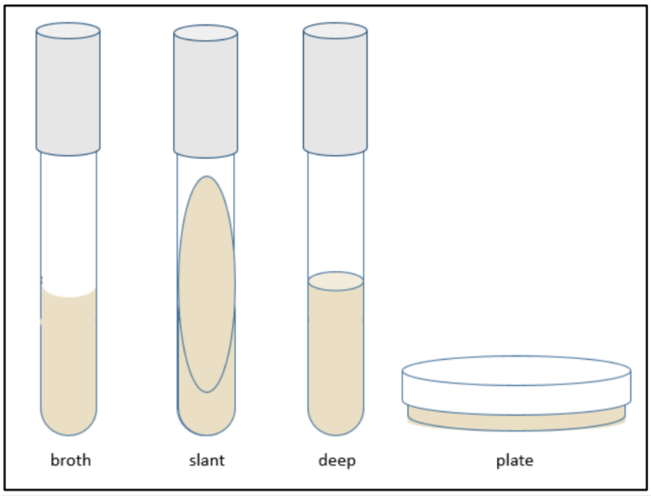
Exp. no	**Date**	**Name of the experiment**
1	03-10-2022	Preparation of media for cultivation of bacteria
2	12-10-2022	Isolation of bacteria in pure culture by streak plate method
3	21-10-2022	Culture characteristics of a bacteria
4	03-11-2022	Preparation of bacterial smear and Gram's staining
5	12-11-2022	Enumeration of bacteria: standard plate count
6	22-11-2022	Antimicrobial sensitivity test
7	13-12-2022	Maintenance of stock cultures in agar stabs
8	21-12-2022	Maintenance of stock cultures in glycerol stock cultures
9	30-12-2022	Isolation and identification of bacteria from soil samples

Experiment 1: Preparation of media for cultivation of bacteria

Background

The survival and continued growth of microorganisms depend on an adequate supply of nutrients and a favourable growth environment. For survival, most microbes must use soluble, low-molecular weight substances that are frequently derived from the enzymatic degradation of complex nutrients. A solution containing these nutrients is a culture medium. All culture media are liquid, semisolid, or solid. A liquid medium lacks a solidifying agent and is called a broth medium. A broth medium is useful for cultivating high numbers of bacterial cells in a small volume of medium, which is particularly helpful when an assay requires a high number of healthy bacterial cells. A broth medium supplemented with a solidifying agent called agar results in a solid or semisolid medium.

Agar, an extract of seaweed, is a complex carbohydrate composed mainly of galactose, and is without nutritional value. Agar serves as an excellent solidifying agent because it liquefies at l00°C and solidifies at 40°C. A completely solid medium requires an agar concentration of 1.5% to 1.8%. A concentration of less than 1% agar results in a semisolid medium. A semisolid medium is useful for testing a cell's ability to grow within the agar at lower oxygen levels and for testing the species' motility. A solid medium is advantageous because it presents a hardened surface on which microorganisms can be grown using specialized techniques for the isolation of discrete colonies. Each colony is a cluster of cells that originates from the multiplication of a single cell and represents the growth of a single species of microorganism. Such a defined and well-isolated colony is a pure culture. Also, while in the liquefied state, we can place solid media in test tubes, which then cool and harden in a slanted position, producing agar slants. These are useful for maintaining pure cultures. The slanted surface of the agar maximizes the available surface area for microorganism growth while minimizing the amount of medium required.



Similar tubes that, following preparation, harden in the upright position are designated as agar deep tubes. Agar deep tubes are used primarily for studying gaseous requirements of microorganisms, since gas exchange between the agar at the butt of the test tube and the external environment is impeded by the height of the agar. Liquid agar medium can also be poured into Petri dishes, producing agar plates, which provide large surface areas for the isolation and study of microorganisms.

Materials required

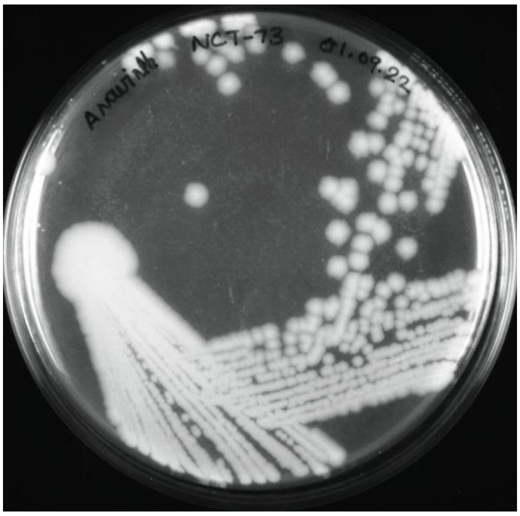
Dehydrated nutrient powder, agar powder, beakers, glass rods, spatula, plastic weigh boats, Erlenmeyer flask, graduated cylinders, petri plates, weighing balance, autoclave, laminar air flow.

Protocol (for preparing 5 plates of nutrient agar)

1. Using graduated cylinders, measure 100 ml of distilled water and pour into an Erlenmeyer flask.
2. Using a weighing boat, weigh 0.5 g of peptone, 0.3 g of yeast extract, 0.5 g of NaCl and 1.5 g of agar powder and mix them in Erlenmeyer flask.
3. Adjust the pH to 7 $\pm$ 0.2 at 25°C.
4. Heat the mixture to fully dissolve all components.
5. Pack 5 Petri plates and load them into the autoclave along with media mixture.
6. Autoclave the dissolved mixture at 121°C for 15 minutes at 15 lbs pressure.
7. Following autoclaving, cool down the media in 65°C water bath.
8. Prepare laminar air flow by surface sterilizing the work space using 70% ethanol and UV sterilizing the laminar space for 15 minutes.
9. Move the autoclave sterilized Petri plates into laminar space.
10. Remove the media from water bath and pour ~20ml into each Petri plate.
11. Leave the plate for media solidification.
12. Following solidification, mark the Petri plates. Now plates are ready for inoculation and cultivation of microorganism.

Result

A nutrient agar media was prepared and used for a cultivation of a bacterial strain.



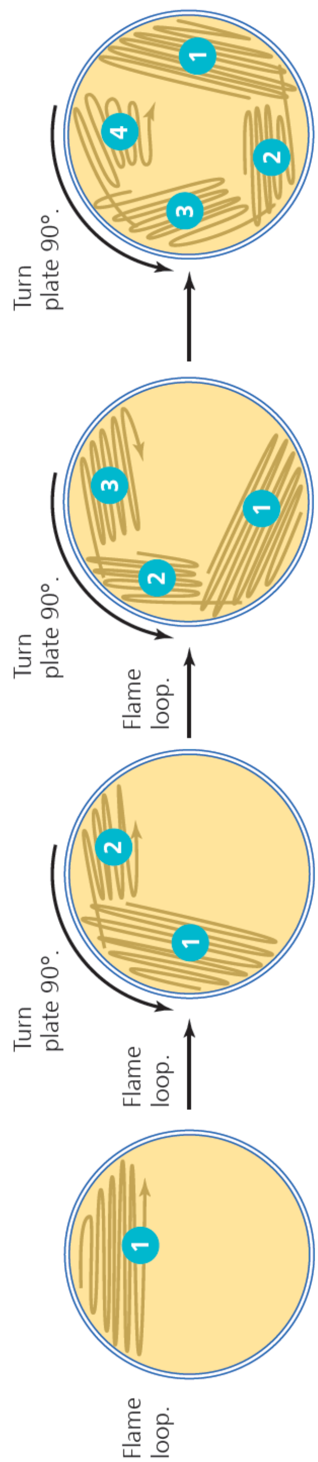
Experiment 2: Isolation of bacteria in pure culture by streak plate method

Background

In nature, microbial populations do not segregate themselves by species, but exist with a mixture of many other cell types. In the laboratory, we can separate these populations into pure cultures. These cultures contain only one type of organism and allow us to study their cultural, morphological, and biochemical properties.

Principle

The techniques commonly used for isolation of discrete colonies initially require that the number of organisms in the inoculum be reduced. The resulting diminution of the population size ensures that, following inoculation, individual cells will be sufficiently far apart on the surface of the agar medium to separate the different species. This necessary dilution can be accomplished by streak plate technique. The streak-plate method is a rapid qualitative isolation method. It is a dilution technique that spreads a loop full of culture over the surface of an agar plate as a means to separate and dilute the microbes and ensure individual colony growth. There are many different procedures for preparing a streak plate; the four-way, or quadrant streak is used here.



Materials required

Inoculation loop, laminar with Bunsen burner, Solid nutrient agar plate, bacterial culture.

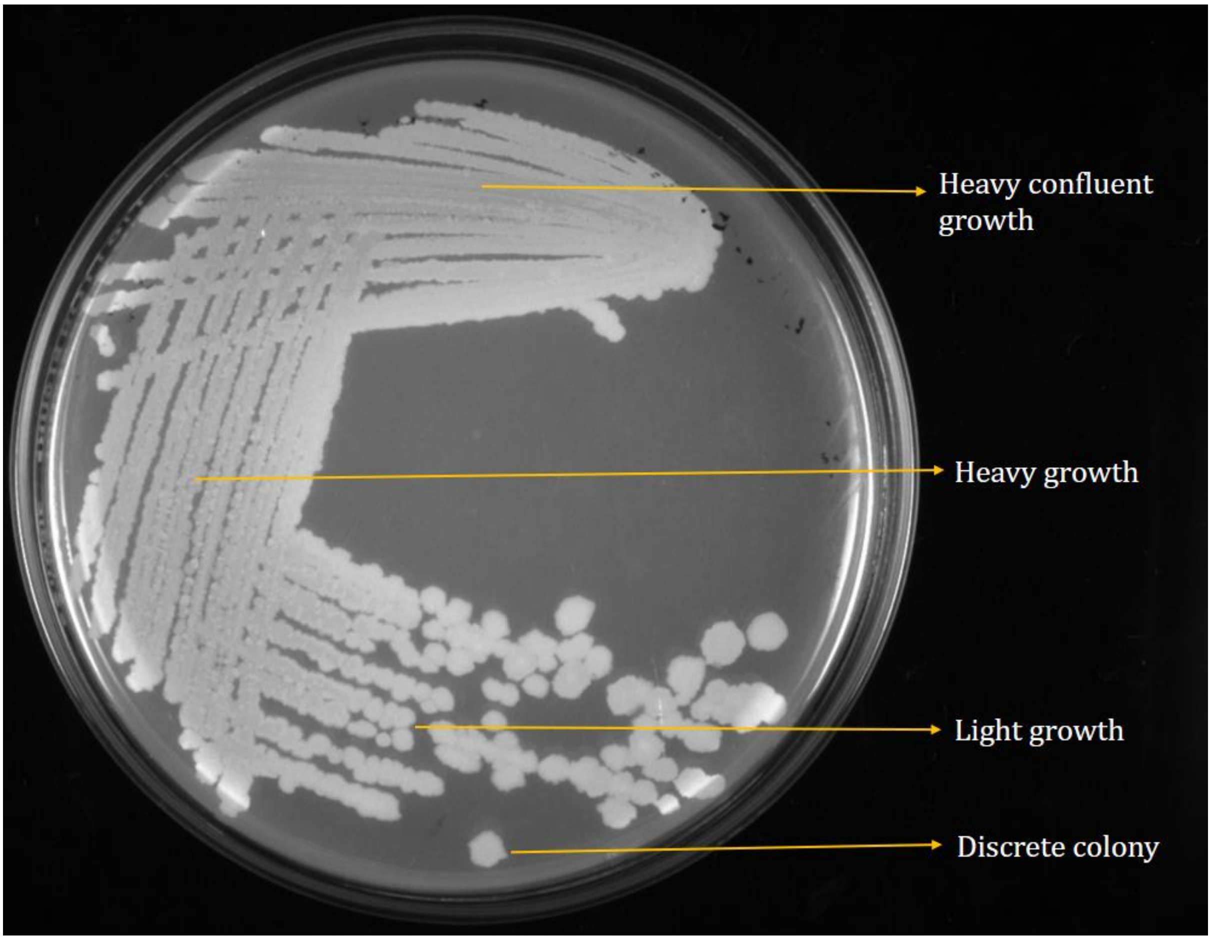
Protocol

1. Place a loop full of culture on the agar surface in Area 1. Flame the loop, cool it by touching it to an unused part of the agar surface close to the periphery of the plate, and then drag it rapidly several times across the surface of Area 1.
2. Re-flame and cool the loop, and turn the Petri dish 90°. Then touch the loop to a corner of the culture in Area 1 and drag it several times across the agar in Area 2. The loop should never enter Area 1 again.
3. Re-flame and cool the loop and again; turn the dish 90°. Streak Area 3 in the same manner as Area 2.

4. Without re-flaming the loop, again turn the dish 90° and then drag the culture from a corner of Area 3 across Area 4, using a wider streak. Don't let the loop touch any of the previously streaked areas. The purpose of flaming of the loop at the points indicated is to dilute the culture so that fewer organisms are streaked in each area, resulting in the final desired separation.

Result

A quadrant streak plate was prepared and discrete colonies were observed in the last quadrant.



Experiment 3: Culture characteristics of a bacteria

Background

Colony morphology is the visual culture characteristics of a bacterial colony on an agar plate. Observing colony morphology is an important skill used in the microbiology laboratory to identify microorganisms. Colonies need to be well isolated from other colonies to observe the characteristic shape, size, color, surface appearance, and texture.

Principle

When grown on a variety of media, microorganisms exhibit differences in the macroscopic appearance of their growth. We use these differences, called cultural characteristics, to separate microorganisms into taxonomic groups. The Bergey's Manual of Systematic Bacteriology outlines the cultural characteristics for all known microorganisms. They are determined by culturing the organisms on nutrient agar slants and plates, in nutrient broth, and in nutrient gelatin. The patterns of growth in each of these media are described below.

Nutrient Agar Slants

These have a single straight line of inoculation on the surface and are evaluated by

1. **Abundance of growth**: The amount of growth is designated as none, slight, moderate, or large.

2. **Pigmentation**: Chromogenic microorganisms may produce intracellular pigments that are responsible for the coloration of the organisms as seen in surface colonies. Other organisms produce extracellular soluble pigments that are excreted into the medium and also produce a color. Most organisms, however, are nonchromogenic and will appear white to gray.

3. **Optical characteristics**: Optical characteristics may be evaluated by the amount of light transmitted through the growth. These characteristics are opaque (no light transmission), translucent (partial transmission), or transparent (full transmission).

4. **Form**: The appearance of the single-line streak of growth on the agar surface is designated as

a. **Filiform**: continuous, threadlike growth with smooth edges

b. **Echinulate**: continuous, threadlike growth with irregular edges

c. **Beaded**: non-confluent to semi-confluent colonies

d. **Effuse**: thin, spreading growth

e. **Arborescent**: treelike growth

f. **Rhizoid**: root-like growth.

5. **Consistency**:

a. **Dry**: free from moisture

b. **Buttery**: moist and shiny

c. **Mucoid**: slimy and glistening

Nutrient Agar Plates

These demonstrate well-isolated colonies and are evaluated by

1. **Size**: pinpoint, small, moderate, or large

2. **Pigmentation**: color of colony

3. **Form**: The shape of the colony is described as follows:

a. **Circular**: unbroken, peripheral edge

b. **Irregular**: indented, peripheral edge

c. **Rhizoid**: root-like, spreading growth

4. **Margin**: The appearance of the outer edge of the colony is described as follows:

a. **Entire**: sharply defined, even

b. **Lobate**: marked indentations

c. **Undulate**: wavy indentations

d. **Serrate**: tooth-like appearance

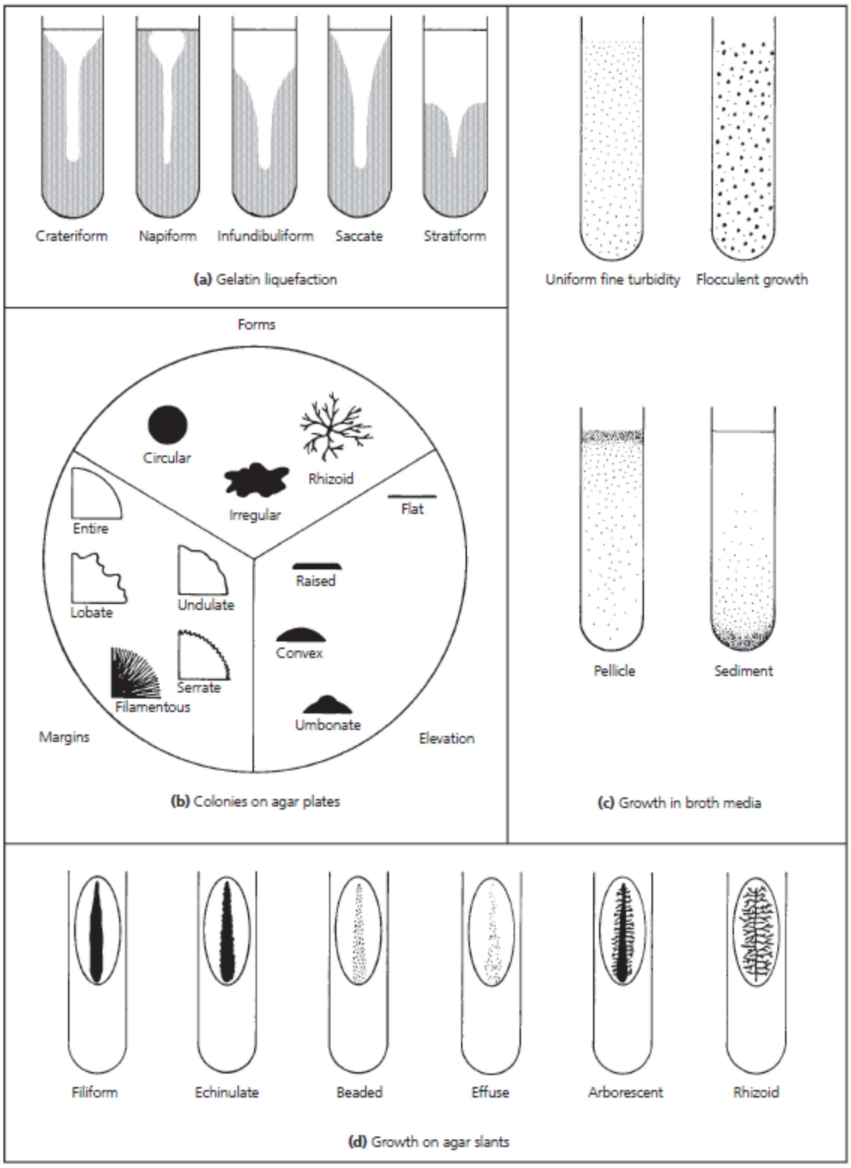
e. **Filamentous**: threadlike, spreading edge

5. **Elevation**: The degree to which colony growth is raised on the agar surface is described as:

a. **Flat**: elevation not discernible

b. **Raised**: slightly elevated

c. **Convex**: dome-shaped elevation



d. **Umbonate**: raised, with elevated convex central region

Nutrient Broth Cultures

These are evaluated by the distribution and appearance of the growth as

1. **Uniform fine turbidity**: finely dispersed growth throughout
2. **Flocculent**: flaky aggregates dispersed throughout
3. **Pellicle**: thick, padlike growth on surface
4. **Sediment**: Concentration of growth at the bottom of broth culture may be granular, flaky, or flocculent.

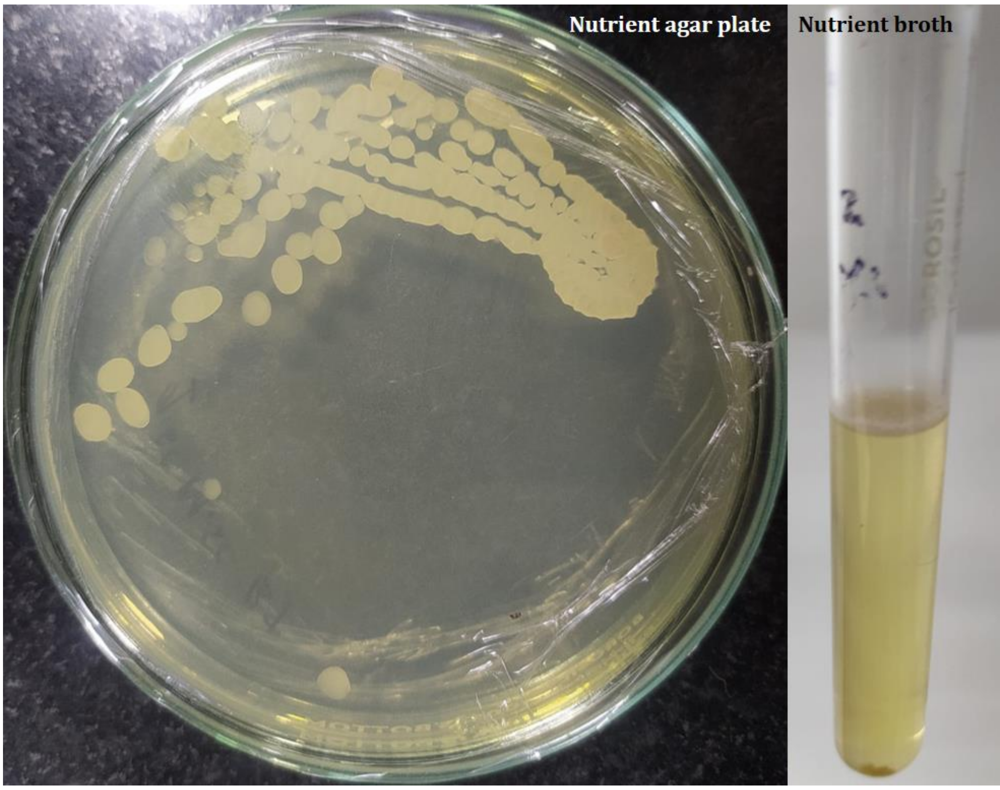
Nutrient Gelatin

This solid medium may be liquefied by the enzymatic action of gelatinase. Liquefaction occurs in a variety of patterns:

1. **Crateriform**: Liquefied surface area is saucer-shaped.
2. **Napiform**: Bulbous-shaped liquefaction at surface
3. **Infundibuliform**: Funnel-shaped
4. **Saccate**: Elongated, tubular
5. **Stratiform**: Complete liquefaction of the upper half of the medium

Result

In nutrient agar plates colonies were moderate to large in size with yellowish white pigmented. Colonies are circular, smooth and has entire margins. Colonies were translucent, effuse and mucoid in nature. The colonies were umbonately elevated. In nutrient broth, pellicle formation was observed on the surface and sediment growth at bottom.



Experiment 4: Preparation of bacterial smear and Gram's staining

Background

Visualizing microorganisms in the living state is quite difficult, not only because they are minute, but also because they are transparent and practically colorless when suspended in an aqueous medium. To study their properties and to divide microorganisms into specified groups for diagnostic purposes, biological stains and staining procedures in conjunction with light microscopy have become major tools in microbiology.

Principle

Bacterial smears must be prepared prior to executing of any of the staining techniques. requires the use of at least four chemical reagents that are applied sequentially to a heat-fixed smear. The first reagent is called the primary stain. Its function is to impart its color to all cells. The second stain is a mordant used to intensify the color of the primary stain. In order to establish a color contrast, the third reagent used is the decolorizing agent. Based on the chemical composition of cellular components, the decolorizing agent may remove the primary stain from the entire cell or only from certain cell structures. The final reagent, the counterstain, has a contrasting color to that of the primary stain. Following decolorization, if the primary stain is not washed out, the counterstain cannot be absorbed, and the cell or its components will retain the color of the primary stain. If the primary stain is removed, the decolorized cellular components will accept and assume the contrasting color of the counterstain. In this way, cell types or their structures can be distinguished from each other on the basis of the stain that is retained.

The most important differential stain used in bacteriology is the Gram stain, named after Dr. Hans Christian Gram. It divides bacterial cells into two major groups, gram positive and gram negative, which makes it an essential tool for classification and differentiation of microorganisms. The Gram stain reaction is based on the difference in the chemical composition of bacterial cell walls. Gram-positive cells have a thick peptidoglycan layer, whereas the peptidoglycan layer in gram-negative cells is much thinner and surrounded by outer lipid-containing layers. Peptidoglycan is a polysaccharide composed of two chemical subunits found only in the bacterial cell wall. These subunits are N-acetylglucosamine and N-acetylmuramic acid. With some organisms, as the adjacent layers of peptidoglycan are formed, they are cross-linked by short chains of peptides by means of a transpeptidase enzyme, resulting in the shape and rigidity of the cell wall.

In the case of gram-negative bacteria and several of the gram-positive, such as the Bacillus, the cross-linking of the peptidoglycan layer is direct because the bacteria do not have short peptide tails. Early experiments have shown that a grampositive cell denuded of its cell wall by the action of lysozyme or penicillin will stain gram-negative. The Gram stain uses four different reagents. Descriptions of these reagents and their mechanisms of action follow.

Primary Stain: Crystal Violet (Hucker's): This violet stain is used first and stains all cells purple.

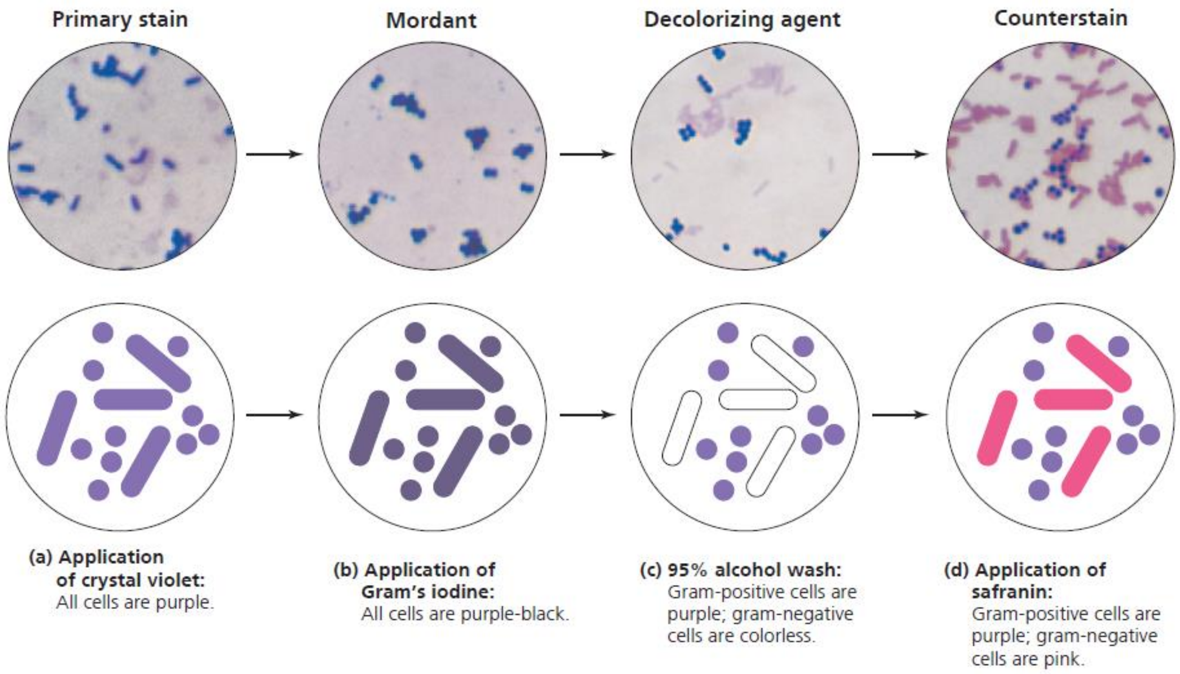
Mordant: Gram's Iodine: This reagent serves not only as a killing agent but also as a mordant, a substance that increases the cells' affinity for a stain. The reagent does this by binding to the primary stain, thus forming an insoluble complex. The resultant crystal–violet–iodine (CV-I) complex serves to intensify the color of the stain. At this point, all cells will appear purple-black.

Decolorizing Agent: Ethyl Alcohol, 95%: This reagent serves a dual function as a protein-dehydrating agent and as a lipid solvent. Its action is determined by two factors, the concentration of lipids and the thickness of the peptidoglycan layer in bacterial cell walls. In gram-negative cells, the alcohol increases the porosity of the cell wall by dissolving the lipids in the outer layers. Thus, the CV-I complex can be more easily removed from the thinner and less highly cross-linked peptidoglycan layer. Therefore, the washing-out effect of the alcohol facilitates the release of the unbound CV-I complex, leaving the cells colorless or unstained. The much thicker peptidoglycan layer in gram-positive cells is responsible for the more stringent retention of the CV-I complex, as the pores are made smaller due to the dehydrating effect of the alcohol. Thus, the tightly bound primary stain complex is difficult to remove, and the cells remain purple. *Note: Be careful not to over-decolorize the smear with alcohol.*

Counterstain: Safranin: This is the final reagent, used to stain pink those cells that have been previously decolorized. Since only gram-negative cells undergo decolorization, they may now absorb the counterstain. Gram-positive cells retain the purple color of the primary stain.

Materials required

Test culture, inoculation loops, gram's reagents, distilled water and Bunsen burner.



Protocol

Smear preparation

Step 1: Prepare the glass microscope slide: Clean slides are essential to prepare microbial smears. Grease or oil from the fingers on slides must be removed by washing the slides with soap, followed by a water rinse and a rinse of 95% alcohol. After cleaning, dry the slides and place them on laboratory towels until ready for use. *Note: Remember to hold the clean slides by their edges.*

Step 2: Label slides: Proper labelling of the slide is essential. Write the initials of the organism on either end of the slide with a glassware marking pencil on the surface on which the smear is to be made. Ensure that the label does not come into contact with staining reagents.

Step 3: Prepare the smear: It is crucial to avoid thick, dense smears. A thick or dense smear occurs when too much of the culture is used in its preparation, which concentrates a large number of cells on the slide. This type of preparation diminishes the amount of light that can pass through and makes it difficult to visualize the morphology of single cells. *Note: Smears require only a small amount of the bacterial culture.* A good smear is one that, when dried, appears as a thin whitish layer or film. The print of your textbook should be legible through the smear.

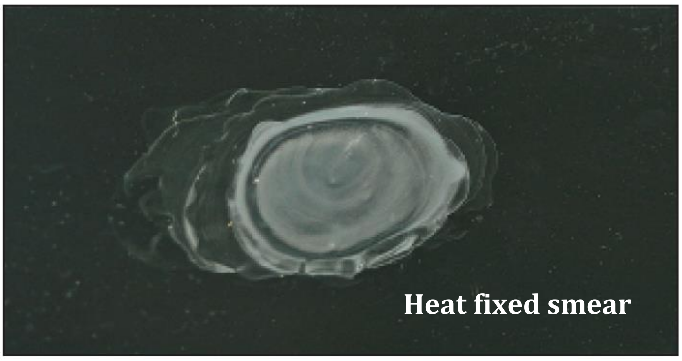
Different techniques are used depending on whether the smear is made from a broth or solid-medium culture.

a. Broth cultures: Resuspend the culture by tapping the tube with your finger. Depending on the size of the loop and the amount of culture growth, apply one or two loop full to the center of the slide with a sterile inoculating loop and spread evenly over an area about the size of a dime. Set the smears on the laboratory table and allow to air-dry.

b. Cultures from solid medium: Organisms cultured in a solid medium produce thick, dense surface growth and are not amenable to direct transfer to the glass slide. These cultures must be diluted by placing one or two loopfuls of water on the center of the slide, in which the cells will be emulsified. Transfer the cells using a sterile inoculating loop or a needle. Only the tip of the loop or needle should touch the culture to prevent the transfer of too many cells. Suspension is accomplished by spreading the cells in a circular motion in the drop of water with the loop or needle. This helps to avoid cell clumping. The finished smear should occupy an area about the size of a nickel and should appear as a translucent, or semitransparent, confluent whitish film. Allow the smear to dry completely.

Note: Do not blow on slide or wave it in the air.

Step 4: Heat fixation: Unless fixed on the glass slide, the bacterial smear will wash away during the staining procedure. This is avoided by heat fixation, during which the bacterial proteins are coagulated and fixed to the glass surface. Heat fixation is performed by the rapid passage of the air-dried smear two or three times over the flame of the Bunsen burner.



Gram's staining

Step 1. Gently flood heat fixed smears with crystal violet and let stand for 1 minute.

Step 2. Gently wash with tap water.

Step 3. Gently flood smears with the Gram's iodine mordant and let stand for 1 minute.

Step 4. Gently wash with tap water.

Step 5. Decolorize with 95% ethyl alcohol. *Note: Do not over-decolorize*. Add reagent drop by drop until the alcohol runs almost clear, showing only a blue tinge.

Step 6. Gently wash with tap water.

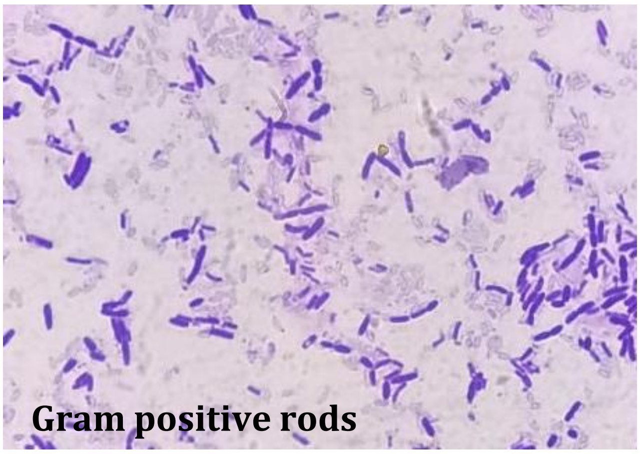
Step 7. Counterstain with safranin for 45 seconds.

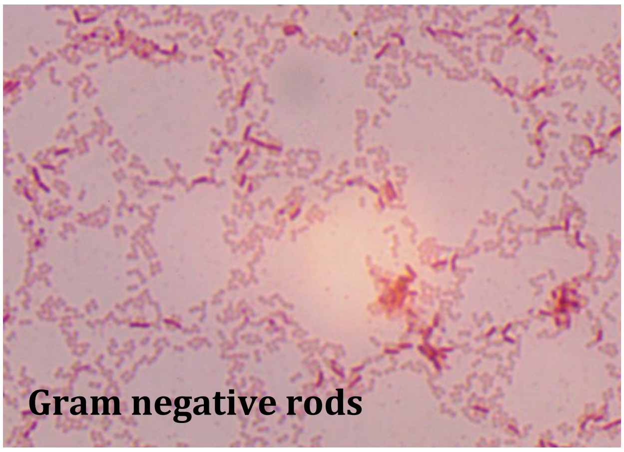
Step 8. Gently wash with tap water.

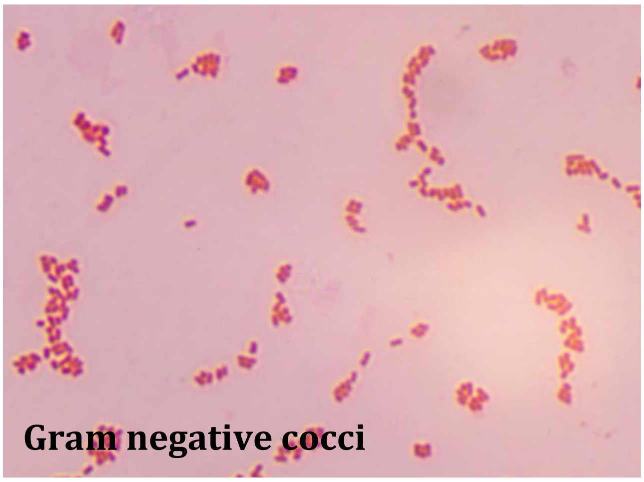
Step 9. Blot dry with bibulous paper and examine under oil immersion.

Result

Gram positive and negative rods have been observed.







Experiment 5: Enumeration of bacteria (from soil): standard plate count

Background

Enumeration of bacteria is defined as the process of determining the number of bacteria in a given sample. Studies involving the analysis of materials, including food, water, milk, and—in some cases—air, require quantitative enumeration of microorganisms in the substances. Many methods have been devised to accomplish this, including direct microscopic counts, use of an electronic cell counter such as the Coulter Counter, chemical methods for estimating cell mass or cellular constituents, turbidimetric measurements for increases in cell mass, and the serial dilution–agar plate method. Serial dilution–agar plate method will be adopted here.

Principle

Microorganisms are ubiquitous and can be present in thousands or millions in a sample, making it difficult to count their numbers. However, serially diluting the cultures makes it easier to determine the count. This method involves serial dilution of a bacterial suspension in sterile water blanks, which serve as a diluent of known volume.

After serial dilution, the aliquots of the diluted sample are plated on an appropriate culture media. Then, the plates are incubated, after which the number of colonies formed is counted. This technique is also known as plate count or colony counts. This method is based on the fact that, on a solid nutrient medium, the viable cells (a cell which is able to divide and form a colony) grow to form colonies that are visible to the naked eye. The resultant colonies are visible to the naked eye making visualization, selection and counting simpler. Plates suitable for counting must contain between 30-300 colonies. To arrive at a suitable dilution that results in this number, serial dilution method is used. The method involves making a tenfold dilution at a time by taking a small amount of the original sample (For eg. 1 mL) and making up the volume (to say 10 mL) by adding an appropriate buffer or normal saline. For every dilution, a specific volume is plated on a suitable medium.

Plate count method can be of two types:

Pour plate method: Molten agar, cooled to 45°C, is poured into a Petri dish containing a specified amount of the diluted sample. Following addition of the molten-then-cooled agar, the cover is replaced, and the plate is gently rotated in a circular motion to achieve uniform distribution of microorganisms. This procedure is repeated for all dilutions to be plated. Dilutions should be plated in duplicate for greater accuracy, incubated overnight, and counted on a Quebec colony counter either by hand or by an electronically modified version of this instrument. This technique allows the growth of anaerobes (beneath the surface) as well as aerobes (on the surface of the plate).

Spread plate method: A known volume of the dilution is plated on the nutrient agar plate. The colonies obtained can be selected and sub cultured easily for further use. The bacterial count in the original sample is estimated as CFU/mL (Colony Forming units/mL).

CFU/mL = CFU/volume of dilution plated × Dilution factor

Materials required

Sample (soil), dehydrated nutrient agar powder, Erlenmeyer flasks, culture tubes, Petri plates, spreader, beakers, 90% ethanol, 0.8% NaCl, micropipettes, test tube stand, autoclave, laminar air flow, bacteriological incubator, water bath set at 50°C.

Methodology

Preparations

Prepare 400ml of nutrient agar (in Erlenmeyer flasks), 5 numbers of 9ml saline blanks, a 99ml saline blank (in Erlenmeyer flask), 20 Petri plates and autoclave.

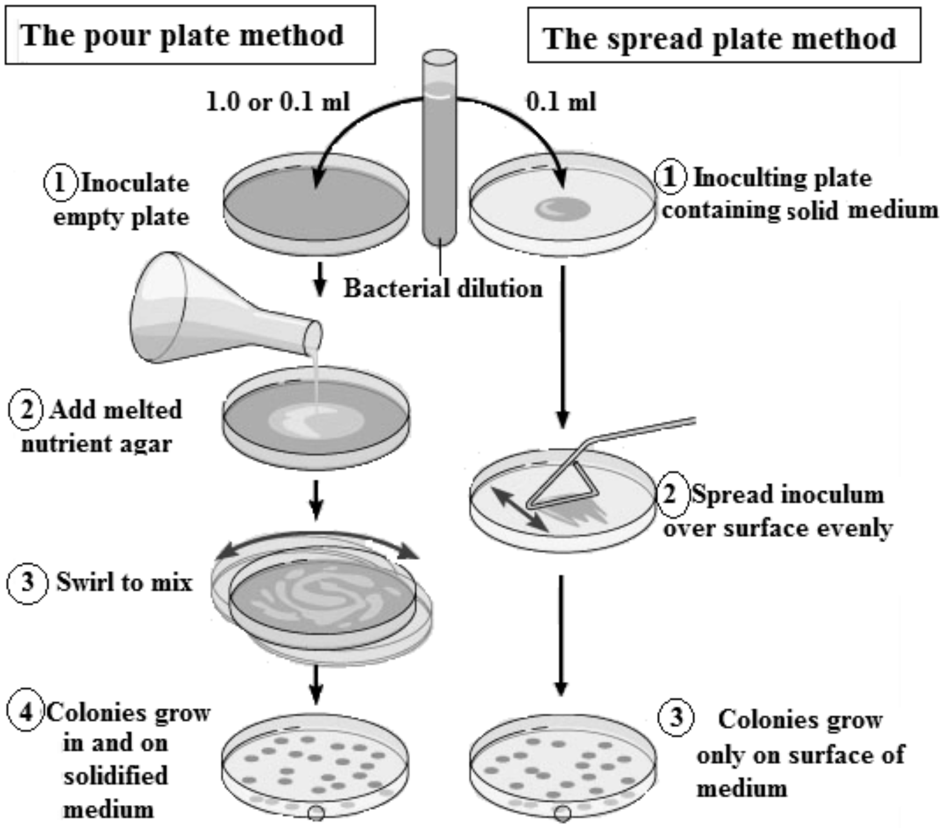
Sample collection

Collect ~2g of garden soil sample in a sterile polyethylene bag using ethanol sterilized spatula. Weigh 1g of soil and keep them inside laminar flow, until further use.

Serial dilution and pour plate

5. Following autoclaving, cool the molten agar in a water bath maintained at 50°C.
6. Transfer the sterilized Petri plates, saline, serial dilution tubes and micropipettes to ready-to-work (surface sterilized (using ethanol) and UV sterilized for 15 minutes) laminar air flow.
7. Cool the sterile 99ml 0.8% saline blank, and add 1g of soil and mix well. This dilutin is regarded as 100 times dilution (10^{-2}).
8. Label the saline blank in flask as dilution 2 and five 9-ml saline blank tubes as dilution 3 through 7.
9. Place the labelled tubes in a test tube rack. Label the Petri dishes in duplicates as P3, P4, P5, P6, P7 (pour plates) and S3, S4, S5, S6, S7 (spread plates).

10. With a sterile pipette, aseptically transfer 1 ml from the 99ml saline blank mixed with soil sample to tube 3. The sample has been diluted 1000 times to 10^{-3}. Discard the tip.
11. Using sterile tip, add 0.1ml each from 3rd tube to 2 empty P1 plates and leave it aside.
12. Mix the mixture in the 3rd tube by gently pipetting up and down. Using a fresh pipette tip, transfer 1ml from tube 3 to tube 4. Now the sample has been diluted 10,000 times to 10^{-4}. Discard the tip.
13. Using sterile tip, add 0.1ml each from 4th tube to 2 empty P2 plates and leave it aside.
14. Using a fresh sterile tip, mix the mixture in the 4th tube by gently pipetting up and down. Using a fresh pipette tip, transfer 1ml from tube 4 to the tube 5. Now the sample has been diluted 100,000 times to 10^{-5}.



15. Using sterile tip, add 0.1ml each from 5th tube to 2 empty P3 plates and leave it aside.
16. Using a fresh sterile tip, mix the tube 5 by gently pipetting up and down. Using a fresh pipette tip, transfer 1ml from tube 5 to the tube 6. Now the sample has been diluted 10, 00,000 times to 10^{-6}.
17. Using sterile tip, add 0.1ml each from 6th tube to 2 empty P4 plates and leave it aside.
18. Using a fresh sterile tip, mix the tube 6 by gently pipetting up and down. Using a fresh pipette tip, transfer 1ml from tube 6 to the tube 7. The sample has been diluted 100, 00,000 times to 10^{-7}. Dilution process is now complete.
19. Using sterile tip, add 0.1ml each from 7th tube to 2 empty P5 plates and leave it aside.
20. Check the temperature of the molten agar medium to be sure the temperature is above 45°C. Remove a media from the water bath and wipe the outside surface dry with a paper towel.

21. Using the pour-plate technique, pour the agar into Plate P1 through P5 1A and rotate the plate gently to ensure uniform distribution of the cells in the medium.
22. Once the agar has solidified, incubate the plates in an inverted position for 24 hours at 37°C.
23. Add and spread 0.1 ml of sterile saline in a nutrient agar plate to act as negative control. Open and leave a nutrient agar plate at the corner of the laminar to act as laminar control. Incubate theses controls along with other plates.

Spread plate technique

The spread-plate technique requires that a previously diluted mixture of microorganisms be used. During inoculation, the cells are spread over the surface of a solid agar medium with a sterile, L-shaped bent glass rod while the Petri dish is hold on hand or spun on a lazy Susan/turntable. The step-by-step procedure for this technique is as follows:

1. Prepare soil sample suspensions as described above and label agar plates accordingly.
2. Place the bent glass rod into a beaker and add a sufficient amount of 95% ethyl alcohol to cover the lower, bent portion.
3. Place an appropriately labelled nutrient agar plate on the turntable. With a sterile pipette, add 0.1 ml of soil suspension on the center of the plate.
4. Remove the glass rod from the beaker, and pass it through the Bunsen burner flame with the bent portion of the rod pointing downward to prevent the burning alcohol from running down your arm.
5. Allow the alcohol to burn off the rod completely. Cool the rod for 10 to 15 seconds.
6. Open the Petri plate lid and touch the agar surface with sterilized L-rod. Move the rod up, down and in side ways to spread the sample.
7. Turn the plate at 90° angle and repeat the spreading process. Again turn the plate at 90° angle and do the spreading. For the last time, turn again the plate at 90° angle and do the spreading.
8. Immerse the rod in alcohol and re-flame.
9. Close the plate and allow the sample to adsorb on the agar surface for 3 minutes. Incubate the plates in an inverted position for 24 hours at 37°C.
10. Add and spread 0.1 ml of sterile saline in a nutrient agar plate to act as negative control and open and leave a nutrient agar plate at the corner of the laminar to act as laminar control. Incubate theses controls along with other plates.

Enumeration: colony counting

Using a Quebec colony counter and a mechanical hand counter, observe all colonies on plates. Statistically valid plate counts are only obtained from bacterial cell dilutions that yield between 30 and 300 colonies. Plates with more than 300 colonies cannot be counted and are designated as too numerous to count—TNTC; plates with fewer than 30 colonies are designated as too few to count—TFTC. Count only plates containing between 30 and 300 colonies. Remember to count all subsurface as well as surface colonies.

The number of organisms per ml of the soil sample is calculated by multiplying the number of colonies counted by the dilution factor:

number of cells per ml = number of colonies * dilution factor

Since the dilutions plated are replicates of each other, determine the average of the duplicate bacterial counts per ml of sample and record in the chart provided in the Lab Report.

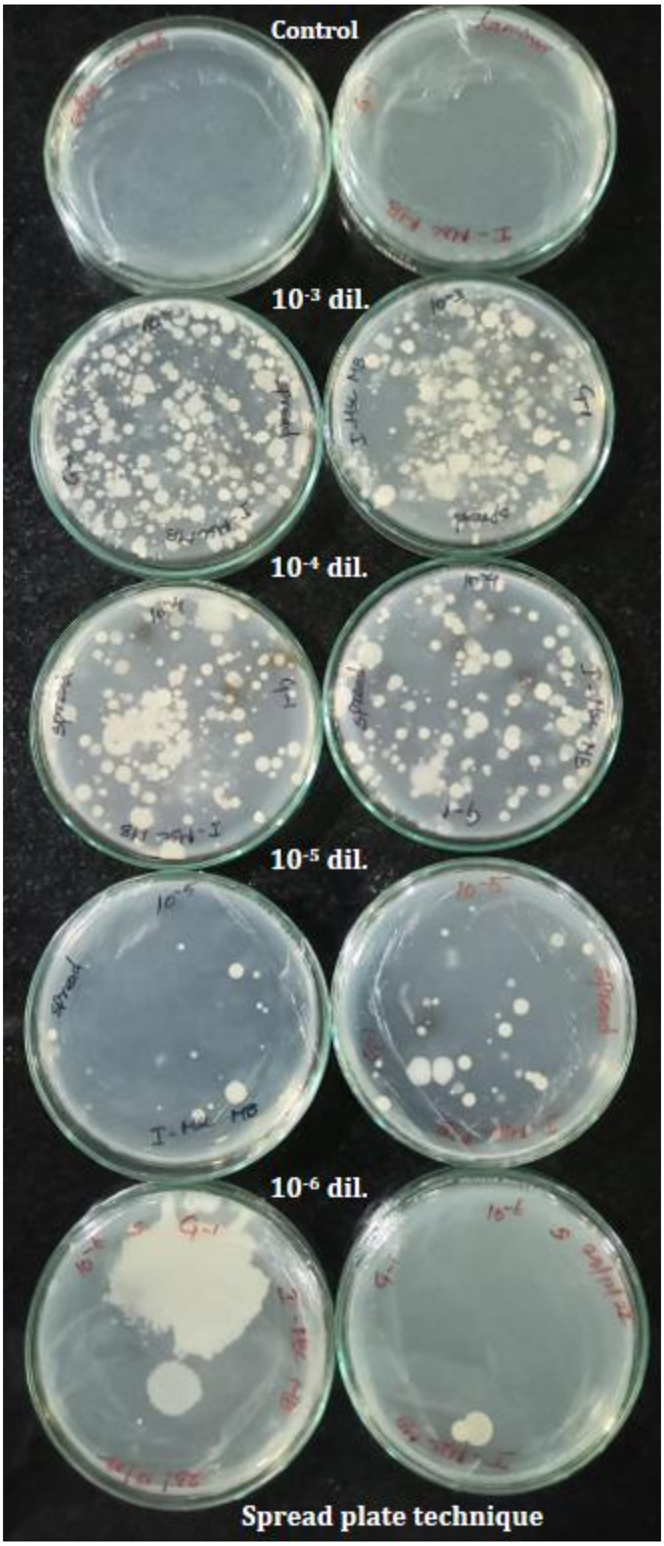
Result

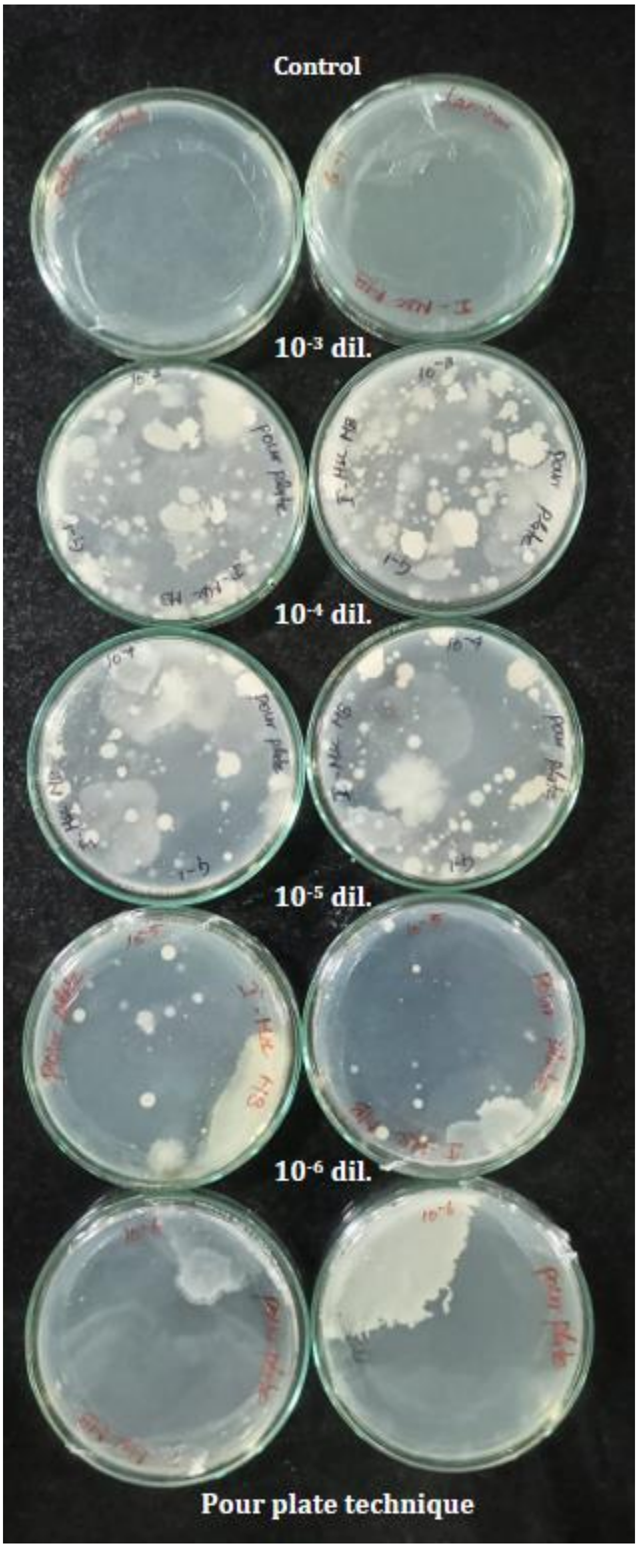
Following 24hours incubation, sample and laminar control remained sterile. Pour and spread plates contained approximately 10^6 CFU/g and 10^7 CFU/g of soil, respectively.

Table: Enumeration of bacteria from soil sample using pour and spread plate technique

Plate	Dilution factor	ml of dilution plated	Number of colonies	Bacterial counts per lm of sample (CFU/ml)	Average count per sample (CFU/ml)
P1	10^{-3}	1	TNTC	-	-
P1	10^{-3}	1	TNTC	-	
P2	10^{-4}	1	121	121	118 or 1.18 x 10^6
P2	10^{-4}	1	115	115	
P3	10^{-5}	1	TFTC	-	-
P3	10^{-5}	1	TFTC	-	
P4	10^{-6}	1	TFTC	-	-
P4	10^{-6}	1	TFTC	-	
P5	10^{-7}	1	TFTC	-	-
P5	10^{-7}	1	TFTC	-	
S1	10^{-3}	0.1	TNTC	-	-
S1	10^{-3}	0.1	TNTC	-	
S2	10^{-4}	0.1	108	1080	1100 or 1.1 x 10^7
S2	10^{-4}	0.1	112	1120	
S3	10^{-5}	0.1	TFTC	-	-
S3	10^{-5}	0.1	TFTC	-	
S4	10^{-6}	0.1	TFTC	-	-
S4	10^{-6}	0.1	TFTC	-	

S5	10^{-7}	0.1	TFTC	-	-
S5	10^{-7}	0.1	TFTC	-	





Experiment 6: Antimicrobial sensitivity test

Background

Chemotherapeutic agents are chemical substances used to treat infectious diseases. Their mode of action is to interfere with microbial metabolism, thereby producing a bacteriostatic or bactericidal effect on the microorganisms, without producing a like effect in host cells. Chemotherapeutic agents act on a number of cellular targets. Their mechanisms of action include inhibition of cell-wall synthesis, inhibition of protein synthesis, inhibition of nucleic acid synthesis, disruption of the cell membrane, and inhibition of folic acid synthesis. These drugs can be separated into two categories:

1. Antibiotics are synthesized and secreted by some true bacteria, actinomycetes, and fungi that destroy or inhibit the growth of other microorganisms. Today, some antibiotics are laboratory synthesized or modified; however, their origins are living cells.
2. Synthetic drugs are synthesized in the laboratory.

Principle

The available chemotherapeutic agents vary in their scope of antimicrobial activity. Some have a limited spectrum of activity, effective against only one group of microorganisms. Others exhibit broad-spectrum activity against a range of microorganisms. The drug susceptibilities of many pathogenic microorganisms are known, but it is sometimes necessary to test several agents to determine the drug of choice. A standardized diffusion procedure with filter paper discs on agar, known as the Kirby-Bauer method, is frequently used to determine the drug susceptibility of microorganisms isolated from infectious processes. This method allows the rapid determination of the efficacy of a drug by measuring the diameter of the zone of inhibition that results from diffusion of the agent into the medium surrounding the disc. In this procedure, filter-paper discs of uniform size are impregnated with specified concentrations of different antibiotics and then placed on the surface of an agar plate that has been seeded with the organism to be tested.

The medium of choice is Mueller-Hinton agar (MHA), with a pH of 7.2 to 7.4, which is poured into plates to a uniform depth of 5 mm and refrigerated after solidification. Prior to use, the plates are transferred to an incubator at 37°C for 10 to 20 minutes to dry off the moisture that develops on the agar surface. The plates are then heavily inoculated with a standardized inoculum by means of a cotton swab to ensure the confluent growth of the organism. The discs are aseptically applied to the surface of the agar plate at well-spaced intervals. Once applied, each disc is gently touched with a sterile applicator stick to ensure its firm contact with the agar surface. Following incubation, the plates are examined for the presence of growth inhibition, which is indicated by a clear zone surrounding each disc. The susceptibility of an organism to a drug is assessed by the size of this zone, which is affected by other variables such as the following:

1. The ability and rate of diffusion of the antibiotic into the medium and its interaction with the test organism.
2. The number of organisms inoculated
3. The growth rate of the organism

Table 42.2 Zone Diameter Interpretive Standards for Organisms Other Than *Haemophilus* and *Neisseria gonorrhoeae*

		ZONE DIAMETER, NEAREST WHOLE MM		
ANTIMICROBIAL AGENT	**DISC CONCENTRATION**	**RESISTANT**	**INTERMEDIATE**	**SUSCEPTIBLE**
Ampicillin				
when testing gram-negative bacteria	10 μg	≤13	14–16	≥17
when testing gram-positive bacteria	10 μg	≤28	—	≥29
Carbenicillin				
when testing *Pseudomonas*	100 μg	≤13	14–16	≥17
when testing other gram-negative organisms	100 μg	≤19	20–22	≥23
Cefoxitin	30 μg	≤14	15–17	≥18
Cephalothin	30 μg	≤14	16–17	≥18
Chloramphenicol	30 μg	≤12	13–17	≥18
Clindamycin	2 μg	≤14	15–20	≥21
Erythromycin	15 μg	≤13	14–22	≥23
Gentamicin	10 μg	≤12	13–14	≥15
Kanamycin	30 μg	≤13	14–17	≥18
Methicillin when testing staphylococci	5 μg	≤9	10–13	≥14
Novobiocin	30 μg	≤17	18–21	≥22
Penicillin G				
when testing staphylococci	10 units	≤28	—	≥29
when testing other bacteria	10 units	≤14	—	≥15
Rifampin	5 μg	≤16	17–19	≥20
Streptomycin	10 μg	≤11	12–14	≥15
Tetracycline	30 μg	≤14	15–18	≥19
Tobramycin	10 μg	≤12	13–14	≥15
Trimethoprim/sulfamethoxazole	1.25/23.75 μg	≤10	11–15	≥16
Vancomycin				
when testing enterococci	30 μg	≤14	15–16	≥17
when testing *Staphylococcus* spp.	30 μg	—	—	≥15
Sulfonamides	250 or 300 μg	≤12	—	≥17
Trimethoprim	5 μg	≤10	—	≥16

Source: Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Disk Susceptibility Tests, Tenth Edition, 2008

A measurement of the diameter of the zone of inhibition in millimeters is made, and its size is compared with that contained in a standardized chart, which is shown in Table given above. Based on this comparison, the test organism is determined to be resistant, intermediate, or susceptible to the antibiotic.

Materials required

Test culture: 0.85% saline suspensions adjusted to an absorbance of 0.1 at 600 nanometer (nm) or equilibrated to a 0.5 McFarland Standard, Mueller-Hinton agar plates, antibiotic discs, forceps, Bunsen burner, sterile cotton swaps, 70% ethanol, millimetre ruler.

Protocol

1. Place MHA plates right-side-up in an incubator heated to 37°C for 10 to 20 minutes, allowing the plates to warm up.
2. Label the bottom of each of the agar plates with the name of the test organism or strain to be inoculated.
3. Using aseptic technique, inoculate all agar plates with their respective test organisms as
4. Follows; Dip a sterile cotton swab into a well-mixed saline test culture and remove excess inoculum by pressing the saturated swab against the inner wall of the culture tube.
5. Using the swab, streak the entire agar surface horizontally, vertically, and around the outer edge of the plate to ensure a heavy growth over the entire surface.
6. Allow all culture plates to dry for about 5 minutes.
7. Distribute the individual antibiotic discs (maximum of 5 discs in 15mm plates) at equal distances (~2.5 cm) with forceps dipped in alcohol and flamed.
8. Gently press each disc down with the wooden end of a cotton swab or with sterile forceps to ensure that the discs adhere to the surface of the agar. *Note: Do not press the discs into the agar.*
9. Incubate all plate cultures in an inverted position for 24 to 48 hours at 37°C.
10. Following incubation, examine all plate cultures for the presence or absence of a zone of inhibition surrounding each disc.
11. Using a ruler graduated in millimeters, carefully measure each zone of inhibition to the nearest millimetre. Tabulate the results.

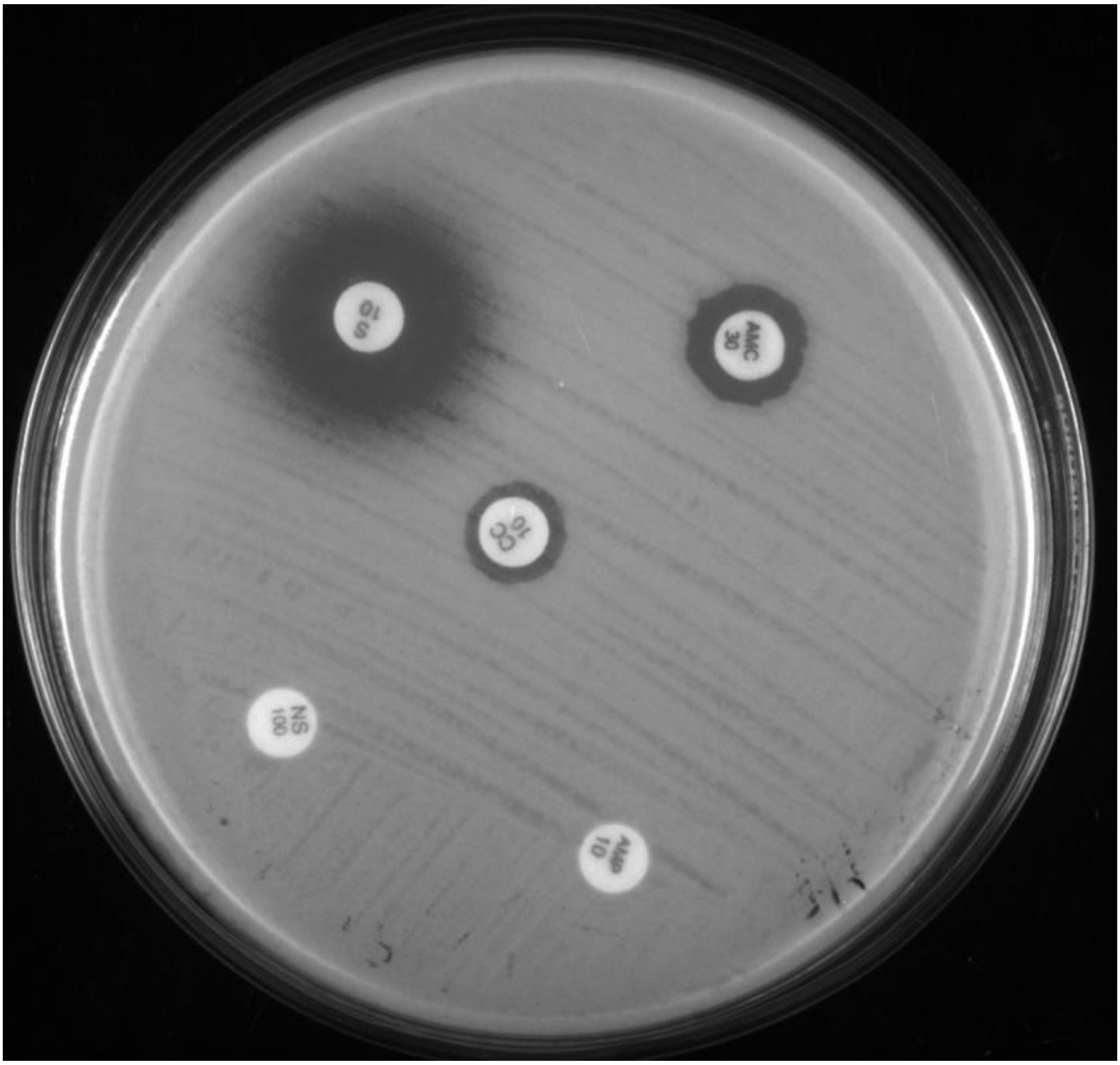
12. Compare your results with Table and determine the susceptibility of each test organism to the chemotherapeutic agent.

Result

The test culture was resistant to ampicillin, clindamycin, nystatin, intermediate to amoxicillin and sensitive to streptomycin.

	Measurements in millimetre				
Antibiotic disc	Streptomycin (S) 10µg	Ampicillin (A) 10µg	Clindamycin (CC) 10µg	Amoxicillin (AMC) 30µg	Nystatin (NS) 100µg
Test culture	S (19)	R	R	I (9)	R

S = sensitive; R = resistant; I = intermediate



Experiment 7: Maintenance of stock cultures in agar stabs

Objective

To prepare and preserve bacterial cultures as media stabs up to 1 year.

Principle

As a general rule, the viable storage period of bacteria increases as the storage temperature decreases. Cryo-protectants are not required when cultures are store above the freezing points. The specific length of time that a culture will remain viable in a given storage condition is dependent upon the nature of the bacterial species/strain. Cell death during storage is inevitable but should be minimized as much as possible. Bacterial cultures that are used regularly (i.e., daily/weekly) used can be stored on agar plates or in stab cultures in a standard refrigerator at 4°C. Storing bacterial cultures in agar plates in a refrigerator is practical only for a short periods of time (<2 weeks), as agar plates is prone to fungal contamination and drying. Consequently, bacterial stocks are best stored in small agar vials at refrigerated conditions up to a year, as the smaller surface area and agar depth reduces the probability of fungal contamination and drying, respectively.

Materials required

24 hours grown bacterial culture in nutrient plate, inoculation loops, laminar air flow, 2ml screw cap tubes, nutrient media, autoclave, permanent markers, autoclave and refrigerator.

Protocol

1. Prepare nutrient agar or tryptic soy agar as per manufacturer's instructions and autoclave.
2. After autoclaving, pour 1ml of media in autoclaved/sterile screw capped 2 ml tubes and let them set as agar deeps (higher volumes may be poured depending on capacity of storage tubes, tubes may be filled till 2/3rd of capacity.
3. Mark the tubes with NCT number, date and tightly seal the marking with cello tapes.
4. Following cooling, stab inoculate the media at several places with overnight growth of the organism on plate cultures using a sterile inoculation loop.
5. Incubate the tube for 12-16 hours at 37°C with slightly loose screw caps.

6. Following incubation, check the surface of the agar stab for growth.
7. After observing the growth, tighten screw caps and store at 4°C for the maximum period of 1 year.
8. For retrieval, remove the portion of the visible growth using a sterile inoculating loop and streak onto fresh culture plates.

Results

Agar media stabs was prepared and preserved for the bacterial strains purified from enumeration plates.

Experiment 8: Maintenance of stock cultures in glycerol stock cultures

Objective

To prepare glycerol stocks of bacteria for long-term storage at -80°C.

Principle

Freezing is an efficient way of storing bacteria. Glycerol allows to reduce the harmful effect of ice crystals of bacteria which can damage cells by dehydration caused by a localized increase in salt concentration leading to denaturation of proteins. Additionally, ice crystals can also puncture cellular membranes. Glycerol as a cryoprotectant depresses the freezing point of bacterial cells, enhancing supercooling. It does so by forming strong hydrogen bonds with water molecules, competing with water-water hydrogen bonding. This disrupts the crystal lattice formation of ice unless the temperature is significantly lowered.

Materials method

Glycerol, 2ml screw cap tubes, bacterial culture, distilled water, pipette, tips, autoclave, inoculation loop and laminar air flow.

Protocol

Glycerol stock preparation

1. Prepare 50% glycerol (v/v) by mixing 5ml glycerol with 5ml distilled water.
2. Mix well and dispense 0.5ml into 2ml screw cap tubes.
3. Label the tubes on the top and its sides (using a label sticker) with appropriate NCT number.
4. Tightly seal the side labels will cello tapes (to make it water proof).
5. Loosen the cap and autoclave the content at 121°C for 15min.
6. Following autoclaving, cool the stock below room temperature before adding the bacterial biomass (or leave the autoclaved glycerol tubes in refrigerator overnight).

Cryopreservation

1. Transfer 3 to 6 loop full of 24 hours grown bacterial biomass into the 0.5ml of 50% glycerol.

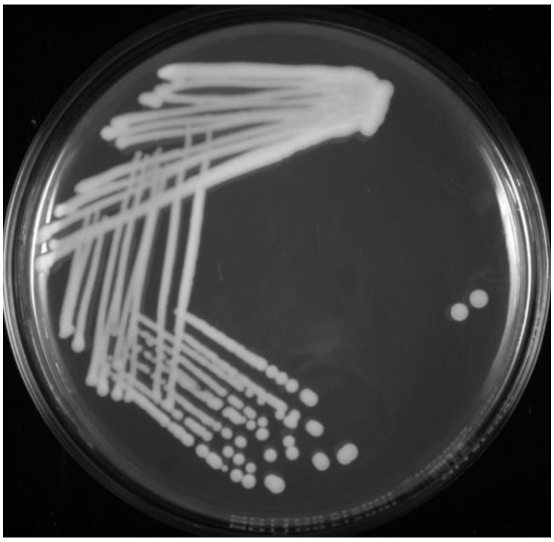
2. Close the cap and vortex the tubes for 30 seconds (for homogenous distribution of bacterial biomass in glycerol).
3. Increase the vortex duration, if homogenization is not achieved in 30 seconds.
4. Put the glycerol stocks in cryo-boxes.
5. Place the cryo-box in -20°C freezer and record its location.

Reviving the bacteria

1. Withdraw the glycerol sock from -20°C in the ice bucket (or on dry ice). Never thaw the glycerol stock (as this can reduce the viability of bacterial cells).
2. If glycerol stock is thawed, never put it back to -20°C (instead use the entire stock for reviving) and make a fresh stock from the revived plate.
3. Using inoculation loop, remove a loop full of frozen bacterial suspension and quadrant streak on to the appropriate nutrient media.
4. Immediately replace the stock on the dry ice (or in ice bucket) and transfer it to -20°C.
5. From the revived plate, 2 more glycerol stock could be made and stored in -80°C (i.e., 3 glycerol stock (2 in -80°C & 1 in -20°C) per bacterial species).

Result

Glycerol stock was prepared, stored and successfully revived for the bacterial strains.



Experiment 9: Isolation and identification of bacteria from soil samples

Objective

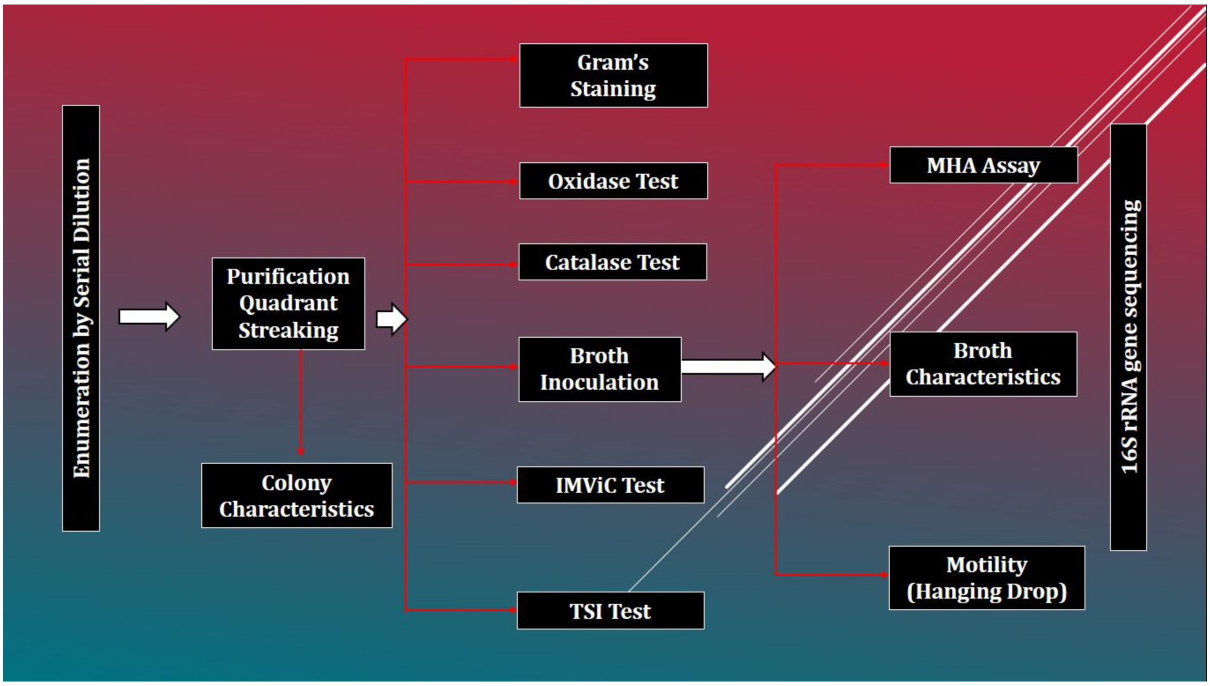
To isolate and identify the bacteria from soil sample.

Principle

Identification of bacteria involves the study of macro-morphological, micro-morphological, bio-chemical characters and molecular characteristics. The macro-morphological characters like colony morphology, texture, pigmentation; micro-morphological characters like cell shape, motility; biochemical characters like catalase, oxidase assays, IMViC and TSI assays should be observed for the pure culture for the identification purposes. The results of these assay were compared with Bergey's manual (9^{th} edition) for identification. The molecular features (16S rRNA gene sequencing) should used for species level resolution.

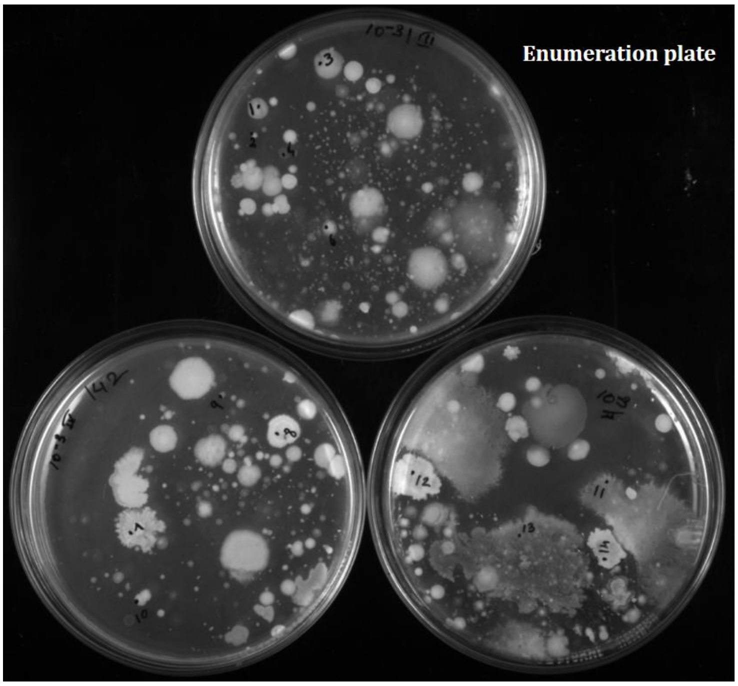
Methodology and work plan

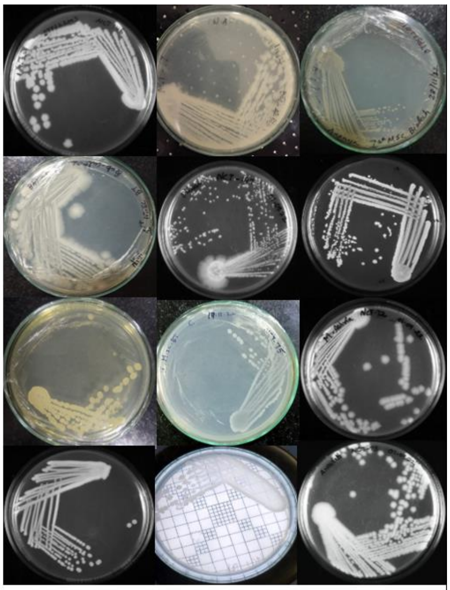
1. Different bacterial types acquired during standard plate count (experiment 5) was purified using quadrant streak method (experiment 2).
2. Following purification, a glycerol stock was prepared for every strain (experiment 8).
3. Re-viability of bacterial strain preserved in glycerol stock was check by quadrant streak onto a fresh nutrient agar plate (experiment 2) and two more stocks was prepared for every bacterial strain. Totally 3 glycerol stocks were prepared for every bacterial strain (2 stocks were kept at -80°C and one at -20°C).
4. The bacterial strains were maintained on agar deeps for daily usage (experiment 7) for biochemical characterization.
5. Further biochemical and morphological characterization was done as per the work plan shown below.



Result

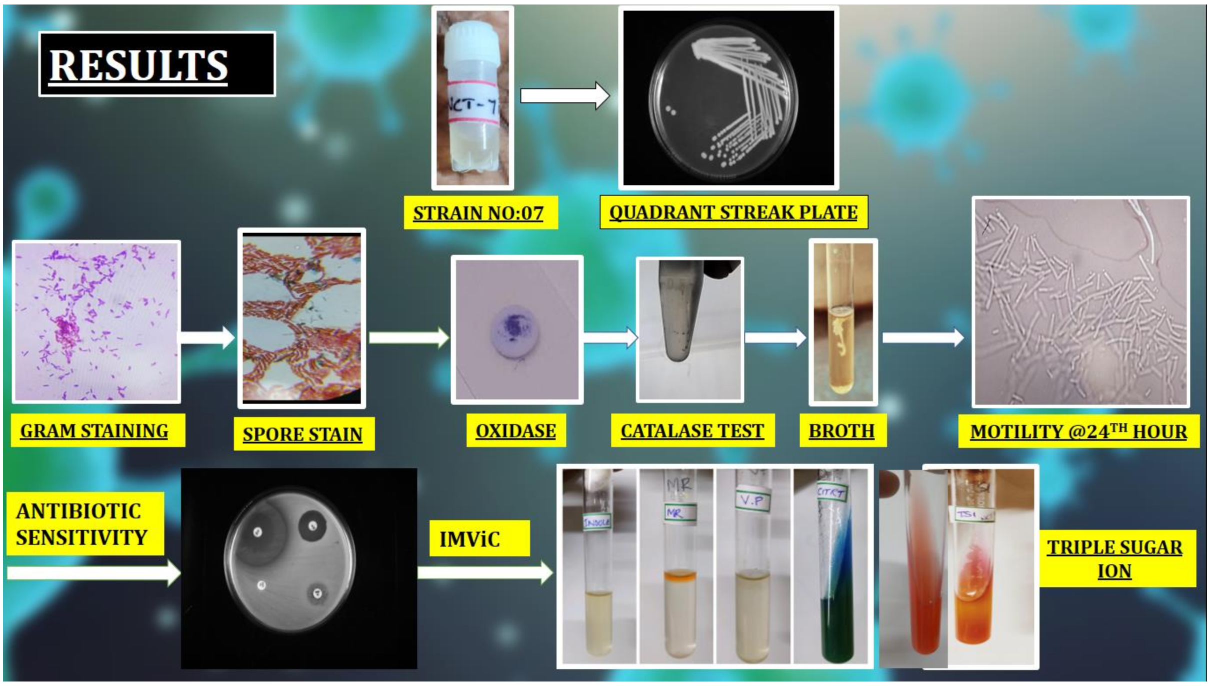
Colonies from enumeration plate selected for identification and its pure cultures were shown below.





Pure cultures

For every pure culture, morphological and biochemical characterization was performed as per the work plan formulated, as shown below.



The results of biochemical characterization were summarized below.

							IMVic				TSI		Motility	Catalase	Oxidase
No.	Student	NCT no.	Grams	Shape	Spore	I	MR	VP	C	Butt	Slant	Gas			
1	Nivetha M	NCT85													
2	Guna	NCT82													
3	Siddik	NCT8													
4	Vishnushree	NCT76													
5	Chelsia	NCT77													
6	Gokul	NCT75													
7	Surendhar	NCT74													
8	Aravindhan	NCT73													
9	Ashwin	NCT72													
10	Ravi	NCT7													
11	Rahul	NCT68													
12	Vani	NCT42													
13	Aranya	NCT39													
14	Ananya	NCT20													
15	Durga	NCT19													
16	Nivetha M	NCT148													
17	Dinesh	NCT147													
18	Jeyaprakash	NCT146													
19	Janani	NCT101													
		Rods	Positives	Motile	Acid	Gas									
		Cocci	Negatives	Non-motile	Alkaline	no gas									

The strains were commercially outsourced for 16S rRNA gene sequencing for species level resolution.

Identification flow charts

Bergey's Manual of Determinative Bacteriology

All of the unknowns will fall into the following groups in Bergey's Manual of Determinative Bacteriology (The pink book on the shelf in the laboratory).

GROUP 4

Description: Gram Negative, Aerobic/Microaerophilic rods and cocci

Key differences are: pigments/fluorescent, motility, growth requirements, denitrification, morphology, and oxidase, read Genera descriptions

Examples: *Acinetobacter, Pseudomonas, Beijerinckia, Acetobacter*

GROUP 5

Description: Facultatively Anaerobic Gram negative rods

Key differences are: growth factors, morph., gram rxn., oxidase rxn., read Genera descriptions

Examples: Family Enterobacteriaceae and Vibrionaceae

GROUP 17

Description: Gram-Positive Cocci

Key differences are: oxygen requirements, morph., growth requirements (45°C and supplements), read Genera descriptions

Examples: *Micrococcus, Staphylococcus, Streptococcus, Enterococcus, Lactococcus, Aerococcus*

GROUP 18

Description: Endospore-Forming Gram positive rods and cocci

Key differences are: oxygen requirements, motility, morph, catalase

Examples: *Bacillus, Clostridium*

GROUP 19

Description: Regular, Nonsporlating Gram positive rods

Key differences are: morph., oxygen require, catalase

Examples: *Lactobacillus, Listeria*

GROUP 20

Description: Irregular, Nonsporlating Gram-positive rods

Key differences are: catalase, motility, morph., read Genera descriptions

Examples: *Actinomyces, Corynebacterium, Arthrobacter, Propionibacterium*

GROUP 21

Description: Weakly Gram-Positive Nonsporlating Acid Fast Slender Rods

Key differences are: acid fast, growth

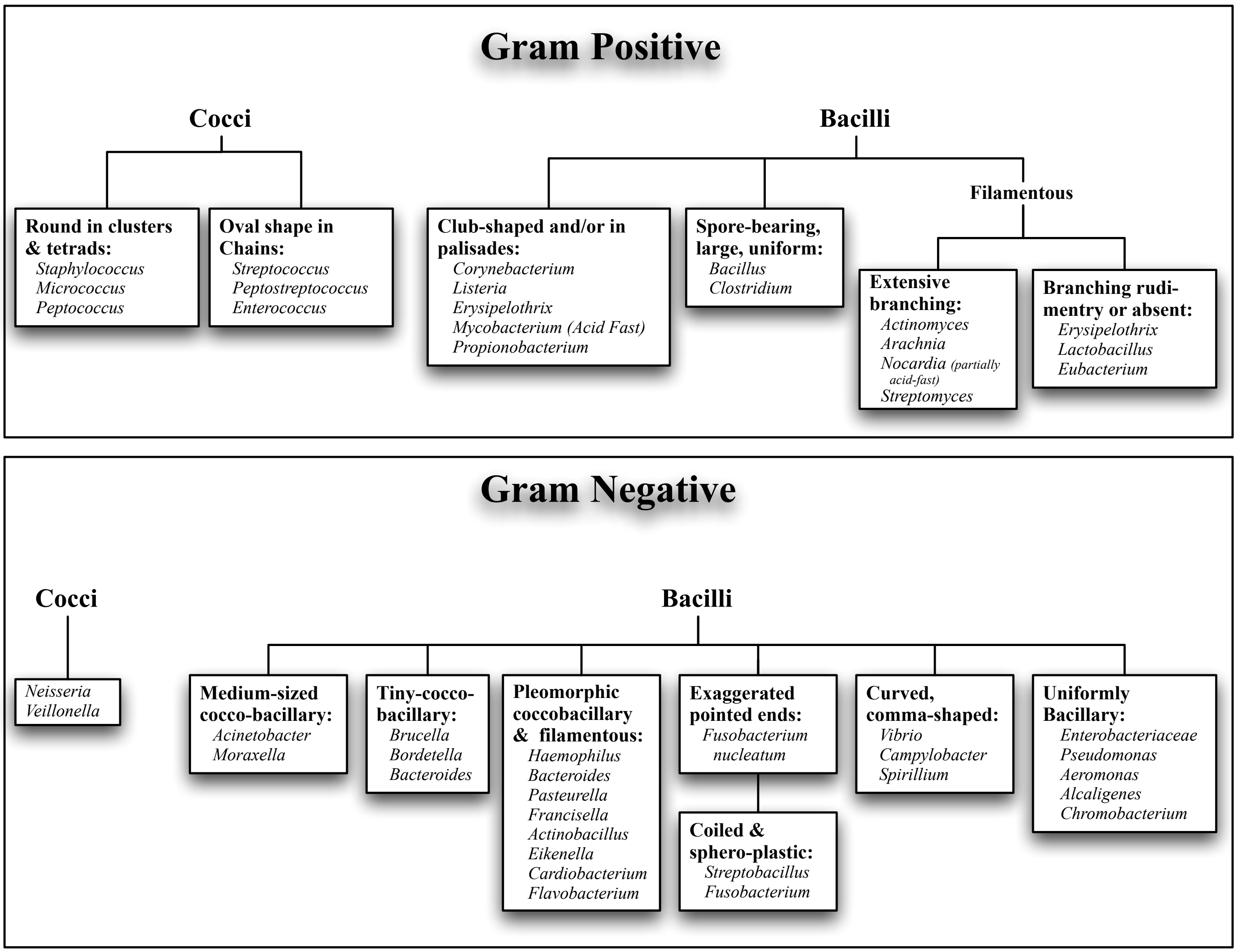
Examples: *Mycobacterium*

Identification flow charts

Differentiation via Gram stains and cell morphology.

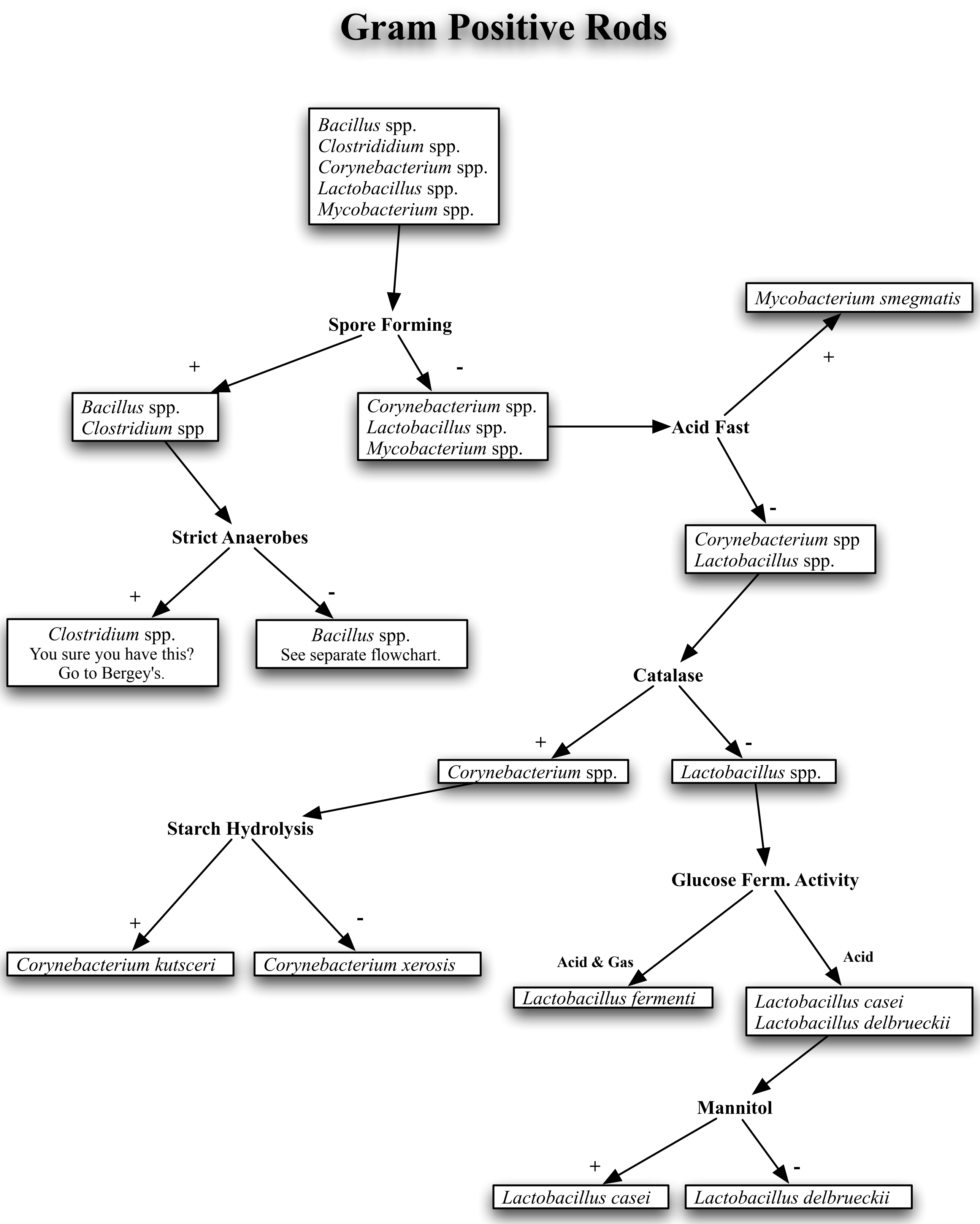
Gram Stain & Morphological Flowchart

Some Examples



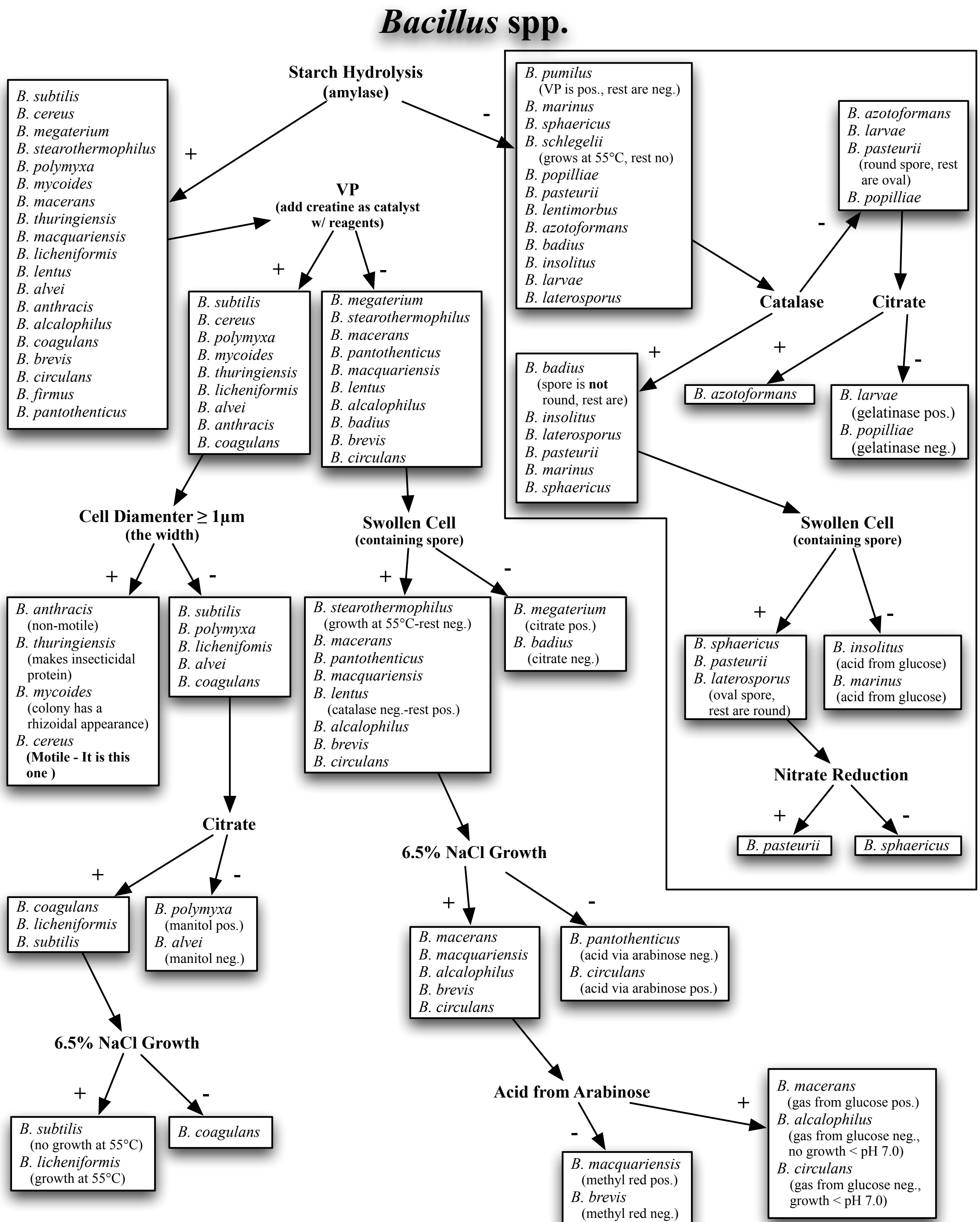
Identification flow charts

Gram Positive Rods ID Flowchart



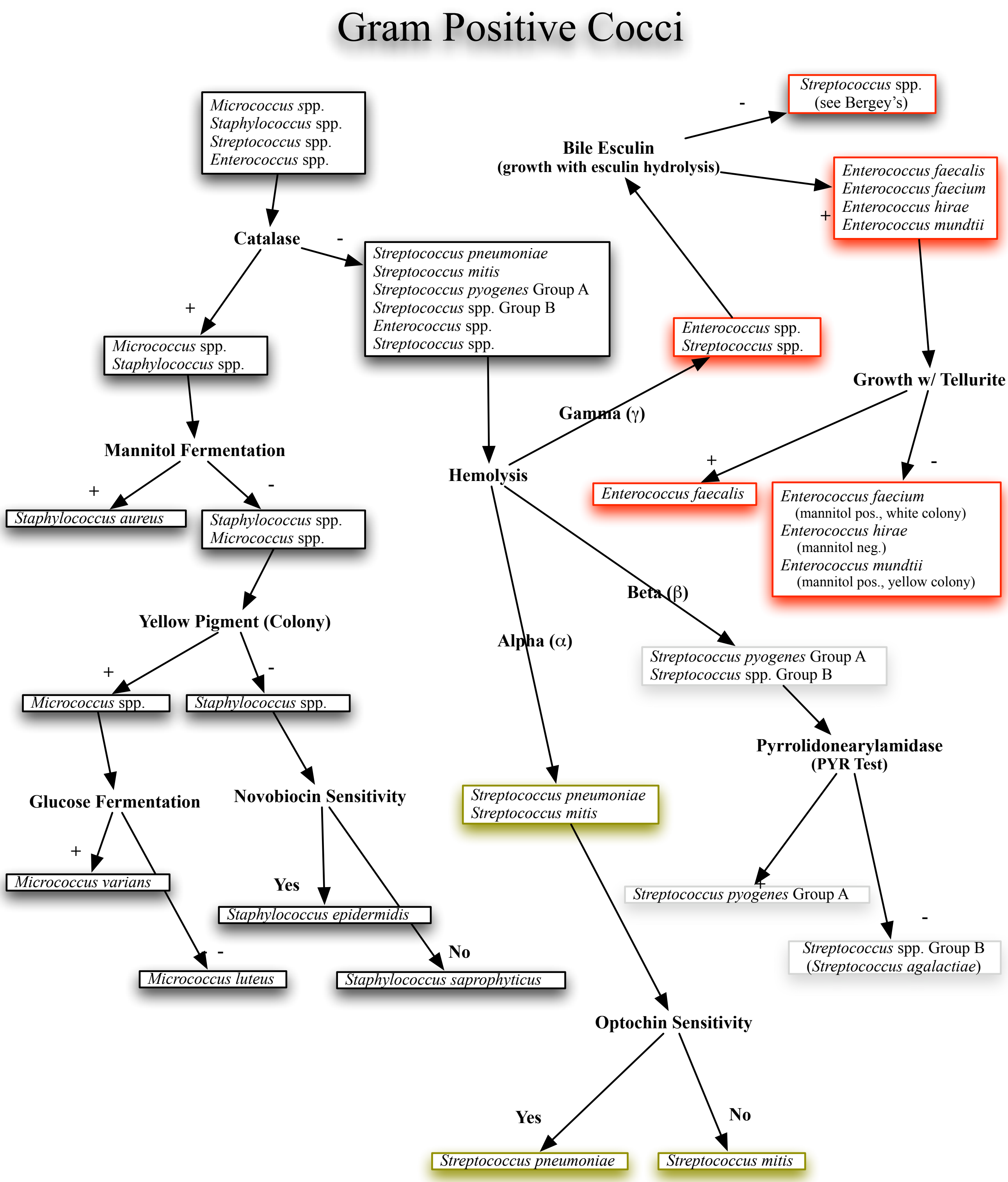
Identification flow charts

Bacillus spp. ID Flowchart



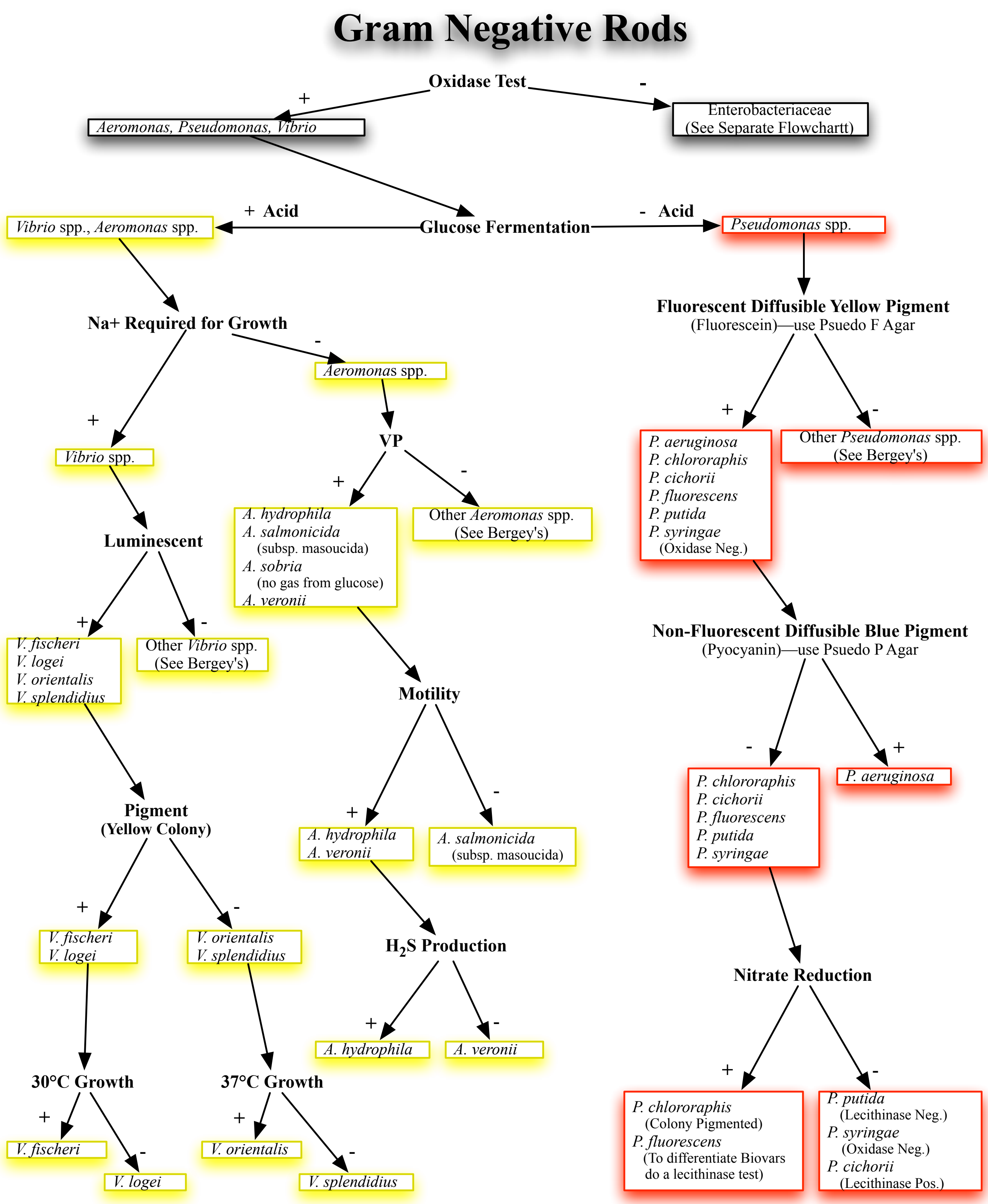
Identification flow charts

Gram Positive Cocci ID Flowchart



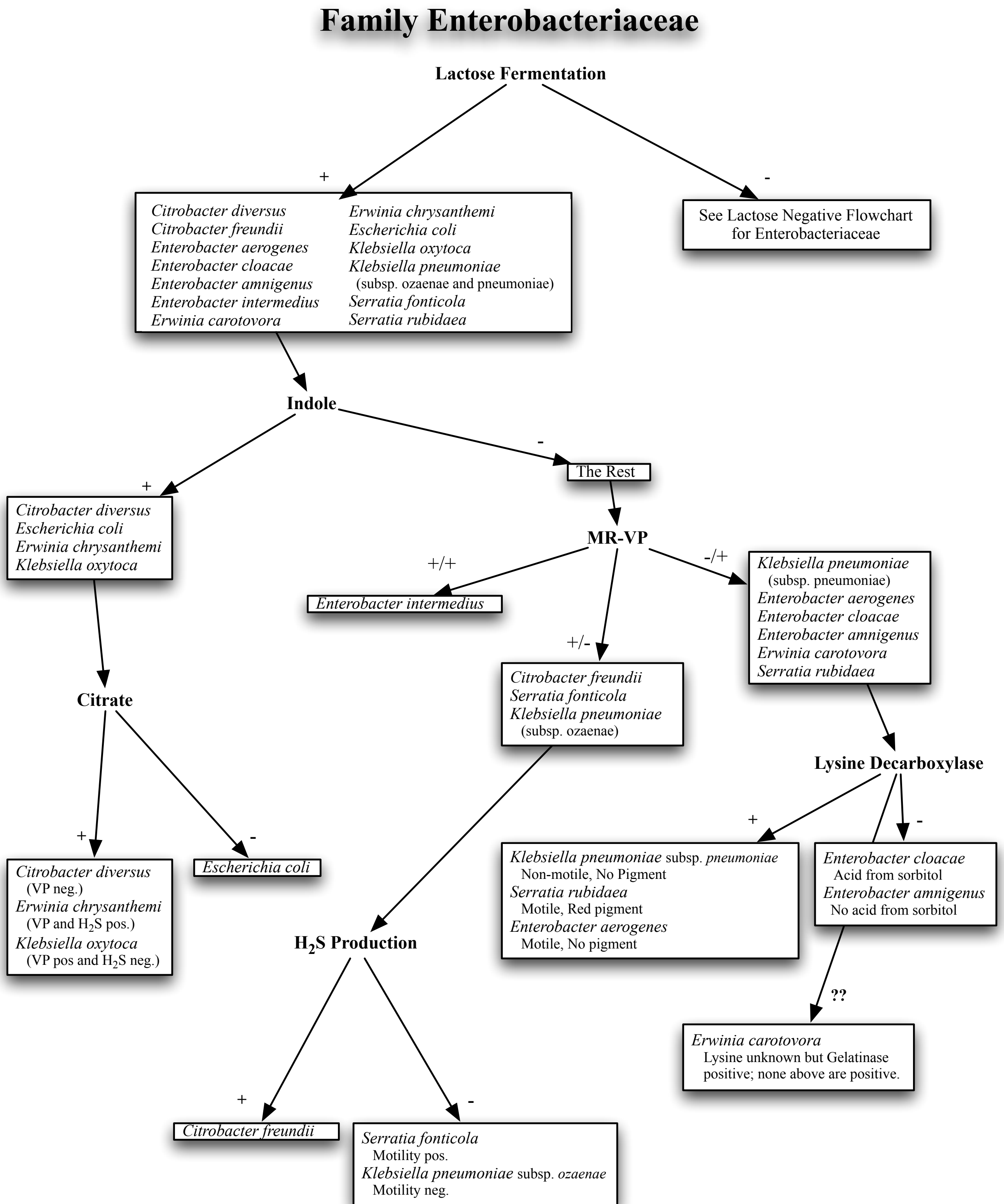
Identification flow charts

Gram Negative Rods ID Flowchart



Identification flow charts

Family Enterobacteriaceae Lactose Positive ID Flowchart



Identification flow charts

Family Enterobacteriaceae Lactose Negative ID Flowchart