



General Microbiology Laboratory



Manual



Medical Technology Department
Islamic University-Gaza

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2009

قال رسول الله صلى الله عليه وسلم

أبغضوا ما أبغضنا وأحبوا ما أحبنا (الترمذي)
والأجود قولي والابحسانة والابحسانة والابحسانة والابحسانة

صدق رسول الله صلى الله عليه وسلم

وصف المساق

اسم المادة: أحياء دقيقة طبية أساسية عملية

رقم المادة: MEDI 3101

الفصل: الأول 2009

عدد الساعات الإجمالية: ساعة واحدة معتمدة (3 ساعات عملية)

وصف المساق: يتناول المساق المواضيع المتعلقة بكيفية استخدام الميكروسكوب والتعامل مع أكثر الصبغات استخداماً في مختبرات الميكروبيولوجي، وكيفية استخدام الفحوصات البيوكيميائية مع التطرق لبعض أنواع البكتيريا المرضية وكيفية تعريفها وعدها ومن ثم شرح مفصل للمضادات الحيوية وتصنيفها وتأثيراتها .

متطلبات سابقة: أحياء عامة عملية

مدرس المساق:

الاسم:

J121

المكتب:

2673

الهاتف:

مدرس مساعد:

المرجع المعتمد:

Microbiology Laboratory Manual prepared by Abdelraouf Elmanama

(Diagnostic Microbiology) author: BAILEY & SCOT'S

مراجع أخرى:

أهداف المساق:

١. استخدام الميكروسكوب بالشكل الجيد الذي يتيح من خلاله تعريف الشرائح البكتيرية.

٢. كيفية الحصول على مزارع بكتيرية نقية وخالية من التلوث.

٣. صبغة البكتيريا بأنواع الصبغات المختلفة واستخدامها في تعريف البكتيريا.

٤. تطبيق الفحوصات البيوكيميائية المختلفة بهدف تعريف البكتيريا.

٥. طرق تشخيص البكتيريا سالبة الجرام من عائلة Pseudomonas و Enterobacteriaceae

٦. طرق تشخيص البكتيريا موجبة الجرام من عائلة Streptococcus و Staphylococcus

٧. استخدام الفحوصات الكيميائية والمصلية في تشخيص أنواع البكتيريا سابقة الذكر

٨. تحديد المضادات الحيوية ذات الحساسية لأنواع البكتيريا المختلفة وتصنيفها وكيفية آلية تأثيرها.

٩. كيفية معرفة العدد التقديري للبكتيريا في العينة الأساسية بواسطة الطرق المختلفة

١٠. تحضير الأوساط الغذائية للبكتيريا

النتائج المتوقعة أن

يحصل عليه الطالب:

الحصول على قدر من المعلومات يضع الطالب على بداية الطريق من خلال تعامله الجيد مع الميكروسكوب

في تشخيص البكتيريا التي تمت صبغتها ومن ثم استخدام الفحوصات البيوكيميائية في تعريف البكتيريا،

ومن ثم عزل البكتيريا الممرضة وتشخيصها بالطرق المتبعة في هذا المجال واختيار المضادات الحيوية

ذات الحساسية المناسبة لها

استخدام الحاسوب:

يتم تدريب الطالب على استخدام الحاسوب في تشخيص البكتيريا الممرضة المعزولة حسب الفحوصات

الكيميائية التي ظهرت معه في المعمل بإدخال البيانات المطلوبة على نوعين من البرامج الخاصة

المستخدمة دولياً لتعريف البكتيريا

درجة 30 امتحان نظري نصفى.

توزيع الدرجات:

درجة 10 بحث على شكل بوربوينت مع الشرح.

درجة 10 حضور و تقارير عملية ونشاط .

درجة 50 امتحان نظري نهائي.

يتم الاتفاق على موعد الامتحان النصفى والتهائى لاحقاً.

تاريخ الامتحانات:

خطة طرح المساق

Course Titles	Hours
1. Introduction	1.5
2.	
3. Introduction to the oil immersion compound microscope	1.5
4. Simple stain, Gram stain	1.5
5. Acid fast stain	1.5
6. The spore stain, and negative stain	1.5
7. Isolation of pure culture and sterile transfer	1.5
8. Bacterial motility.	1.5
9. Amylase Production add Gelatin Liquefaction	1.5
10. Catalase Production	1.5
11. Coagulase Test	1.5
12. Oxidase Production	1.5
13. Methyl Red & Voges-Proskauer Tests (MR-VP)	1.5
14. Tryptophan Hydrolysis (Indole Test)	1.5
15. Citrate Utilization Test, Urease Test	1.5
16. Nitrate Reduction Test	1.5
17. Media preparation & Sterilization	1.5
18. Single Media / Multiple Tests, Triple Sugar Iron Agar	1.5
19. Selective and differential media	1.5
20. Bacterial oxygen requirements	1.5
21. Anaerobic bacteria	1.5
22. The serial dilution method of bacterial enumeration and generation time	3
23. Microbial control agents	1.5
24. Gram positive coccus identification	1.5
25. Pseudomonas identification	1.5
26. Enterobacteriaceae identification	1.5
27. Identification of unknown bacteria	1.5
28. Microbes in the atmosphere	1.5
29. Microbes in the soil	1.5

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معمل الميكروبيولوجي (J121)



احذر

احذر!!!!!! أنت تعمل في بيئة خطيرة بيولوجيا لذا عند الدخول للمعمل والبدء بممارسة الفحوصات العملية ومغادرة المعمل عليك إتباع إرشادات السلامة.

قبل البدء بإجراء الفحص المقرر يجب التزام بالتالي:



تيسر لفقازات

- يمنع منعا باتا الأكل والشرب أو جلب طعام أو شراب إلى المعمل.
- يجب غسل اليدين بالماء والصابون قبل البدء بإجراء الفحص.
- يجب ارتداء القفازات لضمان الوقاية من أي عينات مرضية أثناء التعامل معها.
- يجب ارتداء المعطف الأبيض التنظيف.
- على الأخوات الطالبات وضع غطاء الرأس داخل المعطف مع تغطية أكمام المعطف لأكمام الملابس لتجنب التلوث بأي عينات مرضية.

أثناء إجراء الفحص المقرر يجب التزام بالتالي:



علبة لإسعافات
الأولية

- تحضير الأدوات وكافة المواد اللازمة في منطقة العمل للاستفادة من الوقت.
- يجب عدم التجول في المعمل إلا للضرورة وبحذر وانتباه واحرص على عدم التحدث.
- احرص على التخلص من الأدوات الحادة المستخدمة في الحاوية المخصصة لذلك.
- في حال تعرضك لأي حادث عرضي أو انسكاب أي من المحاليل الكيميائية أو العينات المرضية يجب إبلاغ المدرس المشرف على المعمل للقيام بالإجراءات اللازمة للحفاظ على سلامتك.

عند الانتهاء من إجراء الفحص المقرر يجب التزام بالتالي:



تيسر الباطو

- تأكد من إعادة جميع الأدوات المستخدمة إلى مكانها المخصصة.
- تأكد من إغلاق كافة الأجهزة التي تم استخدامها.
- احرص على نظافة منطقة العمل المخصص بك.
- قم بخلع المعطف أولا ثم القفازات.
- يجب وضع المعطف المستخدم في الحقيبة الخاص به لتجنب ملامسة القرطاسية.
- إعادة المقعد للمكان المخصص به.

ملاحظة/ يمنع التجمع في المعمل قبل الوقت المحدد أو الخروج منه دون إذن المشرف على المعمل.

شكرا لكم على حسن اهتمامكم

قسم التحاليل الطبية - الجامعة الإسلامية - غزة

أنا الطالب/ة قد قرأت ما ورد من إرشادات وعليه أتعهد بالالتزام
التوقيع:

Introduction

Welcome to the microbiology laboratory. The goal of the laboratory is to expose students to the wide variety of lives in the microbial world. Although the study of microbiology includes bacteria, viruses, algae and protozoa, this lab will concentrate primarily on the bacteria.

Microbiological techniques are important in preparing the students for the much harder task of identifying the pathogenic microorganisms in a clinical and environmental specimen. In this manual, I started each experiment with a brief theoretical introduction revealing the theoretical basis on which the experiment is based on, so that there will be a strong conjunction between the practical and theoretical sessions. Included in this manual also, the safety precautions which are essential for every one in the field of microbiology.

Bacteria belong to the kingdom Monera. This kingdom contains more biological diversity than all other kingdoms combined. Most people tend to associate bacteria with disease, but less than ten percent of all bacteria cause disease. Many bacteria cannot even live at the temperatures found in and on the human body. In this lab, most of the bacteria with which we will be working are **non-pathogenic** (do not cause disease). However, some of the bacteria are **opportunistic**; that is, they can cause disease in an ill or injured person. Therefore, treat all bacteria as if they are **pathogenic** (cause disease).

Laboratory Safety Rules

These rules are for the safety of the students, instructors and support staff. Please read and follow them. Failure to follow safety rules may result in removal from the class.

1. *Wear a lab coat in lab.* We will be working with a variety of materials that can cause permanent stains on some fabrics. Also, a lab coat can help protect from accidental contamination by microorganisms.
2. *No eating or drinking during lab.* Many pathogens spread by ingested food and drink. In addition, food can carry microorganisms that might contaminate laboratory cultures.
3. *Keep long or fluffy hair tied up and out of the way.* Hair can contaminate and be contaminated by microbial cultures.
4. *Always wear shoes in lab.*
5. *Thoroughly wash your hands with soap and water before and after lab.* Thorough and frequent hand washing easily and effectively controls the spread of many pathogens.
6. *Clean the lab bench with disinfectant before and after lab.* This helps to prevent contamination of cultures, books, clothing, etc.

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7. *Keep the lab bench free of unnecessary materials.* Don't use the lab bench as a storage area for coats, books, etc.
8. *Do not take cultures from the lab area.*
9. *Dispose of all contaminated materials in autoclave bags.* When in doubt, ask the instructor.
10. *Immediately report all accidents and spills to the instructor.* Cover spills with disinfectant-soaked paper towels for at least 15 minutes before disposing of them.
11. *Read all assigned materials before the lab session.* Experiments will go smoother and have greater chances of success when you know what you will be doing ahead of time.
12. *Treat all microbial cultures as if they are pathogens.* Better safe than sorry.
13. *When in doubt, ask the instructor.* The only stupid questions are those that are intended as such.

NOTES:

1. Personal belongings are not to be stored in the laboratory.
2. You will be assigned to a group consisting of four students and you will work together in a semester long project.
3. Please read the safety instructions posted in the lab.

Glossary of terms

Aerobic: Requires oxygen (opposite of anaerobic).

Agar: Powder added to media for solidification.

Air-dry: Drying of slide suspension in air before heat fixing and staining.

Analog: Similar structure, but not identical.

Antibody: Specific, protective protein produced by the immune system in response to an antigen.

Antigen: Foreign, non self immunogenic material that elicits an immune response.

Atrichous: Without flagella, nonmotile.

Autoclave: Moist heat method of sterilization using pressure.

Axial filament: A structure for motility used by the Spirochment bacteria.

BHI: Brain heart infusion, a really good enrichment medium.

Broth: media without agar.

Brownian movement: Vibrations of an object seen in a microscope, not true motility.

Candle jar: Candle burns in a closed container producing a carbon dioxide incubator, containing 2-10%O₂ and around 10% CO₂.

CFU: Colony- forming unites

CAN: Columbia naladixic acid media, selective (for Gram positive) and differential medium.

Coliforms: Gram- rods which ferment lactose, non spore forming.

Colony: A visible mass of bacteria growing on solidified medium, a clone.

Differential stain: Uses 2 or more dyes which allow differentiation between different bacteria groups or structures.

Counter stain: The 2nd dye added to a smear, taken in after the wall is decolorized, e.g. safrinin, methylene blue.

Declorizer: The reagent used to remove the primary dye from the cell wall in a differential stain e.g. acid alcohol, acetone- alcohol..

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Primary dye: The 1st dye used in a differential stain, e.g. malachite green, crystal violet.

EMB: Eosin methylene blue medium, selective (for Gram negative) and differential medium.

Exoenzyme: Enzyme excreted away from the cell.

Facultative anaerobe: Uses oxygen when present but can either ferment or an aerobically respire without it.

Fastidious: Hard-to- grow bacteria, requiring grow factors or particular nutrients.

Microaerophilic: Likes a reduced oxygen concentration.

Obligate aerobe: Requires oxygen to grow.

Fecal coliforms: Gram- rod which ferment lactose, non spore forming, GI flora in animals, in feces.

Genus: Category of organisms with like features and closely related, divided into species.

Heat- fix: Use of flame to

1. Coagulate proteins of suspension, causing adherence to slide.
2. Kill the microbes.

IMVIC: Acronym= indole, methyl red, Voges- proskauer, citrate.

MIC: Minimal inhibitory concentration of antibiotic that inhibits a bacterium.

NA\NB: Nutrient agar or nutrient broth.

Pathogenic: disease- causing.

PCA: Plate count agar medium general all- purpose enrichment.

Phenotype: Expression of gene as a trait.

Plate count agar: Variation of nutrient agar, for optimizing counts of bacteria in sample.

Streak plate: Procedure where pre-made agar plates have a sample of bacterium placed on tope of the agar and spread via a glass rod.

Zone of inhibition: Area of no bacterial growth around a chemical on a disc indicates sensitivity.

Exercise 1: Introduction to the oil immersion compound microscope.

Introduction

Many students are probably familiar with the compound microscope from using it in previous biology classes. Figure 1 represents a typical compound microscope.

A basic microscope consists of two lenses and the associated hardware to make viewing of specimens easier. The uppermost lens, called the **ocular**, is the part through which a person looks. The lower lens is the **objective**. Usually, several objective lenses are mounted on a **turret**, allowing rapid changing of objective lenses. The body tube holds the ocular and objective lenses in place. Most microbiological specimens are mounted on glass slides and placed on the **stage**.

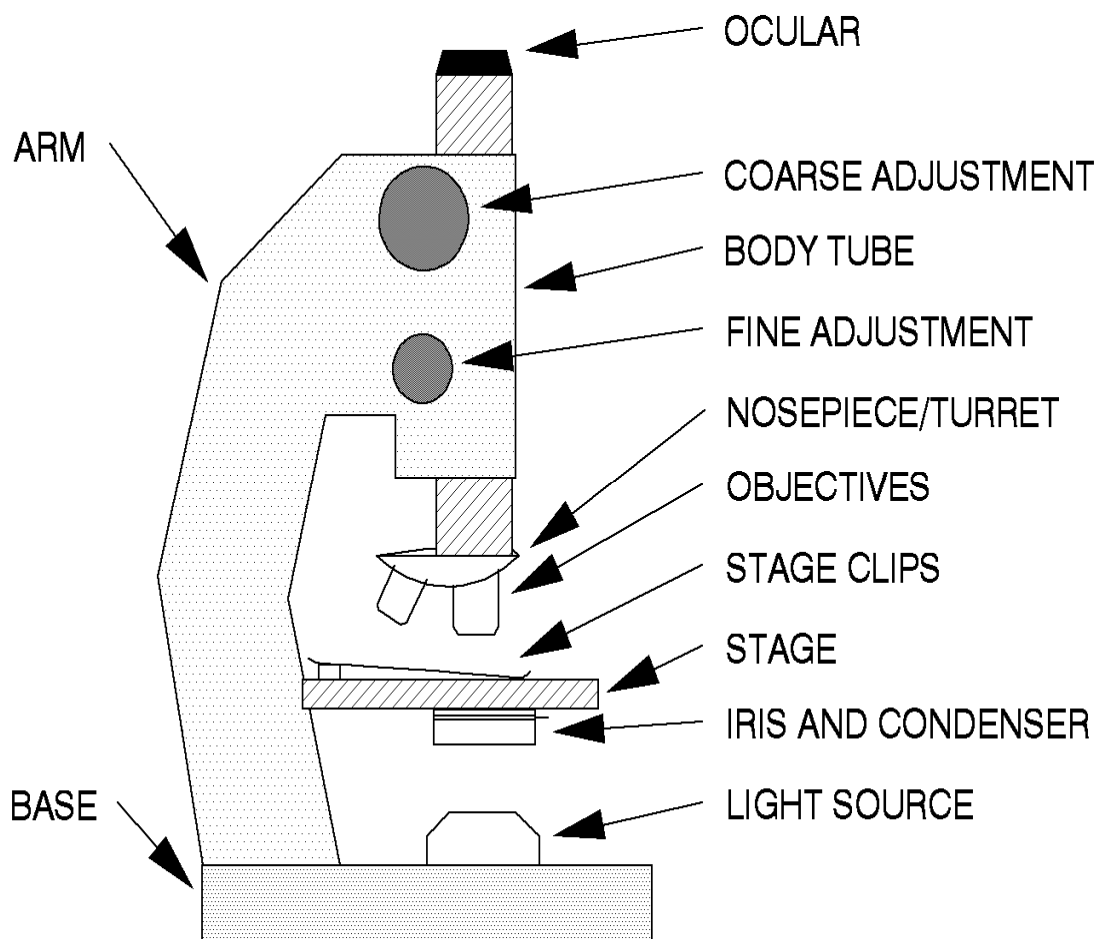


Figure (1): A typical compound microscope. Individual microscopes may vary somewhat from this illustration.

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Usually, clips or clamps hold the slide firmly to the stage. A **light source** and a **condenser** lens are located beneath the stage. The condenser focuses the light through a hole in the stage. The condenser usually includes an **iris** that varies the amount of light passing through the specimen. After passing through the specimen, the light goes through the objective and ocular lenses, and then into the eye of the observer.

As light passes through various substances (glass, air, specimens, etc.), it bends. This bending of light is called **refraction**. The **refractive index** of a substance is a measurement of the extent that the substance bends light. Excessive refraction can cause distortion of the image. At magnifications of less than 500 x, the distortion is minimal. But at higher magnifications, the distortion becomes so great that image details are lost. An oil immersion lens helps to remedy this problem by eliminating the air gap between the specimen and the objective lens. A drop of special immersion oil is placed on the microscope slide, and the oil immersion objective lens is maneuvered so that it is touching the oil. Immersion oil has the same refractive index as glass so that the light passes through the slide, specimen, oil and objective lens as if they were a single piece of glass.

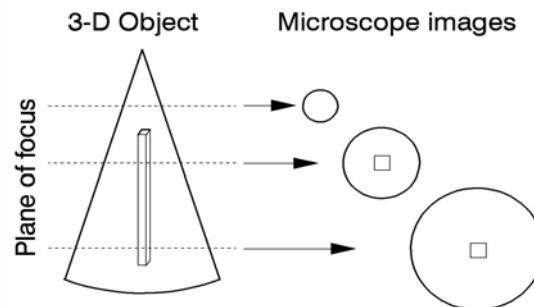


Figure (2): Changes in image composition coincide with changes in depth of focus. Depth of focus is inversely proportional to magnification and aperture diameter.

In this lab, you will become familiar with the use of the microscope (particularly oil immersion microscopy) and will compare the relative size and shape of various microorganisms.

Most bacteria range in size between 0.5-2.0 micrometers (μm)

There are three common shapes of bacteria: the **coccus**, the **bacillus**, and the **spiral**.

Figure 3 represents a typical shape of bacteria.

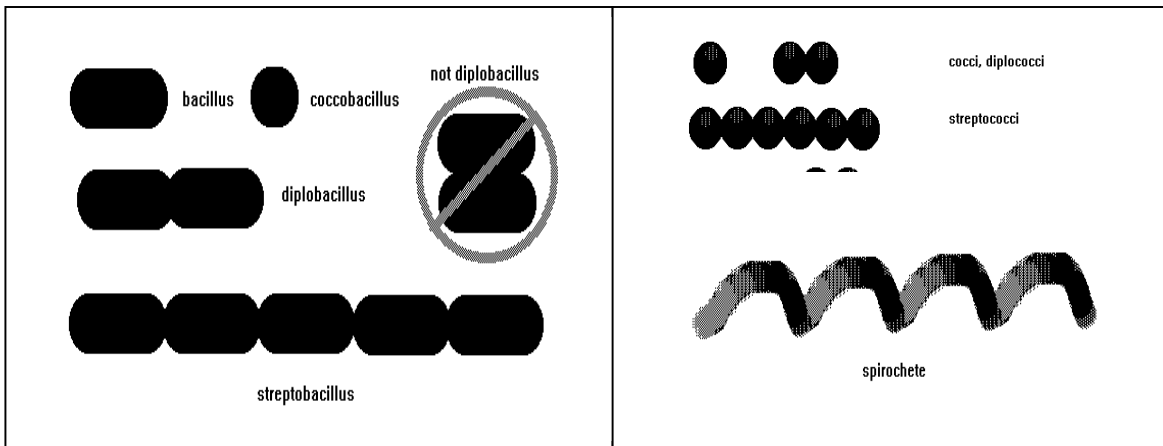


Figure (3): represents a typical shape of bacteria.

Some Concepts to Consider

Resolution: Resolution is the ability to distinguish between two points; The closer the two points, the higher the resolution.

Magnification: Relative enlargement of the specimen, the total magnification of the image is calculated by multiplying the magnification of the ocular by the magnification of the objective.

Depth of focus: thickness of a specimen that can be seen in focus at one time; as magnification increase the depth of focus decrease.

Field of vision: the surface area of view; the area decrease as magnification increase.

Numerical aperture (N.A.): the amount of light reaching the specimen; As N.A. increase the resolution increase.

Materials

Each student/team:

Microscope.
Immersion oil.

Lab supplies:

Prepared stained slides of bacteria.
Selected other prepared slides.

Procedure

1. Obtain a prepared slide of mixed bacteria. Mount the slide onto the stage of the microscope.
2. Start with the lowest power objective in place. Using the course adjustment knob, move the objective lens to its lowest point. Look through the ocular and focus upward with the coarse adjustment until an image comes into view. Use the fine adjustment to obtain maximum clarity. **From this point on, do not use the coarse adjustment; doing so can result in damage to the lens, slide or both.** Adjust the iris to allow enough light for maximum visibility and contrast. Usually, this will be about half the maximum iris opening. Too much light can wash out the details of the image.
3. Move the slide to a point of interest. Move the next objective lens into place and adjust the fine focusing knob, and adjust the iris as necessary. Repeat this step with the highest power, non-oil lens.
4. Note that as the power of the objective lens increases, the distance between the objective and the specimen (working distance) decreases. Also, as magnification increases, the **field of view** (visible area) and **depth of field/focus** (visible thickness) decrease. Moving the fine adjustment up and down allows viewing of other areas along the depth of thickness of the specimen (Figure 8).
5. To use the oil-immersion lens, move the turret halfway between the high-power air (non-oil) lens and the oil lens. Place a drop of immersion oil directly on the slide. Move the oil-immersion lens into place and adjust the fine focusing knob. Adjust the iris as necessary. Make sure that the immersion oil does not get on the air lenses. Make note of the differences and similarities between the organisms.

After using the oil lens for a specimen, wipe the lens with a piece of lens paper. **Do not uses anything but lens paper to clean microscope lenses.** Usually, lens-cleaning fluids are not necessary unless the lens is exceptionally dirty.

Operation of compound microscope

- Clean your lenses with lens paper.
- Set your microscope on the scanning or red lens.
- Focus using the coarse adjustment.
- Change to low power, yellow. Find a portion of the cells are spread apart.
- Switch to high power. Only use the fine adjustment knob.
- When you believe that you have completed this process continue below remember to clean your microscope when you are done and store with the scanning lens in place.

Oil Immersion

- Repeat focus for the bacteria slide.
- Make sure that your focus is perfect for high power.
- Switch the objective to half way between the high and the oil (white).
- Place a drop of oil on the slide.
- Turn oil objective lens into the oil.
- Check your image and only use fine to adjust.

Exercise 2: Bacterial Stains

In our laboratory, bacterial morphology (form and structure) may be examined in two ways:

1. By observing living unstained organisms (wet mount).
2. By observing killed stained organisms.

Besides being very small, bacteria are also almost completely transparent, colorless and featureless in their natural states. However, staining can make the structures of bacteria more pronounced.

A stain (or dye) usually consists of a **chromogen** and an **auxochrome**. Reaction of a benzene derivative with a coloring agent (or **chromophore**) forms a chromogen. The auxochrome imparts a positive or negative charge to the chromogen, thus ionizing it. The ionized stain is capable of binding to cell structures with opposite charges.

Basic stains are cationic; when ionized, the chromogen exhibits a positive charge. Basic stains bind to negatively charged cell structures like nucleic acids. Methylene blue, crystal violet and carbolfuchsin are common basic stains.

Acidic stains are anionic; when ionized, the chromogen exhibits a negative charge. Acidic stains bind to positively charged cell structures like proteins. Picric acid, eosin and nigrosin are common acidic stains.

Positive stains: Dye binds to the specimen.

Negative stains: Dye does not bind to the specimen, but rather around the specimen.

There are three type of staining in Microbiological lab.

1. Simple stain.
2. Differential Stain: (Gram stain , Acid fast Stain)
3. Special stain: (Capsular stain, Endospore stain, Flagellar stain).

Exercise 3.1: Simple Stains

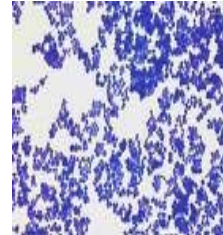
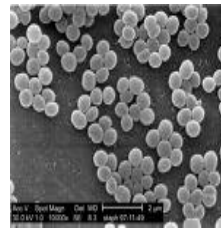
In this exercise, we will use simple stains to show the general structures of some bacteria. Usually, a single basic stain is used in the procedure. Simple stains do not usually provide any data for identification of the bacterium; they simply make the bacterium easier to see.

- To observe basic external structures of cell with bright field scope (cellular morphology).

Materials

Each student/team:

Microscope
Glass slides
Carbolfuchsin
Nigrosin
Methylene blue



Lab supplies:

Nutrient broth cultures of *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus epidermidis* (all 24- to 48-hour).

Procedure

1. Obtain broth cultures of the bacteria listed above.
2. Using an inoculating loop, remove a loopful of suspension from one of the tubes.
Remember to use sterile technique.
3. Smear the bacteria across the center of the slide with the loop. If the bacterial suspension is very thick, add a drop of water and mix the bacteria and the water on the slide.
4. Allow the smear to completely air dry.
5. Heat-fix the smear by quickly passing the slide through a Bunsen burner flame three times. This causes partial melting of the cell walls and membranes of the bacteria, and makes them stick to the slide. Do not overheat the slide as this will destroy the bacteria.
6. Cover the smear with a few drops of one of the stains. Allow the stain to remain for the following periods of time:
 - Carbolfuchsin- 15-30 seconds.
 - Methylene blue- 1-2 minutes.
 - Nigrosin- 20-60 seconds.
7. Gently rinse the slide by holding its surface parallel to a gently flowing stream of water.
8. Gently blot the excess water from the slide with bibulous paper. Do not wipe the slide. Allow the slide to air dry.
9. Observe the slide under the microscope with air and oil lenses. **A cover slip is not required.** Repeat this process with the other bacteria and stains. Note the differences between the various types of stains and their appearances.

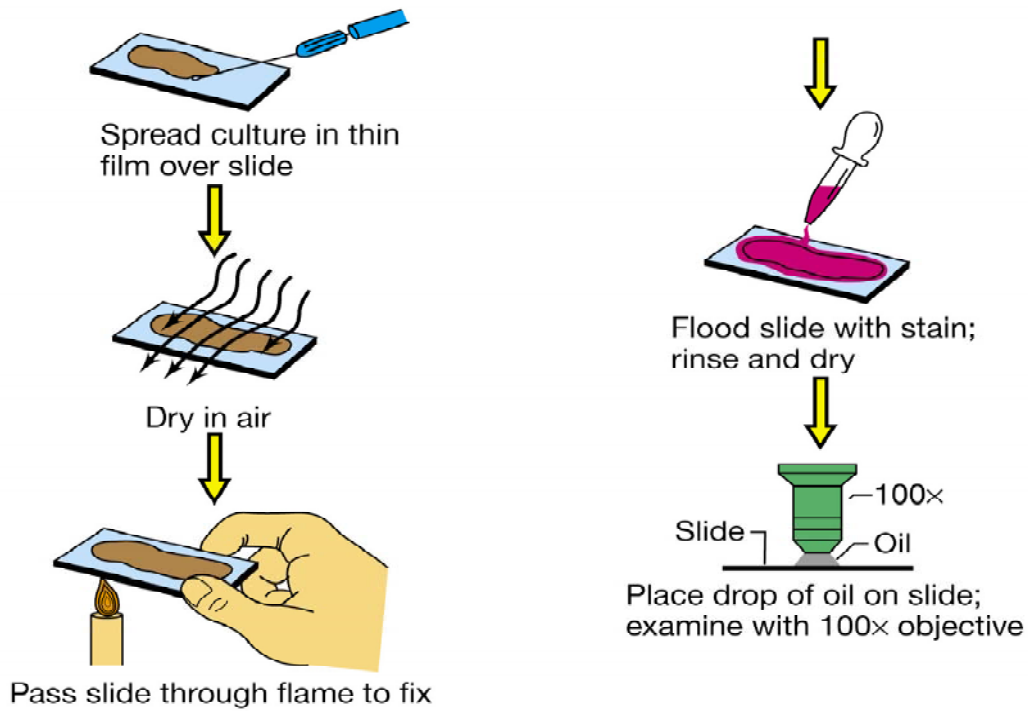


Figure (4): Steps for simple staining technique.

Answer the following questions:

1. What are the uses of simple stain?
2. What is the purpose of normal saline?
3. What is the purpose of fixation?
4. List down cationic stains?

Laboratory Report

Date: Section: Group:

Name: ID:

Lab Title:

Objective of the Lab

Results

Discussion of results

Exercise 2.2: A. Gram stains.

Introduction

In the previous, we examined bacteria with the aid of simple stains. In this experiment, we will use a differential staining method called the Gram stain (named after its inventor). The Gram stain differentiates bacteria into two broad groups. Gram positive bacteria have thick cell walls. Gram negative bacteria have thinner cell walls, but also have an outer cell membrane (part of the capsule) that covers the cell wall. The Gram stain is the most common differential staining procedure. Almost all bacteria are described by their Gram stain characteristics.

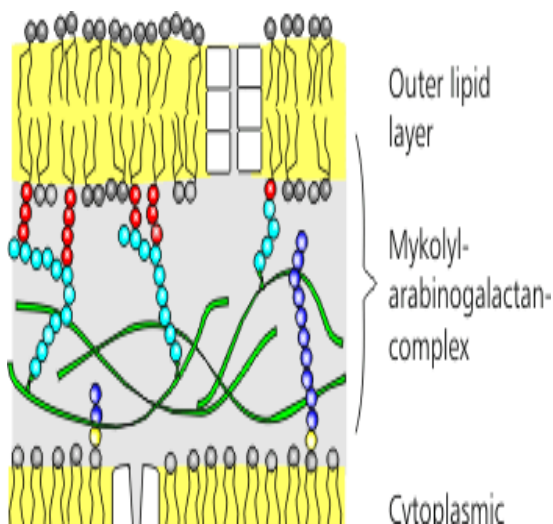


Figure (5): Gram Negative cell wall

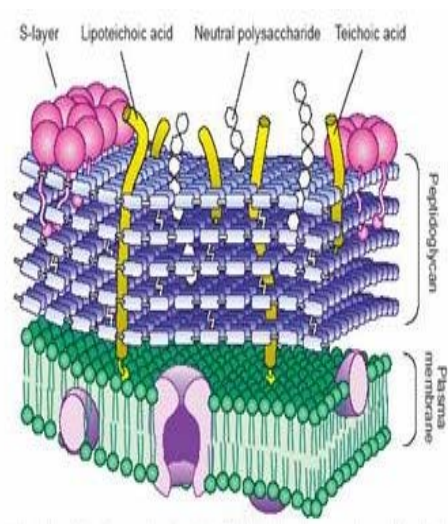


Figure (10): Gram positive cell wall

The Gram stain, like most differential staining procedures, has at least three components: a **primary stain**, a **mordant** and/or **selective treatment**, and a **counterstain**.

The primary stain colors the target cells or cell components in question. Here, the primary stain is crystal violet, and the target cells are the thick-walled bacterial cells. The mordant reacts with the primary stain and the target cells so that the target cells retain the stain.

In the Gram stain, a solution of iodine and potassium iodide (collectively called Gram's iodine) is the mordant.

A selective treatment is an additional step that causes the target cells to retain the primary stain while removing the primary stain from the non-target cells. A 95% ethanol rinse is used in the Gram stain to remove excess crystal violet.

General Microbiology Manual

The counterstain is a contrasting stain, which colors everything that wasn't colored by the primary stain. Safranin is usually used to counterstain in the Gram stain procedure.

Materials

Each student/team:

Crystal violet stain. (purple)
Safranin stain. (red)
Gram's Iodine.
95% denatured ethanol.
Glass slides.

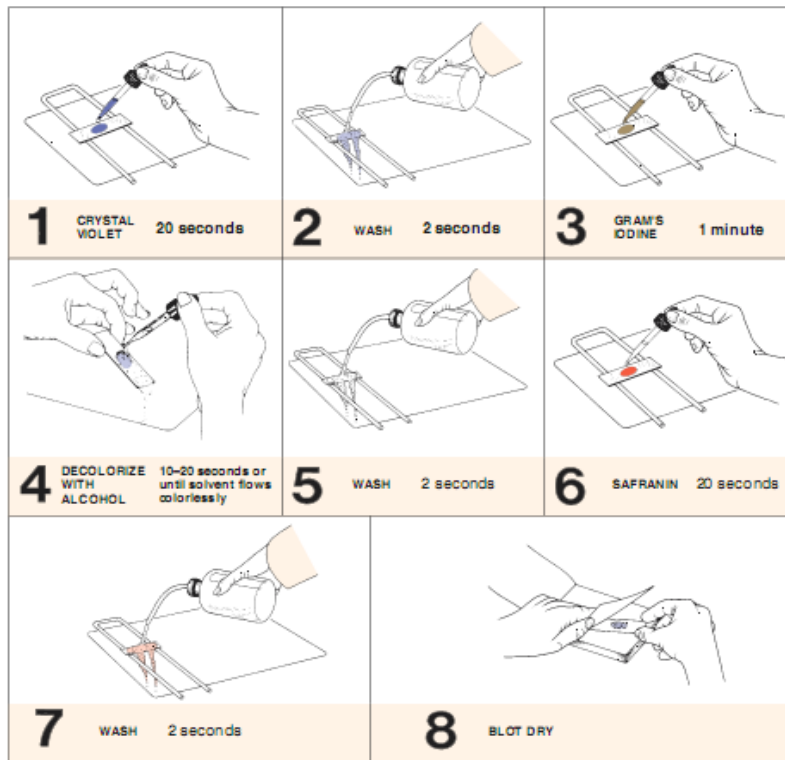


Lab supplies:

Nutrient broth cultures of *Bacillus cereus* and *Escherichia coli* (both 24- to 48-hour).
Broth cultures from Exercise 4.

Procedure

1. Spread a loopful of each culture onto separate glass slides (dilute very heavy suspensions with a loopful of water). Allow the slides to air dry.
2. Heat-fix each slide. Be careful not to overheat the slides.
3. Cover the bacteria with a few drops of crystal violet. Allow it to set for 30-60 seconds.
4. Gently rinse the slides with water.
5. Cover the bacteria with a few drops of Gram's iodine. Allow it to set for 60 seconds.
6. Gently rinse the slides with water.
7. Rinse the slides with 95% ethanol, drop by drop, just until the alcohol rinses clear (decolorization). Be careful not to over-decolorize.
8. Cover the bacteria with a few drops of safranin. Allow it to set for 30 seconds.
9. Gently rinse the slides with water. Blot (not wipe) excess water with tissue paper. Allow the slide to air dry.
10. Observe the slides under oil immersion. Gram positive cells are dark purple, while Gram negative cells are pink. Gram stain characteristics cannot be determined with air lenses. Some bacteria may appear to be Gram positive with air lenses when they are actually Gram negative.



REAGENT	GRAM-POS.	GRAM-NEG.
NONE (Heat-fixed Cells)		
CRYSTAL VIOLET (20 seconds)		
GRAM'S IODINE (1 minute)		
ETHYL ALCOHOL (10-20 seconds)		
SAFRANIN (20 seconds)		

Figure (6): Steps for Gram staining technique.

General Microbiology Manual

Some error during gram stain

- Never ever used old culture during gram stain.
- Never ever used sample for patient take antibiotic.

Time of decolorization

- Over: G (+) change to G (-).
- Low: G (-) change to G (+).

Time of fixation

- Over: G (+) change to G (-).
- Low: No samples remain on the slide after wash.

Answer the following questions:

1. Mention the different between G^+ and G^- ?
2. Why a Gram negative bacterium doesn't keep the stain?
3. Mention the error happened during Gram stain?
4. List 2 reasons why a bacterium that should be gram positive might turn out gram negative?
5. Which of the steps in the gram stain is the **MOST CRITICAL**, and why?
6. Do not heat the slide to speed drying why?
7. Can differentiate species based on the Gram stain results alone?

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Exercise 2.2: B. Acid - fast stain.

Introduction

The acid-fast stain is another differential staining method. In this case, the target cells are usually members of the genus *Mycobacterium*. The cell walls of these bacteria contain an unusually high concentration of waxy lipids, thus making conventional simple stains and Gram stains useless.

The genus *Mycobacterium* contains two important human pathogens, *M. tuberculosis* and *M. leprae*, which cause tuberculosis and leprosy, respectively.

Carbolfuchsin, a phenolic stain, is the primary stain in the acid-fast test. It is soluble in the lipids of the mycobacterial cell wall.

Heating the specimen, or adding a wetting agent such as Tergitol, increases the penetration of the carbolfuchsin. Both procedures are described later in methods section of this experiment.

Following application of the carbolfuchsin, the specimen is cooled and decolorized with a solution of 3% hydrochloric acid and 95% ethanol (**acid-alcohol**).

Since carbolfuchsin is more soluble in waxy cell lipids than in acid-alcohol, the acid-alcohol removes the carbolfuchsin from non-acid-fast organisms, but not from acid-fast organisms. Following decolorization, the sample is counterstained with methylene blue.

Materials

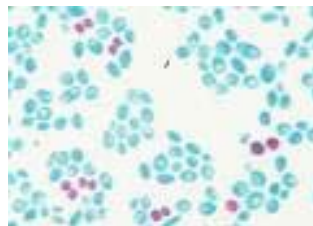
Each student/team:

Carbolfuchsin stain with Tergitol.

Methylene blue stain.

Acid-alcohol.

Glass slides.



Lab supplies:

Nutrient broth cultures of *Mycobacterium smegmatis* and *Escherichia coli* (both 48- to 72-hour).

Procedure

1. Prepare a smear of each organism and a combined smear of both organisms on separate glass slides. When making the combined smear, be careful not to cross-contaminate the stock cultures.
2. Allow the slides to air dry, and then heat fix the organisms.
3. Apply enough of carbolfuchsin with Tergitol to cover the bacteria. Allow it to set for five minutes.
4. (Alternate) If Tergitol is not available, apply enough carbolfuchsin to cover the bacteria. Place the slide on a pre-warmed hot plate set on low for five minutes. **Do not allow the stain to evaporate.** Add additional stain, if necessary. Remove the slide and allow it to cool.
5. Rinse the slide with acid-alcohol, drop by drop, just until the alcohol runs clear.
6. Gently rinse the slide with water.
7. Apply enough methylene blue to cover the bacteria. Allow it to set for two minutes.
8. Gently rinse the slide with water.
9. Blot (don't wipe) the slide dry with bibulous paper. Allow the slide to air dry.
10. Examine the slide under oil immersion. Positive organisms will appear pink or red; negative organisms will appear blue.

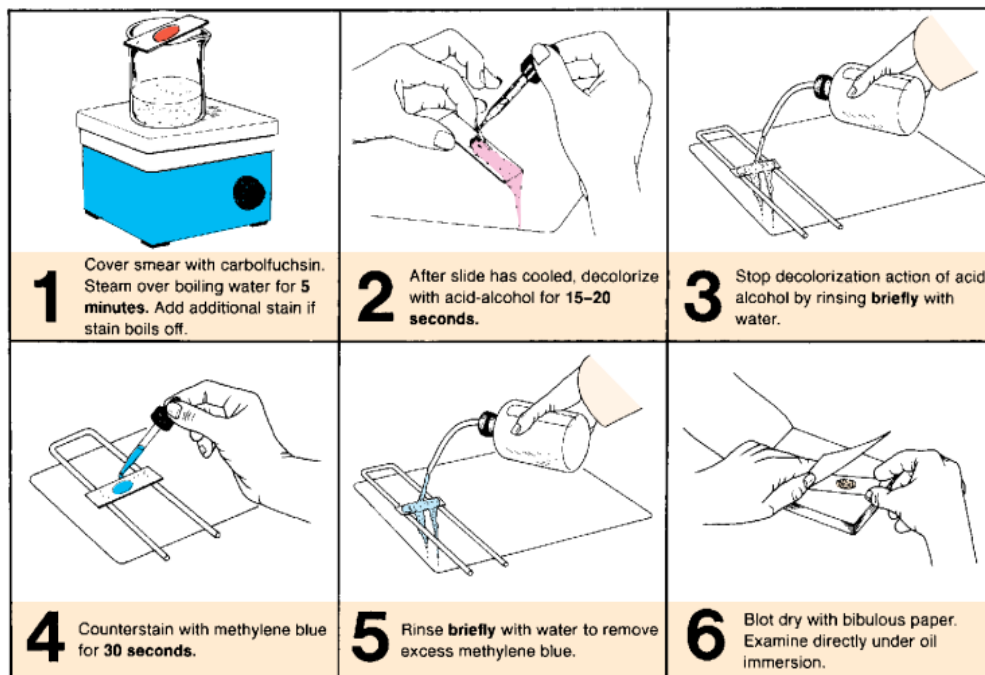


Figure (7): Steps for Acid fast staining technique.

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Answer the following questions:

1. What is chemically unique about the *Mycobacterium* genus that causes it to be acid-fast?

Exercise 2.3: A. The spore stain (Schaeffer-Fulton method)

Introduction

Many species of bacteria can exist in two very different states. In favorable environments, they exist as metabolically active **vegetative cells**. When the environment becomes unfavorable, these cells undergo the process of **sporogenesis**, and form intracellular **endospores**. When the vegetative cell degenerates and dies, the endospore is released as a **spore**. When conditions become favorable, the spore **germinates** to become a vegetative cell again.

- The **endospore** is highly resistant differentiated bacterial cells that are highly resistant to heat, boiling and drying out and are difficult to destroy, Stable for years.
- Do not confuse bacterial spores with fungal spores, which are very different structures. Several thick spore coats surround bacterial spores, making the spores very resistant to heat, cold, desiccation, radiation and a variety of chemical agents (including many microbiological stains). Special staining procedures must be used to visualize spores with the compound microscope.

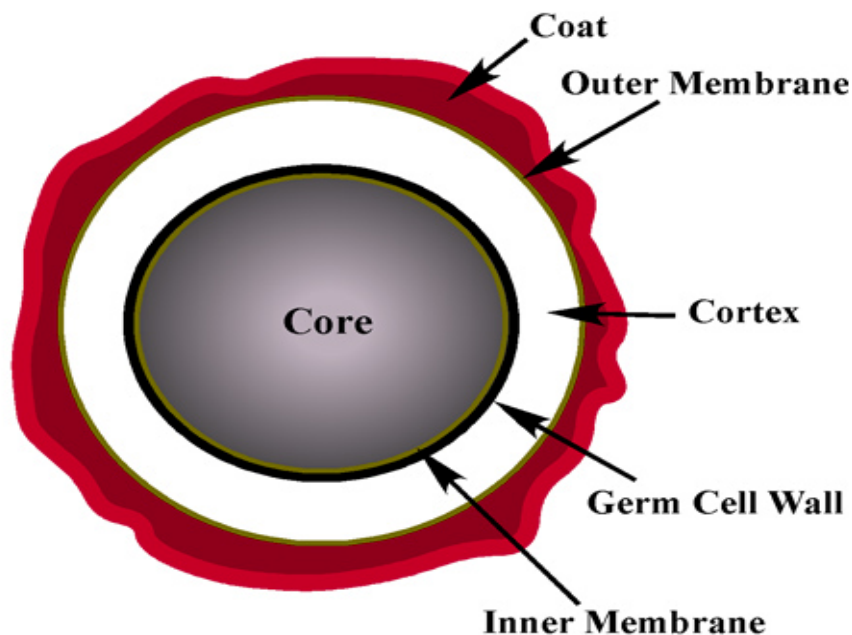


Figure (8): Endospore structure..

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Malachite green is the primary stain. In addition, the stain must be heated to penetrate the spore coat. The bacteria are decolorized with water. Malachite green is soluble in water unless it is bound to the spore coats. Safranin is the counterstain. When describing spore-forming bacteria, the location of the endospore is usually stated as **central**, **terminal**, or **subterminal** (Figure 11).

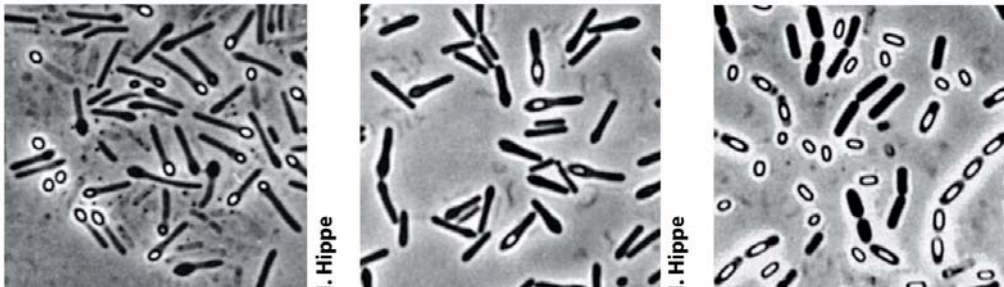


Figure (9): Location of the endospore

Materials

Each student/team:

Malachite green stain.

Safranin stain.

Glass slides.

Lab supplies:

Nutrient broth cultures of *Bacillus subtilis* (or *Clostridium tetani*) and *Escherichia coli* (24 to 72-hour).

Procedure

1. Make smears of each organism on separate slides.
2. Allow the slides to air dry, and then heat fix.
3. Apply a few drops of malachite green to the bacteria. Place the slides directly on a pre-warmed hot plate set on low for 2-3 minutes. **Do not allow the stain to evaporate.** Apply additional stain, if necessary.
4. Remove the slides from the hot plate and allow them to cool.
5. Gently rinse the slides with water.
6. Apply enough safranin to the slides to cover the bacteria. Allow it to set for 30 seconds.
7. Gently rinse the slides with water.
8. Blot (don't wipe) the slides with bibulous paper. Allow the slides to air dry.
9. Examine the slides under oil immersion. The bacteria should appear pink; the spores should appear green.

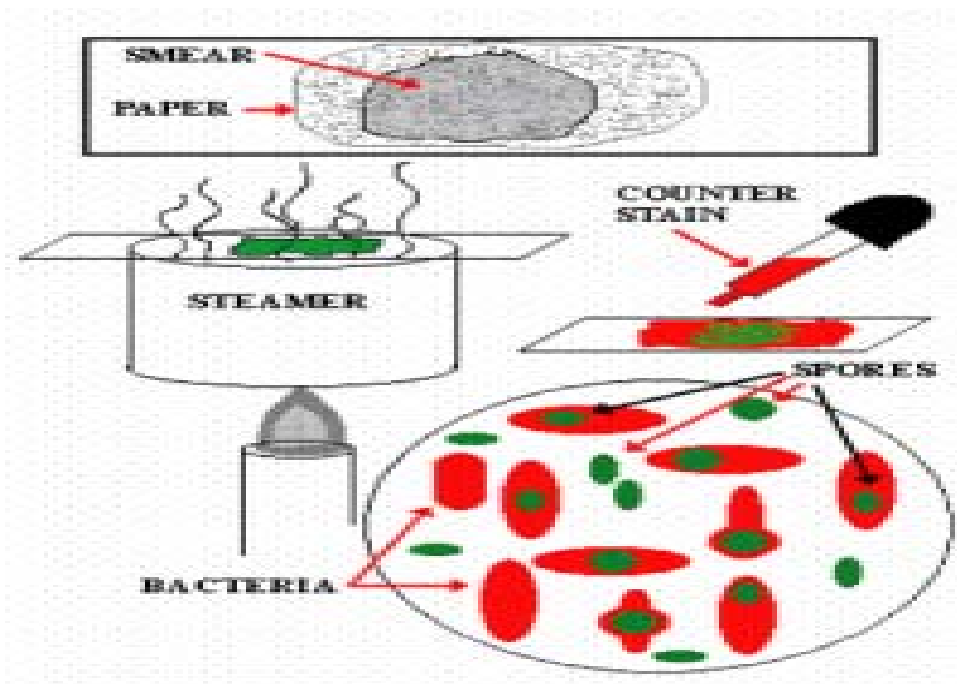


Figure (10): Steps for spore staining technique.

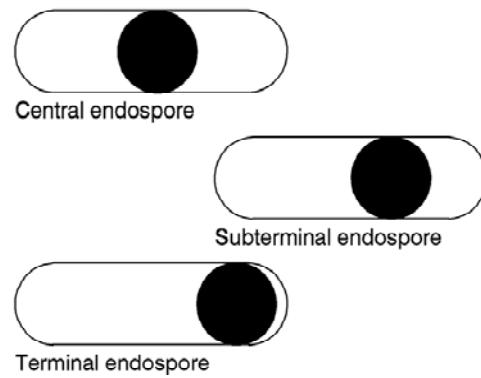


Figure (11): Descriptions of endospore locations within bacterial cells

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Answer the following questions:

1. Defined the endospore?
2. Mention the causes of endospore resistant?
3. What is the purpose of the steam in this stain?
4. What the function of endospore?
5. What is the purpose of the steam in this stain?
6. Why you did not have use a decolorizer in this stain?

Exercise 2.2: B. NEGATIVE STAIN (CAPSULE)

Introduction

When a stain, such as an acid dye, cannot penetrate the outer layers of a microbe, the cell will appear transparent on a colored background. This stain is called a negative or background stain. It is performed by mixing the dye with a suspension of bacteria on a slide and spreading the mixture into a thin layer for viewing.

Capsules are structures composed of carbohydrate or glycoprotein that lay outside of an organism's cell wall and thus are in direct contact with the environment. Many bacteria produce capsules under the right conditions.

Functions of a capsule

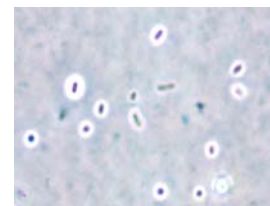
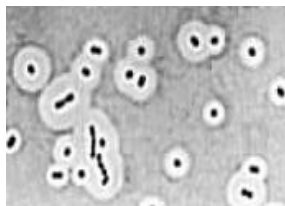
1. Protect the cell from desiccation (drying)
2. Protect the cell from phagocytes (being engulfed by white blood cells)
3. Provide a food reserve when certain organic compounds are in excess.
4. A virulence determinant of pathogenic microbes
5. They serve as binding or adhesion agents for sticking cells together and/or to a surface such as a rock in flowing stream or a tooth

Capsules are not readily stained and therefore are visualized by negative stain techniques. The organisms are prepared as a smear in the presence of an acid dye and allowed to air dry because heat will cause the capsule to shrink. Usually the negative stain (which colors the background) is followed by a simple stain to color the bacterium. The capsule appears as a colorless layer between the bacterium and the background.

Materials

Each student/team:

1. India Ink
2. Methylene blue
3. Microscope slides



Lab supplies:

Bacteria with capsules: *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Pseudomonas putida*.

Procedure

1. Use an inoculating needle to suspend the organism in a drop of India Ink at one end of the slide.
2. Place the short end of a clean microscope slide into the suspension and spread the mixture across the slide to form a thin layer.
3. Allow to air dry. Do not heat fix.
4. Cover the smear with methylene blue for 2-3 minutes. Rinse gently with water and allow to air dry.
5. Examine with oil immersion.
6. Diagram the appearance of the organism.

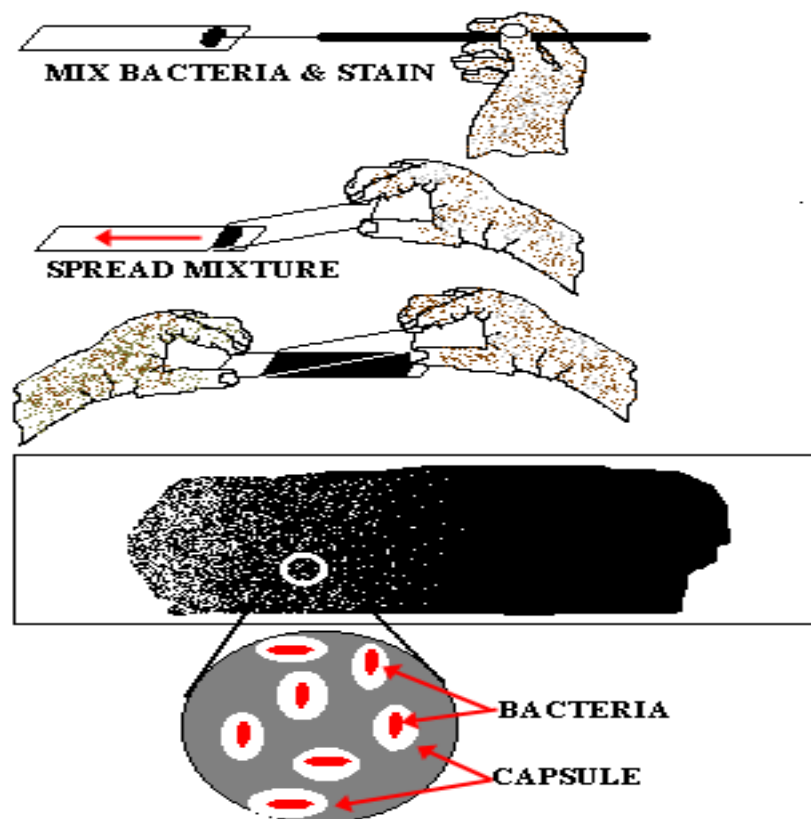


Figure (12): Steps for Negative staining technique.

Interpretation

Capsules appear as clear zones (halos) around the refractile organism.

NOTES:

1. Older cultures are more likely to exhibit capsule production.
2. When performing a capsule stain on your unknown, be sure the culture you take your sample from is at least five days old.
3. This stain is used for direct microscopic examination of capsules of microorganisms.
4. The India ink gives a semi opaque background against which the clear capsules can be easily visualized.

Answer the following questions:

1. Why it is called the negative stain?
2. What the function of capsule?
3. Why we avoid heat fix?
4. Why does the capsule **NOT** take in any dye?

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Exercise 3: A. sterile technique

For the most part, bacterial physiology only can be studied in pure cultures. The best way to obtain a pure culture is to start with a single bacterial cell. This cell then divides quickly, and may produce millions of cells within 24 hours. A single unwanted contaminant cell can do the same thing in an otherwise pure culture, making the culture useless. For this reason, and to protect against disease, strict sterile procedures must be used.

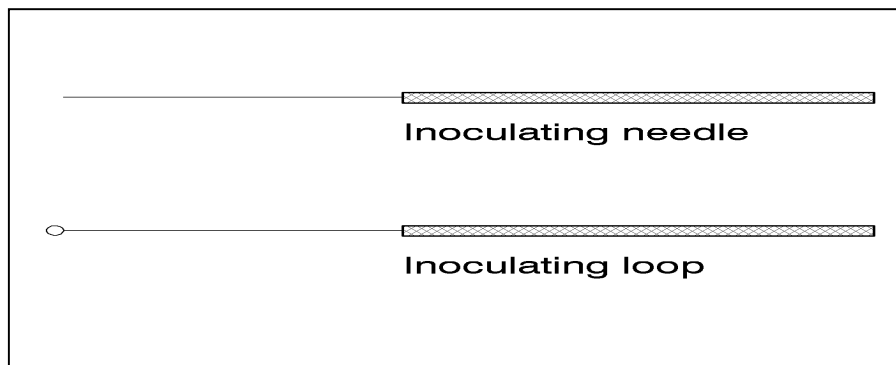


Figure (13): Two common inoculation instruments. Glass instruments are used as well.

The most commonly used device for moving bacteria is the inoculating loop. This is simply a piece of nichrome (an alloy of nickel and chromium) or platinum wire with a loop at one end and a handle at the other. A similar instrument is the inoculating needle, essentially the same as the loop, but with just a straight wire. Sterilize both instruments by holding the wire portions in a flame until they glow red. The instruments should be allowed to cool in the air for 10-20 seconds before using them.

NOTES:

- **Do not blow on the instruments to cool them.**
- **Do not touch the instruments to agar to cool them**
- **Do not lay the loop down once it is sterilized or it may again become contaminated.**

Procedure

1. Flame the loop.
2. Without setting the loop down, open the first culture tube and flame the mouth. Do not set the cap on the bench. The cap should be held in the same hand as the loop.
3. Insert the loop into the culture medium, and then withdraw it.
4. Flame the mouth of the first culture tube again, and replace the cap.
5. Open the second culture tube and flame the mouth. Do not set the cap on the bench. The cap should be held in the same hand as the loop.
6. Insert the loop into the second culture tube and spread the culture suspension (on the loop) **inoculum** into/onto the second culture medium slide.
7. Flame the mouth of the second culture tube, then replace the cap.
8. Flame the loop and set on the bench.

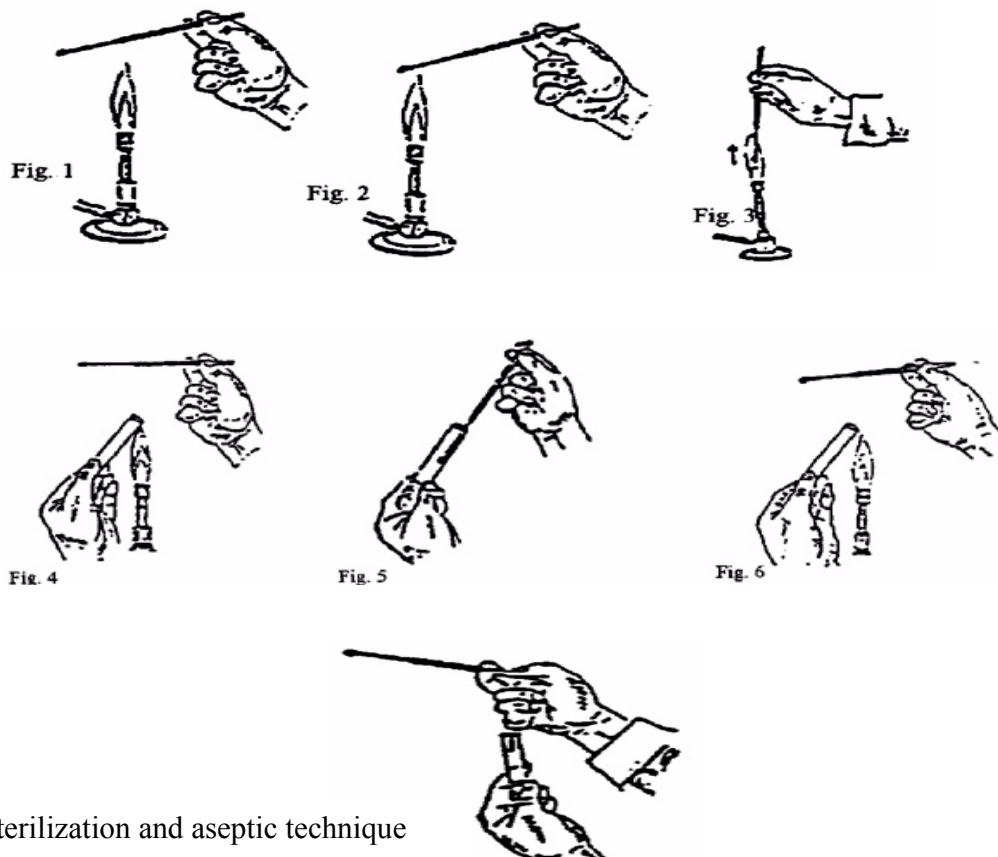


Figure (14): Sterilization and aseptic technique

Important to remember

1. Work on a clear tabletop. Put all unnecessary items away.
2. Wear a lab coat, wash hands before performing any manipulations and after you are through.
3. Disinfect the bench top with an appropriate disinfectant before you begin working and after you are through.
4. Keep all culture closed and tubes upright in a rack until ready for use.
5. Work quickly without disturbances.

❖ Bacteria

1. Are everywhere.
2. On every surface of the body.
3. Including digestive tract.
4. Harmless.
5. Beneficial Pathogenic.
6. Absorb nutrients and release toxins that damage cells and tissues.
7. Bacterial toxins can cause disease even when bacteria are destroyed.
8. Bacteria are Prokaryotes.

Exercise 3: B. Sterile Transfer

Introduction

Often in microbiology, bacteria grown in one medium must be transferred to another in a sterile manner. In this exercise, we will transfer bacteria from a nutrient agar isolation plate to nutrient broth, and nutrient agar plates, deeps and slants.

Nutrient agar plates, slants and deeps contain the same ingredients as nutrient broth, but they also contain agar-agar to make them solid. Agar-agar--usually just called agar--is a carbohydrate produced by some algae. It is similar in appearance to gelatin, but it cannot be digested by most organisms. In solution, agar melts at approximately 70°C, and re-solidifies at approximately 45°C. Agar plates are made by pouring molten agar into petri dishes and allowing it to cool. Agar deeps and slants are produced by pouring molten agar into culture tubes and allowing it to cool. Agar deeps are cooled in an upright position, whereas agar slants are cooled at an angle to produce a diagonal surface

Materials

Each student/team:

- 1 Nutrient agar plate.
- 3 Nutrient agar slants.
- 3 Nutrient agar deeps.
- 3 Nutrient broth tubes.

Lab supplies:

Bacteria growing on plates

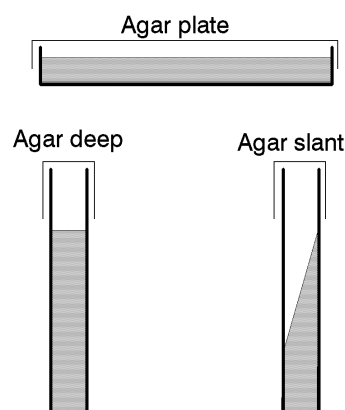


Figure (15): Three common forms of agar media.

Procedure

1. Using a permanent marker, draw three parallel lines on the **bottom** of a nutrient agar plate. Label these 1, 2 and 3. (Note: in almost all instances, petri dishes should be labeled on the bottom. Labels on the lid of the petri dish could be turned so that they no longer represent the locations of samples on the media.)
2. Using the sterilized inoculating loop, touch the loop to one of the isolated colonies from the Exercise 3 plate. Then make a single streak of the bacterium on the agar corresponding to the line you drew on the petri dish. Do not press down so hard as to penetrate the surface of the agar. Also, only open the lid of the dish long enough to make the streak. Never place the lid on the counter top.
3. Repeat step three with two more isolated colonies.
4. **Incubate the plate upside-down** for 24-48 hours at 37°C. Plates are usually placed upside-down to prevent condensation from splattering down onto the bacteria.
5. Obtain three nutrient agar slants. Using an inoculating loop and proper sterile technique, inoculate each of the previous colonies onto a separate slant. Use a zigzag motion to spread the bacteria across the surface of the slant. **Be careful not to penetrate the surface of the agar.**
6. Obtain three nutrient agar deeps. Using an inoculating needle, inoculate each colony into separate deeps by touching the needle to the colony and then stabbing the needle into the agar deep a single time.
7. Obtain three nutrient broth tubes. Using an inoculating loop and proper sterile technique, inoculate each colony by touching the loop to the colony and then stirring the loop inside the broth tube.
8. Incubate the deeps and slants for 48 hours at 37 °C. These transfers may be used again in later labs.

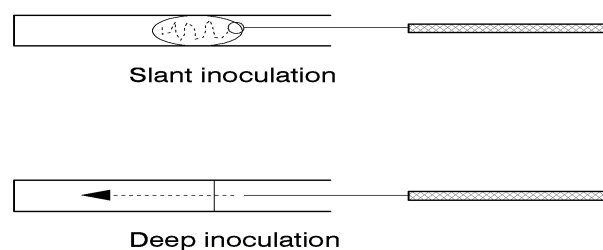


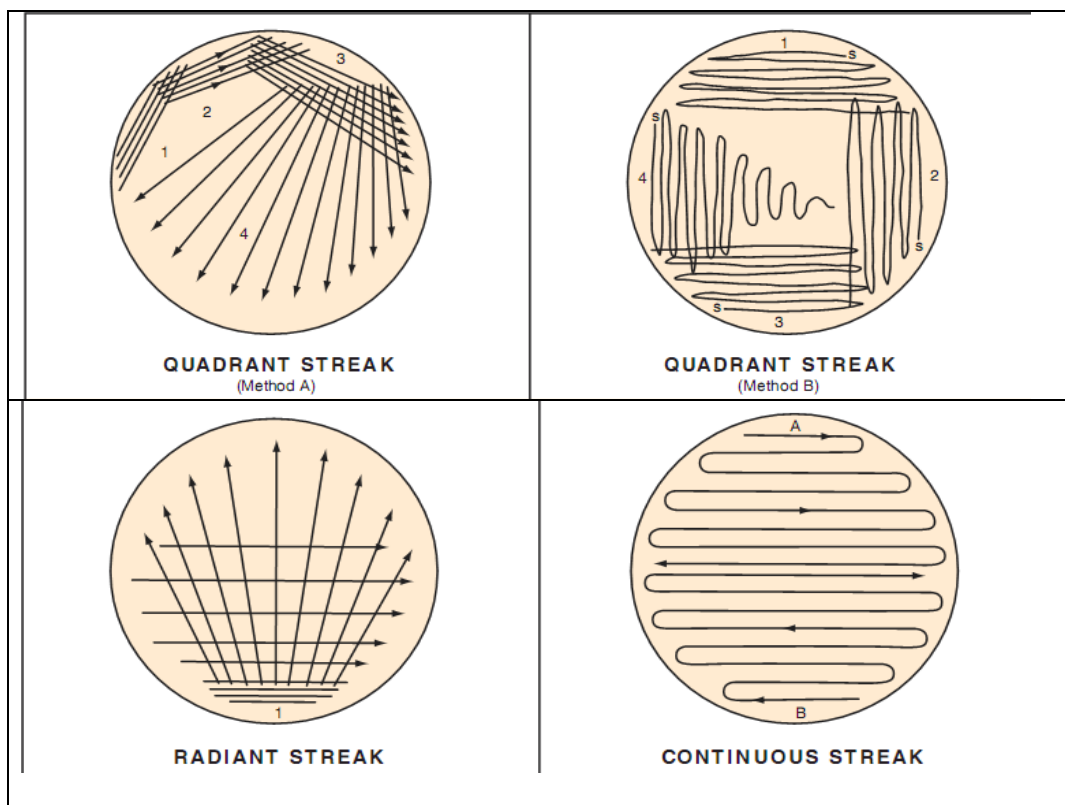
Figure (16): Inoculation of agar slants and deeps

Exercise 3: C. Isolation of pure cultures.

Introduction

Note: this exercise requires three lab periods to complete. The results from this exercise may be used in some of the following exercises.

In previous exercises, we have always started with pure cultures. In nature, bacteria rarely occur as pure cultures. Streak plate methods can separate most mixed cultures. Shows to figure a three-way streak plate. Each streak progressively dilutes the bacteria so that the end of the third streak deposits isolated cells onto the agar. Each cell then divides and grows into a pure colony or **clone**. Once the bacteria have been separated and grown on a streak plate, individual colonies can be transferred to new media and grown as a pure culture.



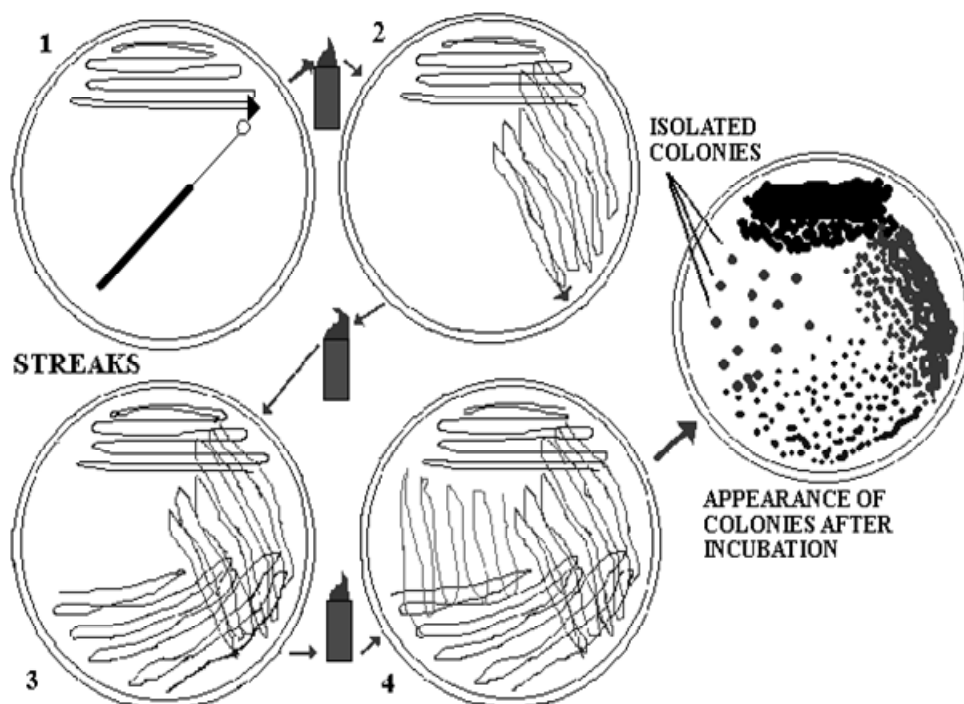
Materials

Each student/team:

- 1 Sterile vial (3-5 mL).
- 1 Sterile rubber band.
- 1 Nutrient agar plate.

Procedure

1. Obtain 1 mL of your own saliva in a sterile vial. Chewing on a sterile rubber band may help to increase saliva production.
2. using proper sterile technique, obtain a loopful of saliva.
3. Streak the saliva on a nutrient agar plate as shown the following figure.
4. Flame the loop and allow it to cool. Make the second streak by first streaking over the end of the first streak, and then continuing the streak without touching the first one.
5. Repeat step four by overlapping the second streak and then finishing the plate.
6. Incubate the plate for 24 to 48 hours at 37°C.



Some Concept:

- **Contaminants:** other microorganisms present in the sample
- **Isolated colois:** a population of millions of cells that are identical and are descendent from a single founder cell
- **Stock Culture:** a culture that already contains cells. It is used a source of cells from which to inoculate new cultures.

Type of media:

1. **Broth tubes:** are tubes containing a liquid medium. A typical nutrient containing broth medium such as Trypticase Soy broth , nutrient broth After incubation, growth may be observed as one or a combination of three forms:
 - **Pellicle:** A mass of organisms is floating on top of the broth.
 - **Turbidity:** The organisms appear as a general cloudiness throughout the broth.
 - **Sediment:** A mass of organisms appears as a deposit at the bottom of the tube.
2. **Slant tubes:** are tubes containing a nutrient medium plus a solidifying agent, agar-agar. The medium has been allowed to solidify at an angle in order to get a flat inoculating surface.
3. **Stab tubes:** (deeps) are tubes of hardened agar medium which are inoculated by "stabbing" the inoculum into the agar.
4. **Agar plates:** are sterile petri plates that are aseptically filled with a melted sterile agar medium and allowed to solidify. Plates are much less confining than slants and stabs and are commonly used in the culturing, separating, and counting of microorganisms.

Answer the following questions:

1. Why plates are incubated upside down?
2. What is the difference between slant and deep tube?
3. What is the purpose of flaming the mouth of the tube?
4. Define the colony, Agar?
5. Why is it essential to have pure cultures for **biochemical tests**?
6. At what temperature does agar solidify?
7. Why do you cross over back the 2nd streak section back into the 1st section and from the 3rd section back across the 2nd section?

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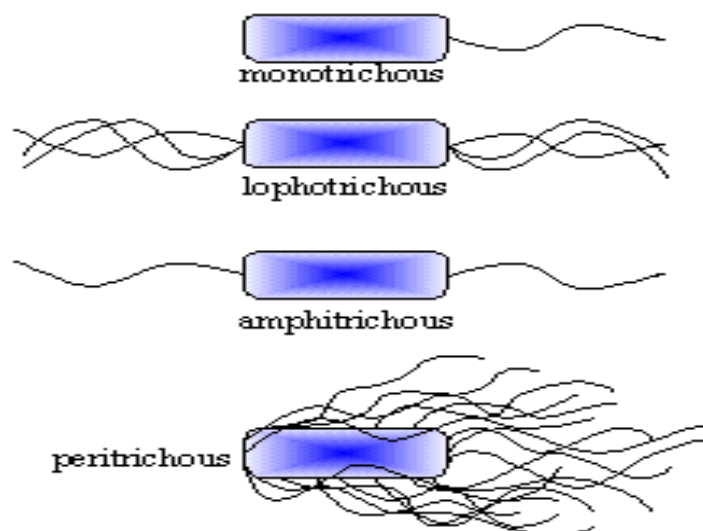
Discussion of results

Exercise 4: Bacterial motility

A large number of bacteria are motile. Most possess one or more flagella on their surface that allow them to swim. The pattern of flagellation is an important feature in identification of motile bacteria. The figure illustrates the commonly observed arrangements of flagella.

Polar flagella occur at one or both ends of the bacterium (*Vibrio cholerae* and some species of *Pseudomonas*). They may be single or in tufts.

Peritrichous flagella are distributed around the surface of the organism (many *Proteus* species). Most motile bacteria move in a straight line for a brief time, then turn and randomly change directions before swimming again.



The straight line movement is called a run and the turn is called a tumble. Runs and tumbles are controlled by the clockwise or counterclockwise rotation of the basal body of the flagellum, the motor that is anchored in the cell membrane. Some bacteria do not tumble, but rather reverse direction when they reverse the rotation of the basal body.

Many flagellated bacteria can move toward useful chemicals and away from harmful ones. This ability to control movement in response to chemical stimuli is termed chemotaxis. Chemotactic bacteria contain receptors in the cell membrane that bind to certain chemicals and cause the basal body to direct either a run or tumble (or forward and reverse directions). When the chemical stimulus is an attractant, such as a rich nutrient source, the basal body is made to rotate so that the bacteria swim in straight lines toward the signal for long periods of time. If the stimulus is a repellent, such as a poison, the basal body reverses direction and causes the bacterium to tumble more often (or reverse direction).

Motility could be detected by:

1. Flagellar stain.
2. Hanging Drop technique.
3. Semi-Solid media Inoculation.

Flagellar Stain

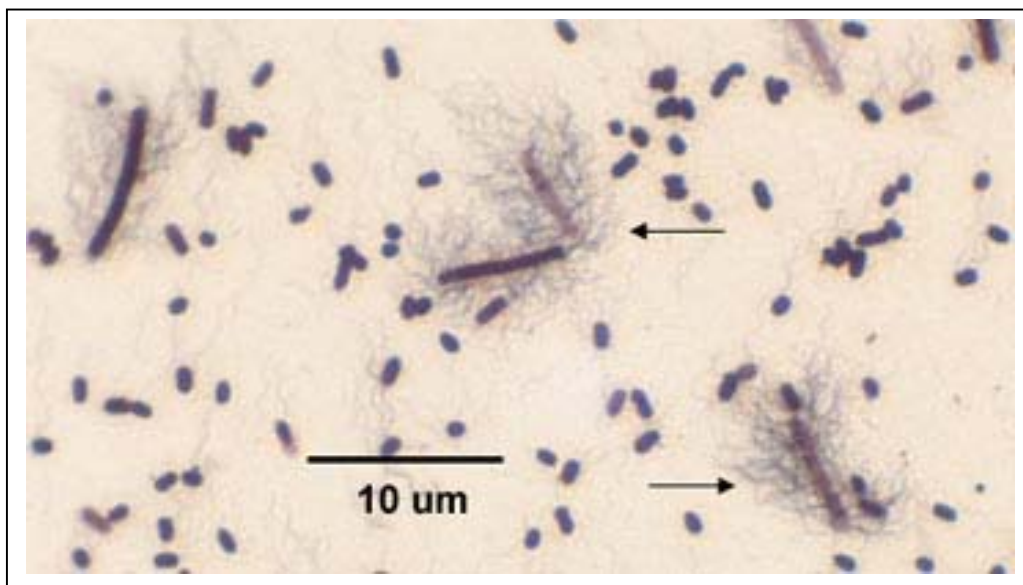
Flagella are too thin to be seen by the ordinary light microscope.

Flagella should be amplified (enlarged). Use a stain that is specifically deposited on Flagella thus increasing diameter.

Some flagellar stains employ rosaniline dyes and a mordant, applied to a bacterial suspension fixed in formalin and spread across a glass slide. The formalin links to, or “fixes,” the flagellar and other surface protein of the cells. The dye and mordant then precipitate around these “fixed” surfaces, enlarging their diameters, and making flagella visible when viewed under the microscope.

Another method, a ferric-tannate mordant and a silver nitrate solution are applied to a bacterial suspension. The resulting dark precipitate that forms on the bacteria and their flagella allows them to be easily visualized under the microscope. This silver-plating technique is also used to stain the very slender spirochetes.

Note: The techniques are somewhat sensitive.



Hanging Drop Technique

This method is commonly used to view living organisms for the rapid determination of motility. The hanging drop is prepared by suspending a fluid sample from a coverslip over a depression well in a specially designed microscope slide. Wet mounts can be used for the same purpose, however, wet mounts tend to dehydrate rapidly. Hanging drops, on the other hand, are sealed within the depression and retain their liquid for longer periods of time. In both methods, the living specimen is unstained. For best results, reduce the amount of light passing through the specimen.

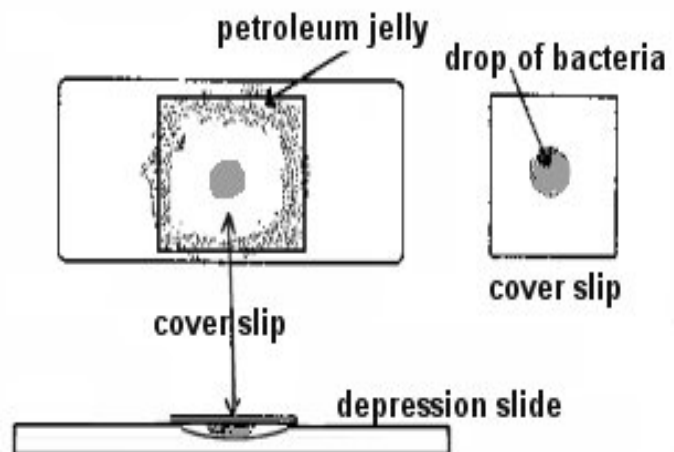
Materials

Each student/team:

Depression slides.
Coverslips (glass).
Petroleum jelly.
Toothpicks.

Lab supplies:

Positive control: *Proteus vulgaris*.
Negative control: *Staph. Epidermidis*.



Procedure

1. Place a drop of the bacterial culture (optimally from a young broth culture) in the middle of a cover slip.
2. Place a thin line of petroleum jelly around the edge of the cover slide.
3. Turn the depression slide upside-down (depressed area facing down) and gently touch the cover slide. The jelly holds the cover slip to the slide and also keeps the suspension from drying out.
4. Now flip the entire microscope slide/cover slip combination over. It should look like the diagram below.

NOTES:

1. You should be able to differentiate true motility from Brownian motility
2. Brownian movement is usually caused by the activity of water molecules. (characterized by back and forth movement)
3. True motility (the bacterial cells runs and tumble).

Motility Agar

Motile bacteria require liquid to move. Thus bacteria can propel themselves in broth or across the surface of a wet agar plate. They will not however move when embedded in 1.5% agar, the minimum concentration found in most agar media. Semisolid agar has a reduced agar concentration (0.4 %) that allows flagellated bacteria to migrate from the site of inoculation. Semisolid media are prepared in tubes and are inoculated through most of their length by stabbing with a needle. Thus after 48 hours of incubation, growth of a motile organism will be observed as a turbid region extending from the stab. Non-motile bacteria will only grow along the stab line.

Materials

Each student/team:

Semisolid nutrient agar in tubes

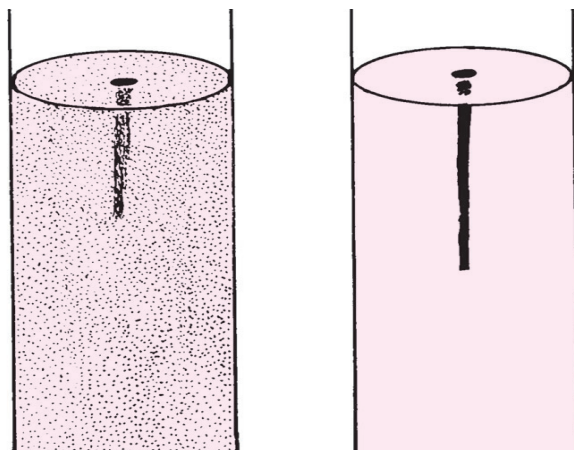
Lab supplies:

Positive control: *Proteus vulgaris*.

Negative control: *Staph. Epidermidis*.

Procedure

1. Using aseptic techniques, inoculate the tube by stabbing with the needle to approximately three-quarters of its depth. Be careful to bring the needle into the center of the medium and not to touch the side of the tube.
2. Incubate at room temperature for 48 hours.
3. Examine for growth.



A

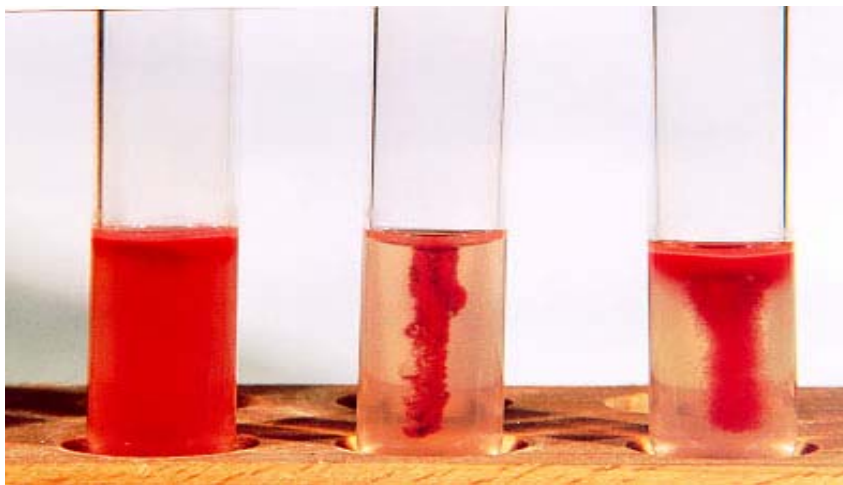
B

Interpretation

(A) Pattern of growth of a motile organism. The entire medium is turbid with the growth of the organism, which has moved away from the stab line.

(B) Pattern of growth of a nonmotile organism. Only the stab line is turbid with growth.

Note: Semi solid media with tetrazolium chloride (color indicator)



From left to right:

+

-

+

Assessing your performance

1. Is there a single narrow stab line the agar?
2. Does the stab go no more than three- quarters the length of the agar?
3. Is the stab line in the center of agar?
4. Is there growth only along the stab line for the negative control?
5. Is there growth spreading from the stab line for the positive control?

Answer the following questions:

1. What advantage might motile bacteria have over non-motile ones?
2. List examples wherein motility test is used in differentiation between similar microorganisms?
3. What the different between solid and semisolid media?

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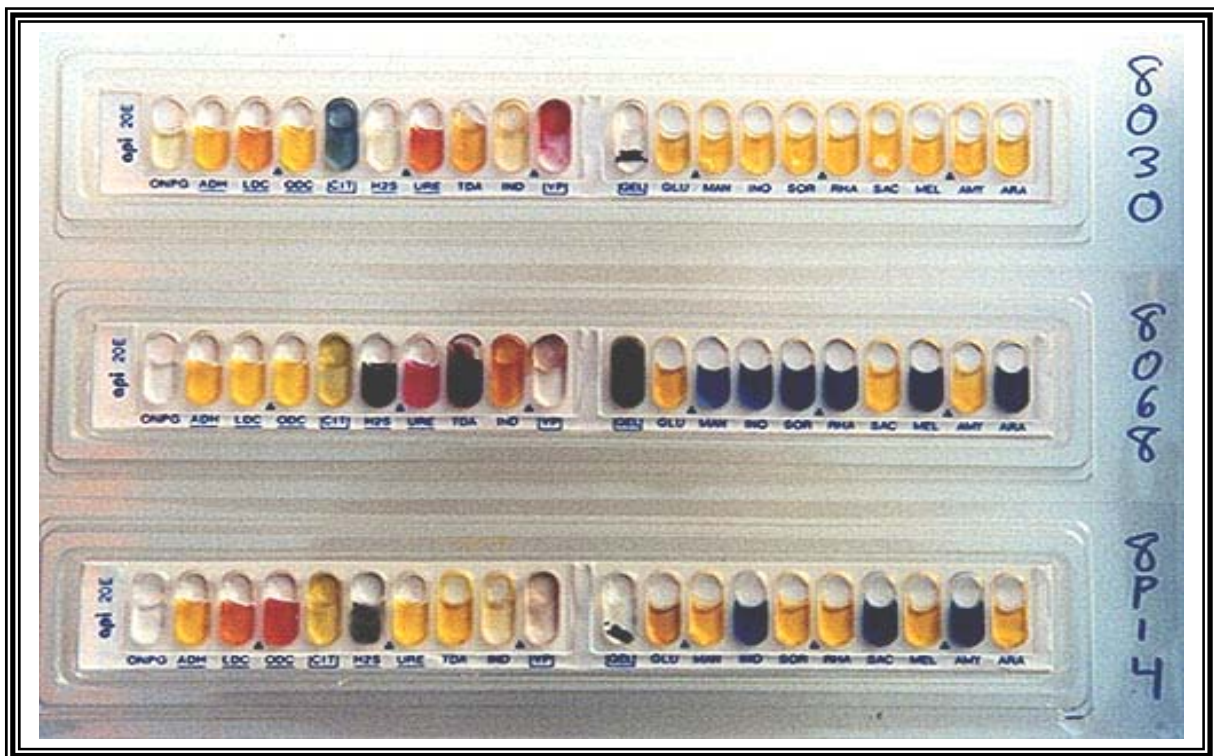
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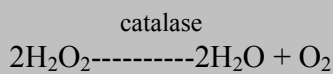
Biochemical tests



Exercise 5: Catalase Production

PRINCIPLE:

Catalase is an enzyme that splits hydrogen peroxide into water and oxygen. Hydrogen peroxide is produced as a byproduct of respiration and is lethal if it accumulates in the cell. All respiring organisms therefore must have some mechanism for detoxification. Catalase is one of the common methods. When hydrogen peroxide is added to a colony of catalase-producing bacteria, it is broken down and the oxygen that is produced can be seen as bubbles.



SIGNIFICANCE:

This test distinguishes **Staphylococci** which is catalase positive from **Streptococci** which is catalase negative. It can also differentiate *Listeria monocytogenes* (positive) from **beta hemolytic streptococci**. Most Neisseria species are catalase positive. It also helps distinguish **Bacillus** species (positive) from **Clostridium** species (mostly negative).

CONTROLS

Positive Control: *E.coli*.

Negative Control: Streptococcus sp.

Method:

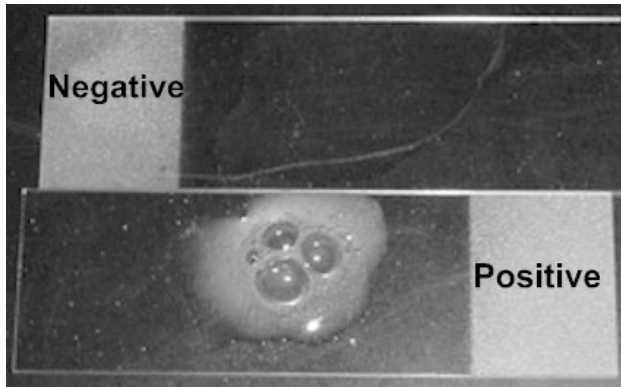
1. TUBE METHOD

1. Inoculate the test organism on agar slant and incubate for 24 hours.
2. Allow 1 mL of 3% hydrogen peroxide to flow over the slant.



B. SLIDE METHOD

1. Add one drop of 3% Hydrogen peroxide on a clean glass slide.
2. Aseptically take a loopful of the test organism and emulsify in the H₂O₂ drop.



❖ **Reading Results:**

- If the organism has catalase, visible bubble production indicates a Positive result.
- If the organism does not have catalase it will not split H₂O₂.

Limitations of the procedure:

- Growth for catalase testing must be from a fresh (18-24 hours) culture. Older colonies may lose their catalase activity, possibly resulting in a false negative result.
- If growth is taken from a blood-containing medium, be careful not to transfer any of the agars since RBCs contain catalase and could result in a false-positive test.

Exercise 6: Coagulase Test

PRINCIPLE:

The presence of a cell surface substance that binds fibrinogen, allows aggregation of microorganisms in plasma containing fibrinogen. This is detected by observation of clumping of microorganisms.

The enzyme coagulase produced by a few of the *Staphylococcus* species, is a key feature of Staph. The enzyme produces coagulation of blood, allowing the organism to wall it is infection off from the host's protective mechanisms rather effectively.

SIGNIFICANCE:

This test is a confirmatory test for the differentiation of the pathogenic *Staphylococcus aureus* from the non-pathogenic *Staphylococcus epidermidis*

Procedure

A. Slide Test:

1. Place a drop of coagulase plasma (rabbit plasma) on a clean, dry glass slide.
2. Place a drop of distilled water or saline next to the drop of plasma as your **negative control**.
3. With a loop, emulsify an amount of the isolated colony being tested in each drop, inoculating the water or saline first. Try to create a smooth suspension.

RESULTS:

- **Positive:** Immediate aggregation visible to the naked eyes,
- **Negative:** No aggregation.

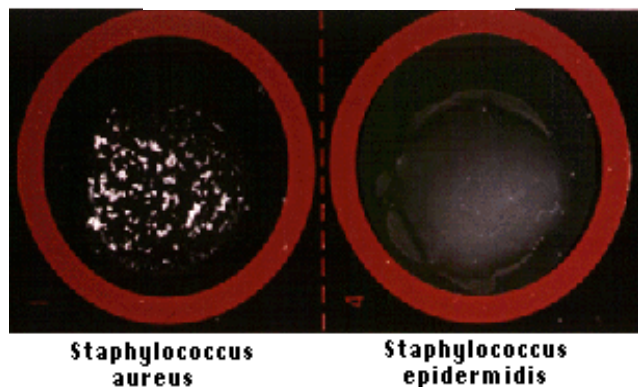


Figure (17): Result of slide method

B. Tube method:

1. Inoculate tube with a 0.5 ml of rabbit plasma with the bacteria inoculum.
2. Place at 37 C and check at 4 hour and after 18 hours by tipping the slide at an angle.
3. Any degree of coagulation is considered a positive test for the free coagulase enzyme.



Figure (18): Result of Tube method

Limitations of the procedure:

- The slide test should be read very quickly, as false positives can occur.
- The slide test should not be performed with organisms taken from high-salt media such as Mannitol Salt Agar, as the salt content can create false positives.
- The tube test is more reliable than the slide test.

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Answer the following questions:

1. Draw a diagram for the differentiation among G + bacilli?
2. Draw a diagram for the differentiation between β -hemolytic *Streptococci* and *Listeria monocytogenes*?
3. What is the nature of the bubbles seen in a + reaction?
4. Name the reagent used for this test and its concentration
5. How do aerobic organisms that cannot produce catalase detoxify hydrogen peroxide?
6. What is the gaseous breakdown product of hydrogen peroxide?
7. What is the substrate and products of the catalase reaction?
8. Draw a diagram to differentiation between *Neisseria* and *Acinetobacter*?
9. What the purpose of coagulase test?
10. Draw a diagram to separation pathogenic from the non-pathogenic *Staphylococcus*

Exercise 7: Amylase Production

Amylase activity is demonstrated using starch agar, a medium containing starch as the carbohydrate source. Starch is a **polysaccharide**--a long chain of glucose molecules linked by **glycosidic bonds**. Amylase breaks the glycosidic bonds (α -1,6-glucosidase), producing small **oligosaccharides** and free glucose.

PRINCIPLE:

Amylase production is tested by growing organisms on starch agar. After incubation, the starch agar is flooded with Gram's iodine. The iodine reacts with starch to produce a dark purple or brown color. If amylase is present, clear zones will appear in the starch agar where hydrolysis has occurred.

CONTROLS:

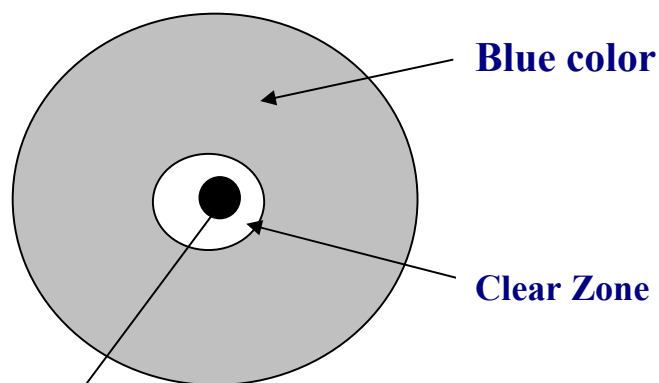
Positive Control: *Bacillus subtilis*

Negative Control: *E.coli*



Procedure

1. Streak each organism across a small portion of the agar surface.
2. Incubate at 37 °C for 48 hours.
3. Cover the surface with iodine. Rotate to distribute the iodine into a thin layer. Do not flood the plate. Record your results.
4. Iodine will turn blue when it reacts with starch. A clear zone will be seen where starch has been digested.



Test Organism

Figure (19): Result of Amylase Production

Exercise 8: Gelatin Liquefaction

PRINCIPLE:

Gelatin is liquefied by the virtue of the production of an enzyme called gelatinase. Gelatin is an incomplete protein; it lacks tryptophan. However, the ability to hydrolyze gelatin is a well established bacterial classification characteristic. Gelatin is produced by the hydrolysis of **collagen**, a large protein found in the connective tissues of animals. Normally, gelatin produces a gel in water below 25°C. When gelatin is hydrolyzed, it loses its ability to form a gel. In this experiment, you will use nutrient gelatin in place of nutrient agar. If a given microbe produces gelatinase, the nutrient gelatin will liquefy.

SIGNIFICANCE:

This test is used to differentiate Gram-negative species. *Serratia*, *Pseudomonas*, and *Vibrio* are positive for this test. The practicality of this test was not appreciated until, the development of the rapid procedures.

Procedure

1. Inoculate gelatin deeps using bacteriological needle for up to 30 days.
2. To determine whether liquefaction has occurred, place the tube in the refrigerator for 30 minutes. Remove and check the tube for liquefaction. If negative, continue incubation until liquefaction occurs.

RESULTS:

Positive: Strong: Liquefaction occurs within 3 days.

Positive: weak: Liquefaction occurs in 4-30 days.

Negative: No liquefaction after 30 days.

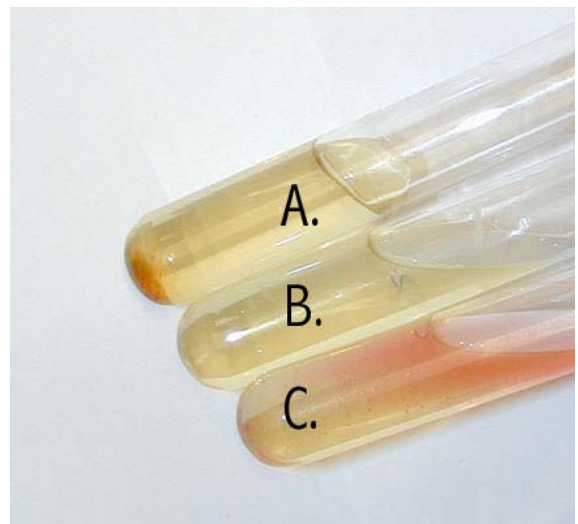


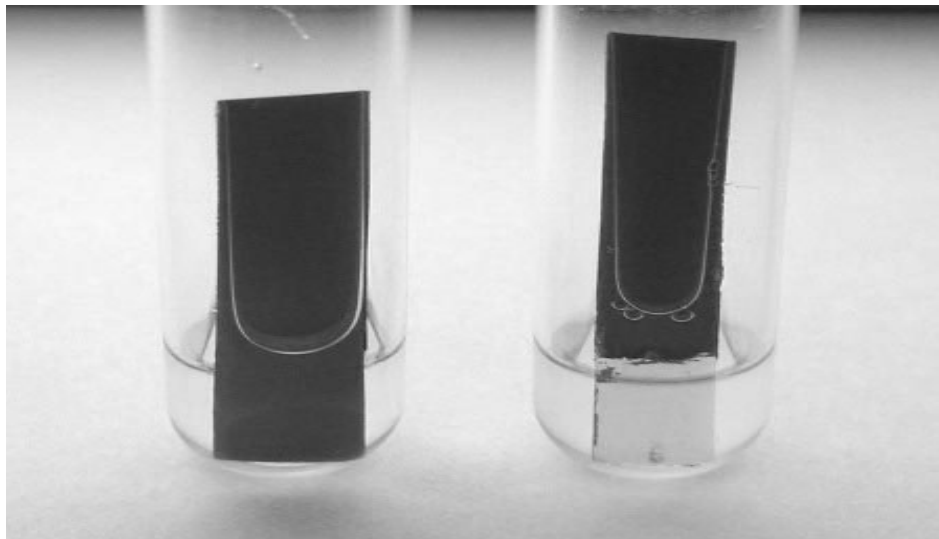
Figure (20): Result of Gelatin Liquefaction: The tubes to the right depict a gelatinase negative (A) and gelatinase positive (B and C) reactions.

X-ray film or gelatin strip

An alternative method for detecting gelatinase production is the use of X-ray film that is coated with a green gelatin emulsion. Organisms that produce gelatinase remove the emulsion from the strip.

Procedure

1. Inoculate each of the two cultures into a separate tube of 0.5 ml saline. The suspension should be very turbid.
2. Insert a strip of the X-ray or gelatin film into each saline suspension.
3. Incubate the tubes at 35°C. Observe at 1, 2, 3, 4, and 24 hours for removal of the gelatin emulsion from the strip with subsequent appearance of the transparent strip support.



Answer the following questions:

1. What the significant of the amylase?
2. Why we place the tube in the refrigerator for 30 min?
3. What the important roles of charcoal in gelatin liquefaction test?

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Exercise 9: Bacterial metabolism--carbohydrate fermentation.

Introduction

Most bacteria produce energy (ATP) by one or more of three mechanisms:

1. Aerobic respiration.
2. Anaerobic respiration.
3. Fermentation.

Aerobic respiration is an oxidative process which uses oxygen as a final electron acceptor.

Anaerobic respiration is similar to aerobic respiration, but it uses an inorganic molecule other than oxygen as the final electron acceptor.

Fermentation uses an organic molecule as a final electron acceptor.

Aerobic respiration produces 36-38 ATP per glucose molecule, and is the most efficient form of energy production. Fermentation is the least efficient means of energy production; it produces only two ATP per glucose molecule. Anaerobic respiration is more efficient than fermentation, but less efficient than aerobic respiration. The ATP yield per glucose molecule varies, depending on the final electron acceptor used.

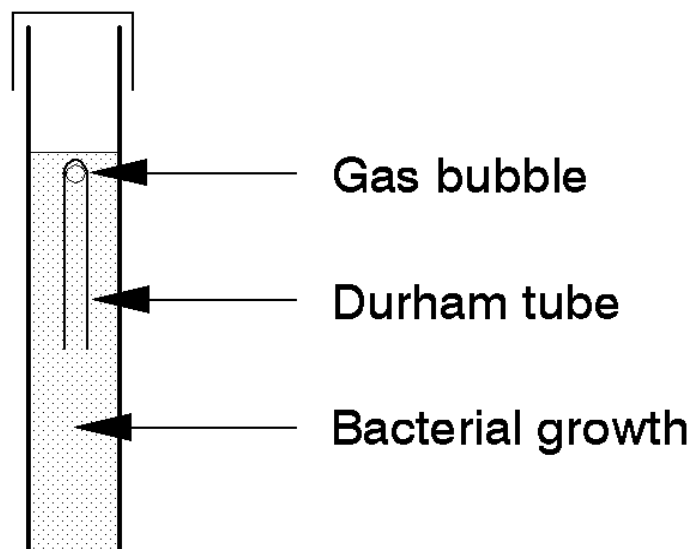


Figure (21): Carbohydrate fermentation tube.

General Microbiology Manual

Fermentation is an anaerobic process. However, many aerobic bacteria are capable of fermentation, and may do so even when oxygen is available. In this experiment, we will examine the capability of bacteria to ferment a variety of carbohydrates. We will also determine the end products of bacterial fermentation.

1. A cid end product.
2. A cid and gas end products.

Most bacteria produce organic acids as by-products of fermentation. Incorporation of a pH indicator into a medium allows detection of acidic fermentation products. **Phenol red** is one of the most commonly used indicators. At acid pH, phenol red changes from red to yellow. Other indicators may be used as well. Many bacteria also produce gases in addition to acids. Gases may be detected by placing an inverted glass tube, called a Durham tube, into broth tubes. If the bacteria produce gas, the Durham tube traps the bubbles (Figure 9.1). Acid and gas production from different carbohydrates are important bacterial identification characteristics.

Materials

Each student/team:

3 (each) Phenol red broths (with Durham tubes) of: glucose (dextrose), lactose, sucrose.

Lab supplies:

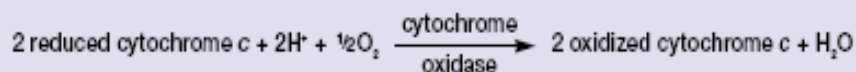
Nutrient broth cultures of *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* (all 24- to 48-hour).

Procedure

1. Inoculate each of the above bacteria into separate tubes of glucose, lactose and sucrose broth.
2. Incubate all of the tubes for 24-48 hours at 37°C.
3. Examine the tubes for acid and gas production.

Exercise 10: Oxidase Test

Oxidases are enzymes that catalyze the reduction of oxygen during respiration. For example, in most gram positive bacteria and many gram negative bacteria cytochrome oxidase performs the final step in electron transport, reducing oxygen to water. Other bacteria, such as the *Enterobacteriaceae*, do not reduce oxygen using this enzyme.



Thus detection of cytochrome oxidase is a valuable tool in differentiating among bacteria. The test utilizes a colorless reagent to detect oxidase.

This chemical (**Tetra methyl-p-phenylenediamine**) in the presence of oxygen and an oxidase enzyme will form a colored compound.

CONTROL:

Positive Control: *Ps. aeruginosa* (on agar)

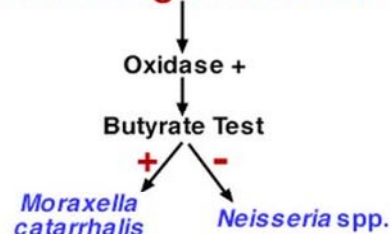
Negative Control: *E. coli*

Procedure

1. Place a piece of filter paper in a Petri plate and soak with the oxidase reagent. Avoid direct contact with this chemical.
2. Using a sterile swab, transfer the bacteria to the filter paper. (**A platinum loop** may be used to transfer organisms but iron in a nichrome loop may interfere with the reaction.) (figure 19)
3. Observe for a color change. A positive reaction appears pink, then maroon and finally black. Do not handle the filter paper when discarding.

NOTE: An alternate procedure is performed by placing some oxidase reagent directly on the colony on the agar.

Gram Negative Cocci



RESULTS:



Figure (22): Result of Oxidase Test by used a filter disk

Limitations of the procedure:

- We keep the Oxidase reagent either frozen or unopened in tubes until needed. If old reagent is sitting out on the bench and is *Purple*.
- Use a young culture, preferably less than 24 hrs old.
- Use fresh reagent, less than a couple of hours old (it is taken out of the freezer).
- Read the reaction within 20 seconds (NOT after), usually it will change in less than 15 seconds. The oxygen will change the reagent color as time passes, so it must be read quickly.

Answer the following questions:

1. Why do you have to read this reaction within 30 seconds?
2. Why does the oxidase reagent need to be fresh?

Exercise 11: Methyl Red and voges- proskauer tests (MR-VP)

These fermentation tests are used to differentiate between certain intestinal bacteria called coliforms. The medium contains dextrose as the carbohydrate source. Some coliforms will ferment the dextrose to acid products that will cause the pH to drop below pH 5. This is called a mixed acid fermentation. After incubation the addition of methyl red, a dye which turns red **below pH 4.4**, will indicate whether such fermentation has occurred. Other coliforms will convert dextrose to less acidic products such as ethanol or butanediol. These bacteria are negative in the methyl red test.

Butanediol fermentation is demonstrated by the Voges-Proskauer test which measures the presence of acetoin (acetyl methyl carbinol), a precursor to butanediol. This test uses the same medium as the methyl red test and both tests are usually performed in parallel. **Barritt's reagents, alpha-naphthol and potassium hydroxide**, are added to a 48 hour culture and the tube is shaken to aerate the solution. The development of a pink or red color after agitation is a positive reaction for the production of acetoin.

CONTROLS:

E. coli (positive MR for negative for VP);
E. aerogenes (positive for VP negative for MR)

Procedure

1. Inoculate the organism. Incubate for 48 hrs or 5 days at 37 °C. (A single uninoculated control should be kept.)
2. Remove 1 ml from each culture to a clean tube for the VP test.
3. Methyl red test: Add a few drops of methyl red to the original culture tube and mix the contents. Record your results.
4. Voges-Proskauer test: Add 0.5 ml (~15 drops) of Barritt's reagent A to the tube and mix. Add 0.5 ml of Barritt's B and mix. Aerate the tube by mixing occasionally over a two hour period or until pink or red color developments. Record your results.

RESULTS:

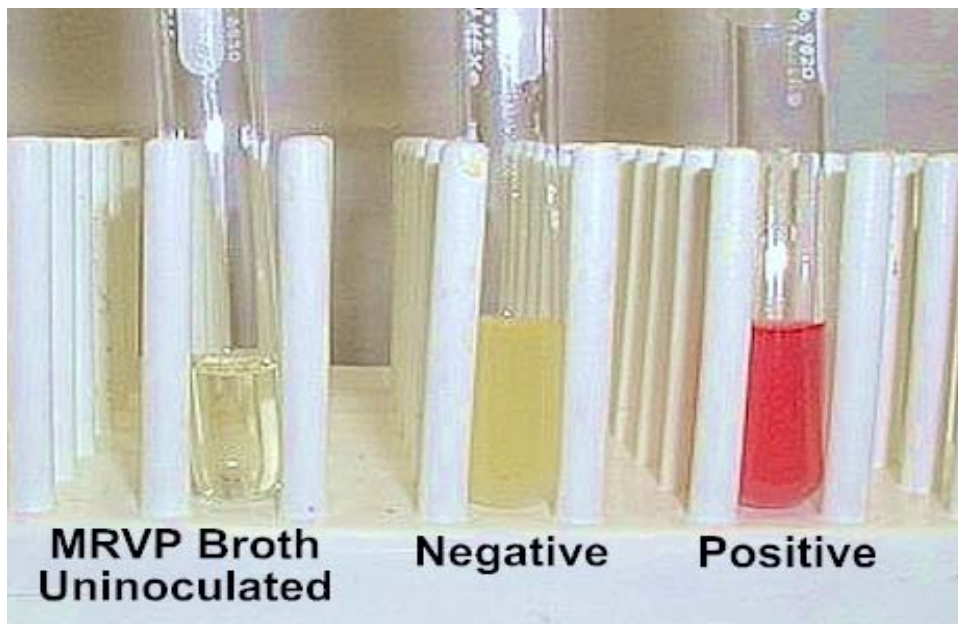


Figure (23): Result of Methyl Red Test



Figure (24): Result of VP Test

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Answer the following questions:

1. What are the products of fermentation?
2. What is the purpose of KOH in MR-VP test?
3. The end product identified in the VP test is a neutral compound called _____.

Exercise 12: Tryptophan hydrolysis (Indole Production)

The ability to degrade amino acids to identifiable end products is often used to differentiate among bacteria. Tryptophan, for example, is hydrolyzed to indole, pyruvic acid and ammonia by tryptophanase. The pyruvic acid can be further metabolized to produce large amounts of energy. The ammonia is available for use in synthesis of new amino acids.

Indole can be detected by reaction with Kovac's reagent (Para-dimethylaminobenzaldehyde in alcohol) to produce a red color.

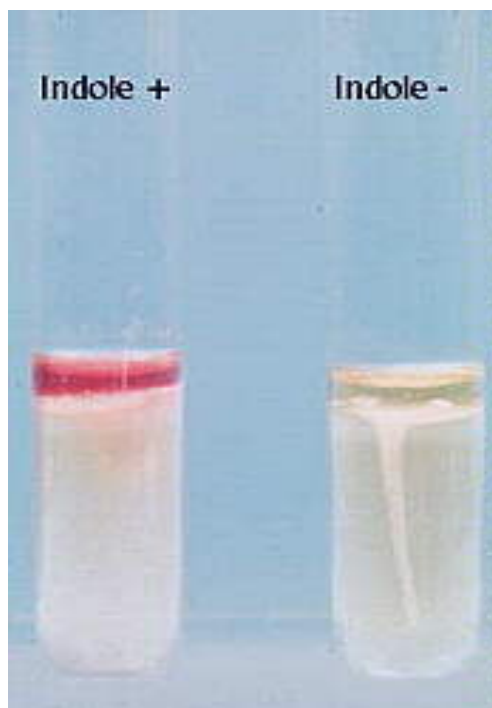
CONTROL:

Positive Control: *E.coli*.

Negative Control: *Enterobacter aerogenes*

Method:

1. Inoculate tryptone broth (1%) with the organism.
2. Incubate at 37 °C for 48 hours.
3. Add 10 drops of Kovac's reagent. A red color in the alcohol (upper) layer is a positive result.



Exercise 13: Citrate Utilization

Some bacteria may be able to use organic compounds other than sugars as their sole source of carbon. The ability to metabolize citrate for example is useful for differentiating among Enterobacteriaceae. **Simmons Citrate agar** is a medium containing citrate as the sole carbon source and ammonium salts as the sole nitrogen source. Organisms that metabolize citrate utilize the ammonium salts releasing ammonia and increasing the pH of the medium. Brom thymol blue is present in the medium as the indicator dye. It is green at neutral pH and deep blue above pH 7.6.

Koser's citrate broth is another medium used to test for citrate utilization. Growth is evidence of a positive reaction.

CONTROLS:

Positive Control : *Enterobacter aerogenes*

Negative Control : *E. coli*

Procedure

1. Using a sterile inoculating needle, streak one organism over the surface of the agar slant, then stab the butt. Repeat with the second organism.
2. Incubate the tubes at 37 °C for 48 hours.
3. Examine for growth. Is there a change in the indicator dye in the agar?

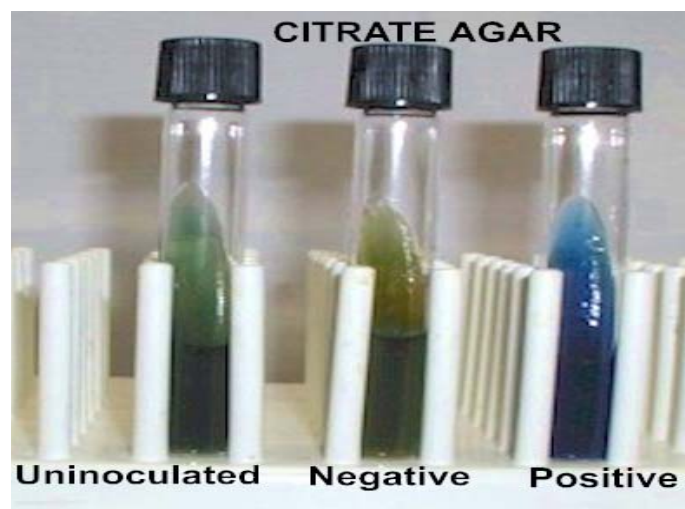


Figure (25): Result of citrate utilize

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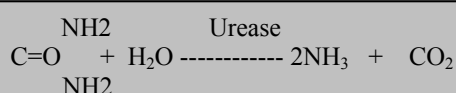
Answer the following questions:

1. What is the substrate for the Indole test? How is Indole produced?
2. Draw the equation of tryptophan breakdown?
3. What are the reagents used in the indole test, methyl red test, and Voges-Proskauer test?
4. Why determine motility and H₂S before adding Kovac's reagent?

Exercise 14: Urease Test

PRINCIPLE:

Urease hydrolyses urea producing carbon dioxide and ammonia, the alkalinity of which causes the indicator **phenol red** to change from yellow to red.



SIGNIFICANCE:

Urease test is used to screen lactose negative gram-negative enterobacteriaceae on differential media plated with materials from stool specimen, helping to differentiate *Salmonella* and *Shigella* species which are urease negative from the urease positive non-pathogen. *Proteus*, and some *Citrobacter* species and some *Haemophilus* species are urease positive.

Procedure

1. 1-Streak the slant of **Christensen's urea medium** with the test organism.
2. 2-Incubate at 35 °C (or the appropriate temperature for the organism) for 24 hours to four days.

RESULTS:

Positive: A bright pink color develops on the slant and may extend throughout the medium

Negative: No change in the original color of the medium.

Notes: Urease-Producing by some *Enterobacteriaceae* like

1. *Proteus*
2. *Klebsiella pneumoniae*
3. *Enterobacter cloacae*
4. *Yersinia enterocolitica*

Medical Application

In the clinical laboratory, members of the genus *Proteus* can be distinguished from other enteric nonlactose-fermenting bacteria (*Salmonella*, *Shigella*) by their fast urease activity. *P. mirabilis* is a major cause of human urinary tract infections.



Urea
(Control)

Urea
12 drops urease

Urea
6 drops urease

Urea
3 drops urease

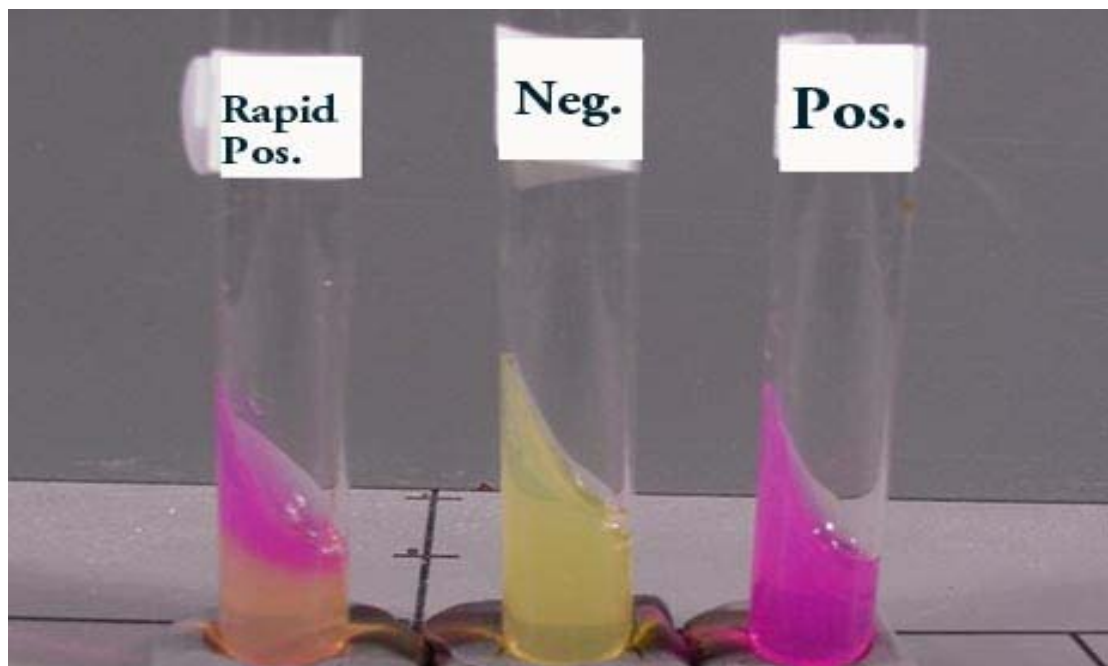


Figure (26): Result of urease test

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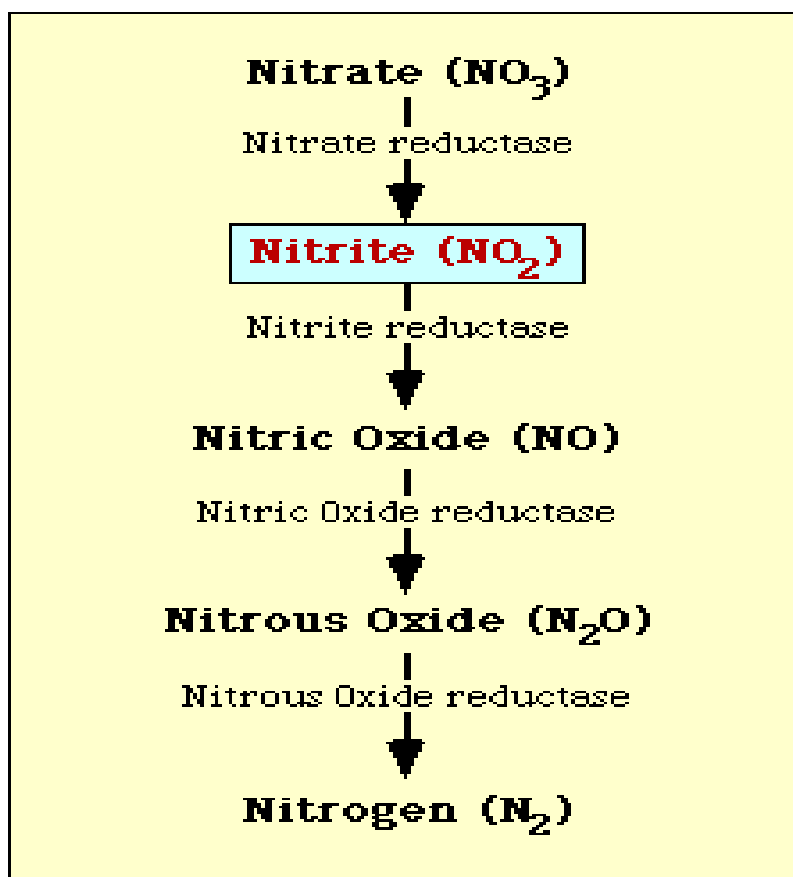
Results

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Exercise 15: Nitrate Reaction Test

PRINCIPLE:

Organisms that possess the enzyme, nitrate reductase reduces nitrate to nitrite. Nitrite combines with an acidified substrate **naphthylamine** to form red colored end product. If the organism has further reduced nitrite to nitrogen gas, the test for nitrate will yield negative (colorless) results. An additional test for the presence of unreacted nitrate must be performed to validate such colorless results. **Metallic zinc** catalyzes the reduction of nitrate to nitrite, thus, with the addition of zinc, a negative test will yield a red color, indicating the presence of unreacted nitrate.



SIGNIFICANCE:

Most Gram-negative bacilli are positive for nitrate reduction and negative for Nitrite reduction. This test is used in the identification of only few gram negative bacilli such as **Moraxella** and **Acinetobacter**, some strains of **Pseudomonas** and **Vibrio** and **Flavobacterium**.

Procedure

1. Inoculate a nitrate broth with the test organism.
2. Incubate at 37 C for 24 hours.
3. Add 5 drops of reagent A (**Sulfanilic acid**) and 5 drops of reagent B (**naphthylamine**) to the broth.

RESULTS:

Positive: A red color develops in 1-2 minutes.
Negative: Colorless

NB: IF NEGATIVE

Add a pinch of zinc dust to the tube
Positive: Colorless
Negative: Red color

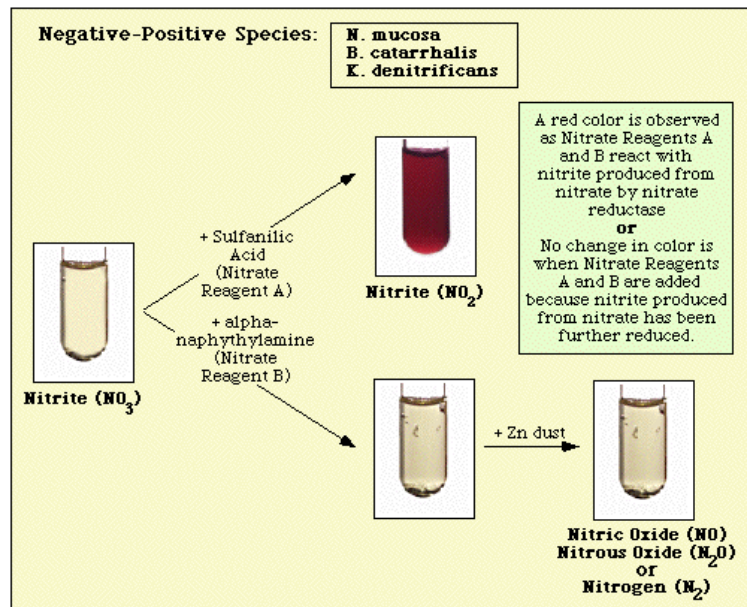
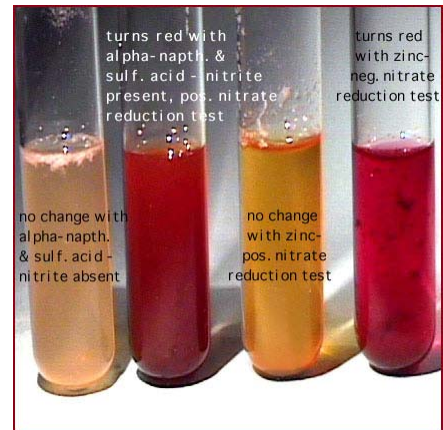


Figure (27): Nitrate reduction procedures

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❖ **Three different bacteria that give three different nitrate reduction results will be learned.**

1. *Staphylococcus epidermidis* is unable to use nitrate as a terminal electron acceptor; therefore, it cannot reduce nitrate.
2. *Escherichia coli* can reduce nitrate only to nitrite.
3. *Pseudomonas fluorescens* are characterized by excretion of diffusible yellow-green pigments that fluoresce in ultraviolet light) often reduces nitrate completely to molecular nitrogen.

Most enteric bacteria are nitrate reducers. Pathogenic examples include *Escherichia coli* (opportunistic urinary tract infections), *Klebsiella pneumoniae* (bacterial pneumonia), *Morganella morganii* and *Proteus mirabilis* (nosocomial infections).

Nonenteric nitrogen reducing pathogens include *Staphylococcus aureus* (staphylococcal food poisoning, bacteremia, various abscesses) and *Bacillus anthracis* (anthrax).

Answer the following questions:

1. Mention the color of Bromthymol blue at different pHs?
2. What the purpose of zinc powder in nitrate reduction test?
3. Name the 2 major end products of nitrate reduction.
4. Is nitrate reduction an aerobic pathway or an anaerobic pathway? Explain
5. Why does the agar become alkaline when bacteria grow on Simmons citrate medium?

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Exercise 16: Media Preparation & Sterilization.

1. Medium (media, plural) – a nutrient blend used to support microbial growth.
2. There are three physical forms of media, broth, solid, and semisolid.
 - a. Solid and semisolid media are **agar** based.
3. Solid media are more versatile in their usage.
 - a. Promote surface growth
 - b. Used to isolate pure cultures
 - c. Ideal for culture storage
 - d. Helpful in the observation of biochemical reactions
 - e. Used to make slants, deeps, and plates (named by medium)
 - i. Examples: EMB slant, EMB plates, nutrient agar slant (NAS), etc.
4. Microorganisms are grown and sub-cultured on different types of chemical media.
 - a. Chemically Defined or Synthetic
 - b. Complex or Non-synthetic
5. A medium is sterilized (living organisms removed) before usage in the lab.
 - a. Sterilization methods include; **autoclaving, dry-heat, filtration, UV exposure and ethylene oxide.**

Culture: Is part of specimen grown in culture media.

Culture Media: is a medium (liquid or solid) that contains nutrients to grow bacteria *in vitro*. Because sometimes we cannot identify with microscopical examination directly, and sometimes we do culture for antibiotic sensitivity testing.

Properties of Media:

Media has to support the growth of the bacteria, should be nutritive (contains the required amount of nutrients), and with suitable pH (neutral to slightly alkaline 7.3-7.4), suitable temperature, and suitable atmosphere. (Bacteria grow at 37 °C)

The media has to be sterile so that we do not get wrong diagnosis by contamination. (Most media are sterilized by autoclaving at 121 °C and 2 atmosphere for 15-20 minutes. With the autoclave, all bacteria, fungi, viruses, and spores are destroyed. Some media can't be sterilized by autoclaving because they contain eggs or carbohydrates. (Autoclaving is to increase boiling from 100 °C to 121 °C).

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Forms of culture:

Culture media can be used in many forms: solid (agar), semisolid, and liquid (broth).

Solid (agar):

Is Broth plus agar (seaweed).

Are prepared by adding a solidifying agent (agar 2-3%).

Prepared mainly in Petri dishes, but also in tubes and slopes.

After growth the bacterial colonies are visible.

Useful in identifying different types of bacteria (can see shape and color of colony). (A colony is the smallest bacterial unit that can be seen with the naked eye). e.g. blood agar, chocolate agar, MacConkey agar.

Semisolid agar (soft agar):

Contains small amounts of agar (0.5-0.7%).

Used to check for motility and also used as a transport media for fragile organisms.

Can have semisolid agar in Petri dishes or in tubes. In tubes it is usually slanted to increase surface area.

Liquid (Broth):

Mostly used for biochemical tests (blood culture, Broth culture).

Growth of bacteria is shown by turbidity in medium.

E.g. Nutrient broth, Selenite F broth, alkaline peptone water.

Properties of agar:

- Some what like gelatin.
- It melts at 97 °C and solidifies at 37 °C.
- Comes as sold powder and then you add water to it.

Bacteria can be divided according to medium requirements into fastidious and non- fastidious bacteria depending on the type of media they grow in. Non-fastidious bacteria (like E. Coli) grow in any type of media, while fastidious bacteria grow in certain types of media, because they need O₂, special atmosphere, etc. (like Neisseria)

Microorganisms according to atmospheric requirement:

Obligate aerobes need O₂ at 20%.

Facultative anaerobes with or without O₂ but better with O₂.

Strict anaerobes need complete absence of O₂.

Microaerophilic need O₂ at 5%.

Cepnophilic need CO₂.

Most human pathogens are facultative anaerobes.

Routine incubation in lab is aerobic with O₂.

The rest need special incubator methods.

Types of Culture Media:

Simple (basal, ordinary) Culture Media: are media that contain the basic nutrients (growth factors) that support the growth of bacteria without special nutrients, and they are used as basis of enriched media. E.g. Nutrient broth, nutrient agar, peptone water. They are for the growth of non-fastidious organisms like *E.coli*).

Enriched Culture Media: are media that are enriched with: Whole blood e.g. blood agar (opaque and red in color)

Lysed blood (heated to 80⁰C) e.g. Chocolate agar (contains lysed blood, and it's opaque and chocolate colored)

Serum e.g. Loeffler's media.
Special extracts e.g. meat.

Vitamins.

Fastidious and non-fastidious organisms grow in it.

Selective Media: it is a media, which contains substances that prevent or slow the growth of microorganisms other than the bacteria for which the media is prepared for.

E.g.

XLD (xylose lactose desoxycholate): contains bile salts that inhibit many fecal normal flora and has a pH indicator and it is selective for salmonella and Shigella, similar to moc

EMB (Eosin Methylene blue): enteric isolation media.

TSI (triple sugar iron agar): slanted tube.

TCBS: selective for cholera (cholera grows in highly alkaline media). It ferments sucrose

CLED (Cystine lactose electrolyte deficient media): used in urine lab diagnosis (diagnosis of UTI).

LJ (L.Jesnsin) : selective for Mb.TB. Has egg, selective media is malachite green, Incubated for 4-6 weeks. Always sloped and with a screw cap.

Differential Media (indicators): Contains indicators, dyes, etc, to differentiate microorganisms. E.g. MacConkey agar, which contains neutral red (pH indicator) and is used to differentiate lactose fermentose and non-lactose fermentose. (E.g. *E.coli* is lactose fermentose and produces acid and causes a decrease in pH and produces red color. Salmonella on the other hand is non-lactose fermentose, and doesn't produce acid, and doesn't change pH and does not change the color).

Description of common media used in Microbiology Laboratory

Alkaline Peptone Broth or Alkaline Peptone Water: enrichment medium for increasing the numbers of target bacteria that can multiply under alkaline condition(e.g., *Vibrio cholerae*), while inhibiting the growth of contaminating flora that cannot multiply at an elevated PH.

Bile Esculin Agar (BSA): selective media for the detection of fecal streptococci (group D) and Enterococcus; tests ability of the organism to hydrolyze Esculin to esculetin. Brownish-black colonies surrounded by surrounded by a black zone are positive. Oxgall (bile) is inhibitory agent. Ferric citrate is indicator.

Blood Agar (BAP) : consist of a basal medium such as TSA enriched with 5% defibrinated sheep blood or in some locations, horse blood. This is the most commonly used medium, and supports the growth of the most of the common fastidious organisms, as well as, all of the less fastidious organisms (e.g. coliforms). BAP will be used to practice streaking technique to obtain isolated colonies to observe differences in colony morphology; to determine various patterns of hemolytic activity; to perform CAMP test for Group B streptococci.

Brilliant Green Agar: selective medium for isolation of Salmonella, except *S. typhi*. Brilliant green is the selective agent. Phenol red is the PH indicator.

Campy – blood agar (Campy- BAP): Brucella agar supplemented with sheep blood and vancomycin (inhibits Gram-positive), trimethoprim (broad spectrum), and cephalothin (inhibits streptococci).

Cary-Blair Transport Medium: semi-solid alkaline medium used to transport sensitive specimens to the laboratory when the specimen cannot be cultured immediately.

Chopped (Cooked) Meat Medium: used for cultivation and maintenance of Clostridium and to evaluate proteolysis.

Chocolate Agar: blood agar prepared by heating blood to 95C until medium becomes brown or chocolate in color heating the blood releases broth X and V growth factors and also destroys the inhibitors of V factor. These factors are required for the growth of most species of Haemophilus and also Neisseria gonorrhoeae.

Coagulase Test Medium: citrated rabbit plasma which clots in the presence of the enzyme coagulase.

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Cystine (Cysteine) Tellurite Blood Agar: broth a differential and selective medium for the isolation of *C. diphtheriae*; however, a few strains of streptococci and staphylococci are able to grow on this medium; *C. diphtheriae* produces gray to black colonies because the Tellurite is reduced intracellularly to tellurium.

Cystine Trypticase Agar (CTA) : carbohydrate-supplemented CTA medium dispensed in tubes is used to detect fermentation of the various carbohydrates and can be used for determination of motility.

Cystine Trypticase Agar (CTA) Sugar Fermentation Media: CTA supplemented with either glucose, maltose or sucrose; tests for utilization of carbohydrates.

Glucose Broth: for anaerobic fermentation (overlaid with mineral oil after inoculation). Phenol red is the PH indicator.

Loeffler Medium: primary isolation medium for *Corynebacterium diphtheriae*; colony morphology is observed.

Lysine Decarboxylase Medium: a yellow color indicates a low PH and that the test is negative (failure to produce an amino by decarboxylation of lysine) . Bromocresol purple is the PH indicator.

MacConkey Agar: an inhibitory and differential medium used to distinguish lactose- fermenting Gram- negative organism from non fermentation
Crystal violet, bile salts and neutral red are inhibitor agent.
neutral red is the PH indicator.

Mannitol Salt Agar (MSA): for selective isolation for coagulase positive, mannitol-fermenting staphylococcus.
Mannitol fermentation by pathogenic staphylococci is indicated by a yellow halo surrounding the colonies.
Sodium chloride is the inhibitor agent.
Phenol red is the PH indicator.

Motility-Indole -ornithine Agar: motility is indicator by the character of the growth in the butt of the tube. Motile organism will produce a general clouding of the medium or a fuzzy stab line. Non motile organisms will give a sharply delineated stab line. The ornithine reaction is indicator by color in the butt of tube. Yellow indicates a negative test (failure to decarboxylate ornithine); purple is a positive test (decarboxylate of ornithine). Kovacs is add to the tube to determine indole reaction. Red indicates a positive reaction (indole production); yellow is a negative test (failure to production indole from tryptophan).

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Mueller Hinton Agar: rich medium consisting of 30% beef infusion, 1.75% acid case peptone, 0.15% starch and 1.7% agar that support the growth of most microorganism. It is commonly used for antibiotic susceptibility testing: disk diffusion antibiotic susceptibility; antibiotic serum level measurements; MBC determination.

Mueller Hinton Broth: culture medium for broth tube antibiotic MIC assay.

Nitrate Broth: some bacteria (e.g. *Pseudomonas aeruginosa*) have respiratory enzyme systems that can use nitrate as a terminal electron receptor. The product of the reaction is nitrite. Some of the organisms that reduce nitrate to nitrite will then reduce the nitrite further. In the scheme below, first test for nitrite by a colorimetric test. If these test is negative, it can mean rather that nitrite was not reduced, or that it was reduced beyond the nitrite stage. This can be resolved by the addition of zinc dust; if nitrate is still present, the zinc will reduce it chemically to nitrite, which will then be revealed by the colorimetric reaction.

Nutrient Agar: contains 0.5% gelysate peptone, 0.3% beef extract, and 1.5% agar, and will support the growth of many organism which are not nutritionally fastidious (e.g. staphylococci, and enteric). (Note: Agar is a substance which melts at 100C and solidifies at about 42C; it has no nutritional benefits, but is only a stabilizer to allow for solidification of the medium).

OF Glucose Medium: to detect fermentation or oxidation of glucose.
Brom thymol blue is PH indicator

OF Maltose Medium: to detect fermentation or oxidation of Maltose.
Brom thymol blue is PH indicator

Phenyl ethyl Alcohol Agar (PEA): for the isolation of staphylococcus and inhibition of Gram-negative bacilli.
Phenyl ethyl Alcohol is the inhibitory agent

Salmonella Shigella (SS) Agar : isolation and differential medium for pathogenic Gram-negative bacilli in particular, Salmonella and Shigella. Inhibitor for Coliforms

Serum Tellurite agar: isolation medium for *Corynebacterium diphtheriae*; *C diphtheriae* (also streptococcus) produce gray to black colonies because the tellurite is reduced intracellularly to tellurium

SF Broth: (*Streptococcus (Enterococcus) Faecalis* broth): selective medium for the detection of fecal streptococci (group D) and Enterococci from water, milk and other material of sanitary importance. Growth of all other cocci is inhibited. Fermentation of glucose is indicated by a color change of broth.
Bromocresol purple is the PH indicator.

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Simmons Citrate Agar: utilization of citrate as the sole source of carbon is indicated by the medium turning a deep blue color because of an alkaline reaction. Non utilization will leave the green color of the slant unchanged.

Brom thymol blue is PH indicator.

Skirrows agar: peptone and soy protein base agar supplemented with lysed horse blood and vancomycin (inhibits Gram positive), polymyxin B (antifungal), and trimethoprim(broad spectrum)

Sugar utilization Medium: supplemented with glucose (with Durham tubes to determine gas production), sucrose mannitol or lactose.

Phenol red is the PH indicator.

TCBS Agar (Thiosulfate-Citrate- Bile salts- Sucrose agar): Differential and selective plating medium for Vibrio adjusted to PH 8.6 (alkaline); Vibrio cholerae colonies appear yellow; other Vibrio spp. Colonies appear green.

Thayer – Martin Agar: chocolate agar supplemented with antibiotic inhibitors for selective isolation of pathogenic Neisseria.

Vancomycin inhibits Gram-positive organisms.

Colistin inhibits Gram-positive enteric organism.

Nystatin inhibits yeast.

Triple Sugar Iron Agar (TSI): this a key medium for use in beginning the identification of a Gram- negative bacilli of the enteric group. It contains glucose (0.1%), Lactose (1%), sucrose(1%). And peptone (2%) as nutritional sources. Sodium Thiosulfate serves as the electron receptor for reduction of sulfur and production of H₂S. Detects fermentation of sucrose, lactose, glucose, as well as production of hydrogen sulfide and /or gas .

Phenol red is the PH indicator; ferric ammonium citrate is H₂S indicator.

Trypticase Lactose Agar: to determine motility and lactose fermentation by anaerobes.

Phenol red is the PH indicator.

Trypticase Nitrate Broth: to determine indole production and nitrate reduction.

Trypticase Salicin Agar: to determine motility and Salicin fermentation by anaerobes.

Phenol red is the PH indicator.

Trypticase Sucrose Agar: to determine motility and sucrose fermentation by anaerobes.

Phenol red is the PH indicator.

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Urease Broth or Urea Agar slant: Prompt hydrolysis of urea by proteus species is indication by a deep pink color appearing in the medium within eight hours. At 18 hours, this color will have spread throughout the whole tube. Many strains of Klebsiella, Enterobacter and Citrobacter will yield apposite reaction, but usually the pink color will be limited to the slant in 24 to 48 hours. Do not reincubate tubes that show any evidence of color change.

Practical

This lab requires 3 continuous hours and you will be directed by your instructors

Exercise 17: Single Media/ Multiple Tests

Media for Isolation of Microbes

- **General (all purpose):** contains basic nutrients for most bacteria. Like (TSA & TSB).
- **Enriched:** contains extra growth factors & nutrients (Fastidious organism)
- **Selective:** contains ingredients that inhibit growth of some bacteria & allow growth of others
- **Differential:** contain indicators that change appearance of media in response to differential use of an ingredient

Several media are designed to yield more than one biochemical reaction. Among the more commonly used media in this category are SIM media, Triple Sugar Iron agar (TSI) and Kligler's Iron agar (KIA).

SIM medium

derives its name from three reactions: production of hydrogen sulfide from sulfur-containing amino acids, indole production and motility. Check for motility by observing migration of the inoculum from the stab line through the semisolid medium. The liberation of hydrogen sulfide is indicated by a blackening of the medium. Indole production is determined after the addition of indole reagent. The medium is used primarily for differentiation of gram negative enteric bacteria.

Kligler's iron agar (KIA) and triple sugar iron agar (TSI)

KIA and TSIA are widely used in the identification of gram negative bacteria particularly the Enterobacteriaceae. The media are identical except that TSI contains sucrose in addition to the dextrose and lactose found in KIA. The media are poured as slants and are inoculated with a stab to the butt followed by a streak of the slant surface. The bacteria therefore are exposed to both an anaerobic environment (butt) and an aerobic one (slant). Phenol red is present as an indicator. Do not tighten the cap on the tube.

If the bacteria are nonfermenters, such as *Pseudomonas*, they can grow on the slant by the aerobic degradation of protein components in the medium.

Lysine Iron agar (LIA)

Here is another multipurpose medium in one tube. Reactions for lysine decarboxylation (Anaerobic alkaline reaction – over neutralizing the acid formed from glucose fermentation), lysine deamination (formation of red- colored products at the top of the medium) and H₂S production (black precipitate).

Motility Indole Ornithine (MIO) Medium

The reactions in this medium are observed as follows:

- Motility: Observe for cloudiness in the medium (growth away from the stab line) . for a non- motile organism be seen along cracks in the medium caused by gas production, but there will be clear pockets of no growth.
- Ornithine Decarboxylation: Observe the lower three-quarters (anaerobic region) of the medium for change in the color of the PH indicator; growth must be present in this part of the tube for correct analysis of result:
- Gray, blue or purple color: positive reaction for Ornithine decarboxylation- formation of a highly alkaline product, over-neutralizing the acid produced from glucose fermentation.
- Yellow color: Negative reaction. Yellow color is due to the default acid production from glucose fermentation.
- Indole Production: About one-half dropperful of Kovacs reagent is add to the medium. A red ring indicates production of indole from the breakdown of tryptophan.

Triple Sugar Iron Agar (TSIA)

PRINCIPLE:

TSIA detects three primary characteristics of a bacterium:

- 1. The ability to produce gas from the fermentation of sugars,**
- 2. The ability ferment lactose and sucrose**
- 3. The production of large amounts of hydrogen sulfide.**

SIGNIFICANCE:

This test is of a great value in the initial identification of the family Enterobacteriaceae

Procedure

1. Inoculate the test organism on TSIA slants by stabbing the butt and streaking the slant completely.
2. Incubate for 24 hours at 37 °C.

RESULTS AND INTERPRETATIONS:

NB: READ THE SLANT FIRST AND THEN THE BUTT.

Yellow=Acid=A

Red=Alkaline=K

Cracks in the butt=Gas=G

Blaking in the butt=+=hydrogen sulfide.

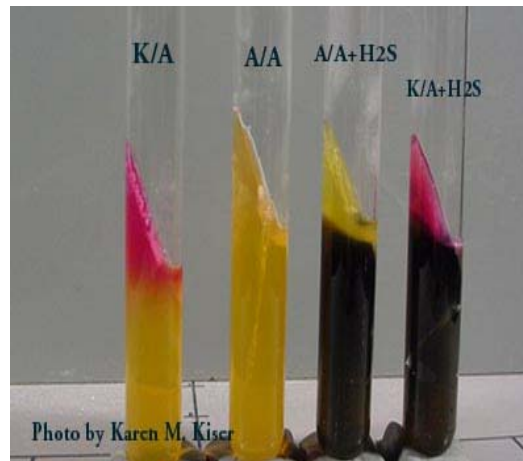
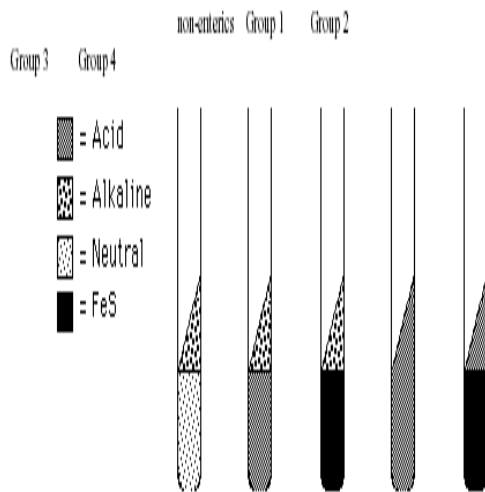


Figure (28): Reactions of TSIA

Results (Slant/Butt)	Symbol	Interpretation
Red/yellow	K/A	Glucose fermentation only; Peptone catabolized
Yellow/yellow	A/A	Glucose and lactose and/or sucrose fermentation
Red/red	K/K	No fermentation; Peptone catabolized
Yellow/yellow with bubbles	A/A,G	Glucose and lactose and/or sucrose fermentation; Gas produced
Red/yellow with bubbles	K/A,G	Glucose fermentation only; Gas produced
Red/yellow with bubbles and black precipitate	K/A,G, H ₂ S	Glucose fermentation only; Gas produced; H ₂ S produced
Red/yellow with black precipitate	K/A, H ₂ S	Glucose fermentation only; H ₂ S produced
Yellow/yellow with black precipitate	A/A, H ₂ S	Glucose and lactose and/or sucrose fermentation; H ₂ S produced

The possible result: A=acid production; K=alkaline reaction; G=gas production; H₂S=sulfur reduction

Exercise 18: Selective and differential Media

Introduction

In addition to general-purpose media, which allow the growth of most types of bacteria, microbiologists use specialized media to identify and/or isolate specific groups of bacteria. In this experiment, we will use **differential** and **selective** media.

Selective media are used to isolate specific groups of bacteria. These usually contain inhibitors which prevent the growth of unwanted bacteria, while allowing the growth of the desired bacteria. Differential media allow microbiologists to distinguish between biochemical and morphologically related groups of bacteria. These media incorporate chemicals that produce some characteristic change in the colonies or media around the colonies of specific types of bacteria within the group. Often, selective and differential capabilities are incorporated into a single medium.

Mannitol salt agar (MSA) contains 7.5% NaCl, a salt level which is toxic to most bacteria except the genus *Staphylococcus*. This is the selective portion of the medium. MSA is also differential. It contains the carbohydrate, mannitol, as well as the pH indicator, phenol red. Staphylococci that ferment mannitol and produce acid exhibit a yellow color around their colonies. Staphylococci that do not ferment mannitol do not produce a color change from the normal red-pink color of the medium.

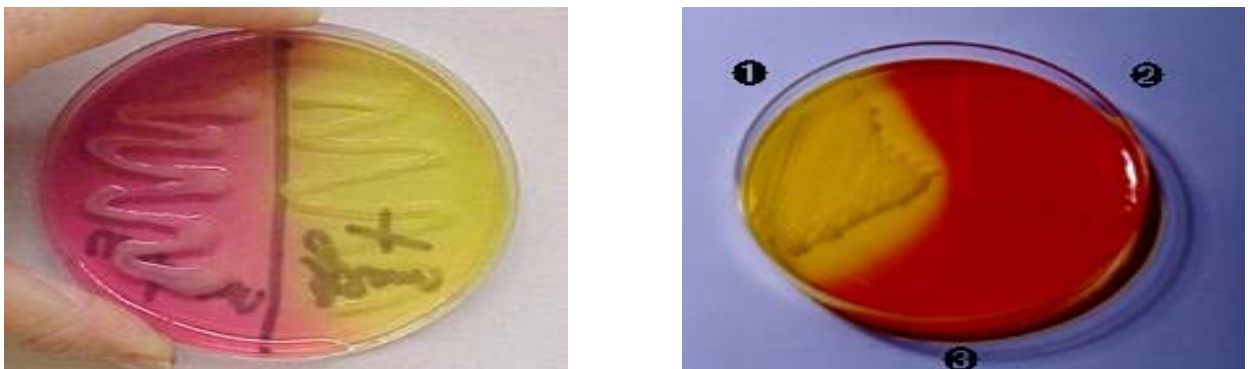


Figure (29): *Staphylococcus aureus* yellow color

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MacConkey agar (Mac) is used for the isolation of Gram negative organisms. It contains crystal violet, which inhibits the growth of Gram positive organisms and also gives the medium a light pink-lavender color. The medium also contains bile salts, lactose, and the pH indicator, neutral red. As well as being selective for Gram negative, Mac is differential within that group. **Coliform bacilli** produce acid from lactose fermentation causing the colonies to turn red from the pH indicator. *E. coli* produces even greater quantities of acid causing the surrounding medium and the colonies to turn red. **Non-coliform bacilli** do not ferment lactose, and appear uncolored or transparent on Mac.

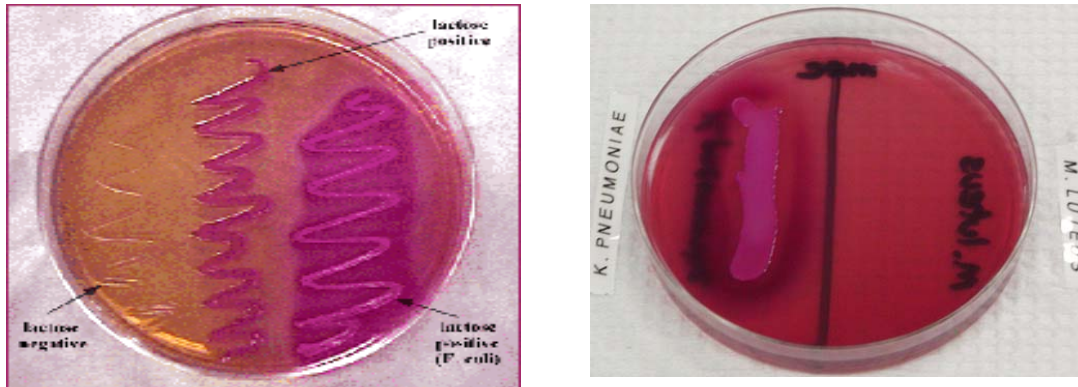


Figure (30): Color of lactose fermenters.

Levine's eosin-methylene blue agar (EMB) is primarily a differential medium. However, it does inhibit the growth of some Gram positive bacteria. EMB is used to differentiate between enteric lactose fermenters (coliforms) and non-lactose fermenters as well as specifically identifying *E. coli*. It is often used to confirm the results of tests with MacConkey agar. The eosin and methylene blue dyes cause lactose fermenters to have pink colonies. *E. coli* incorporates so much of the dye that the dyes precipitate in the cells and give the colonies a metallic green sheen. Non-lactose fermenters are usually transparent and take on the purple color of the medium.



Figure (31): *E. coli* results in a metallic green sheen on EMB



Figure (32) *Enterobacter cloacae* form pink colony on EMB.

Materials

Each student/team:

- 1 Mannitol salt agar plate.
- 1 MacConkey agar plate.
- 1 Eosin-methylene blue plate.

Lab supplies:

Nutrient broth cultures of *Salmonella typhimurium*, *Escherichia coli*, *Enterococcus (Streptococcus) faecalis* and *Staphylococcus aureus* (all 24- to 48-hour).

Procedure

1. Using a grease pencil or marker, divide the bottom of each of the listed plates into four sections. Label the plates as shown in Figure 10.1.
2. Using a loop and proper sterile technique, inoculate each plate with *E. coli*, *Staphylococcus aureus*, *Salmonella typhimurium* and *Streptococcus sp.* using a single streak as shown in Figure 10.2.
3. Incubate all of the plates at 37°C for 48 hours. Record the results.

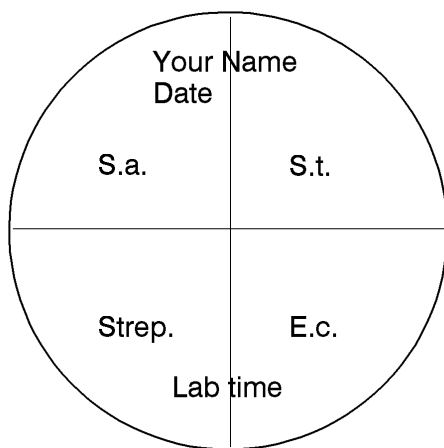


Figure (33): Division of differential media plates

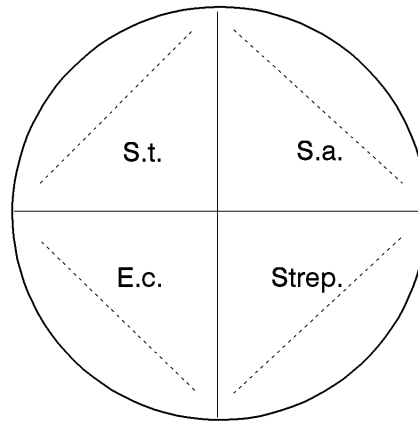


Figure (34): Inoculation of differential media plates.

Laboratory Report

Date: Section: Group:

Name: ID:

Lab Title:

Objective of the Lab

Results

Discussion of results

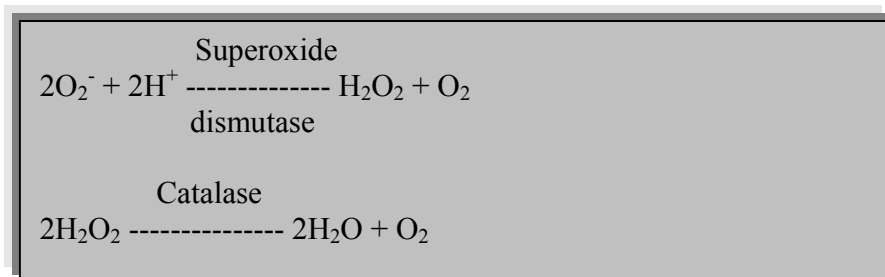
Answer the following questions:

1. What would the medium look like if the bacterium is K/A +H₂S +CO₂.
2- If the organism uses glucose only, without lactose or sucrose, TSIA reaction would be ____.
2. How do we determine the production of Hydrogen Sulfide?
3. What is unique about MSA agar?

Exercise 19: Bacteria oxygen requirements

Introduction

Bacteria can exist in a wide variety of environments, including environments that lack oxygen. In fact, oxygen is toxic to some bacteria. This toxicity is usually due to the accidental formation of superoxides (O_2^-) and hydrogen peroxide (H_2O_2), which react with other cell components. Cells that live in oxygen-containing environments have the enzymes superoxide dismutase and catalase (among others) that convert superoxides and peroxides to oxygen and water. Bacteria are often classified according to their oxygen requirements.



Detoxification of superoxides by superoxide dismutase and catalase. Superoxides are first converted to hydrogen peroxide, and then to water. Peroxides are detoxified by catalase or peroxidases.

Obligate aerobes require oxygen to live. Their metabolic pathways require oxygen as the final electron acceptor (aerobic respiration), and they have no alternate means of producing ATP.

Microaerophiles require oxygen for growth, but they cannot tolerate normal atmospheric levels of oxygen. Too much oxygen seems to interfere with their metabolic enzymes.

Obligate anaerobes do not possess superoxide dismutase or catalase, and therefore cannot cope with the toxic by-products of oxygen metabolism. Obligate anaerobes use anaerobic respiration and/or fermentation to generate ATP.

Aerotolerant anaerobes utilize anaerobic respiration and/or fermentation to produce ATP. Also, they possess catalase and superoxide dismutase, and so are not affected by the toxic by-products of oxygen.

Facultative anaerobes utilize oxygen and aerobic respiration whenever possible. Additionally, they can undergo anaerobic respiration and/or fermentation to survive without oxygen.

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The oxygen requirements of bacteria can be tested by inoculating them into a medium with an oxygen gradient. For this lab, the oxygen gradient will be produced by heating an agar medium deep to drive off any dissolved oxygen. Over time, the top of the deep will become oxygenated, while the bottom will remain anaerobic. After inoculation (Figure 11.2), growth only at the top of the deep indicates an obligate aerobe. Growth just under the surface of the deep indicates a microaerophile. Growth only at the bottom of the deep indicates an obligate anaerobe. And growth throughout the deep indicates either a facultative anaerobe or an aerotolerant anaerobe.

Materials

Each student/team:

- 3 Brain-heart infusion agar deeps.
- 1 Hot plate with water bath.
- 1 Ice water bath.
- 1 Thermometer.

Lab supplies:

- Ice.
- Nutrient broth cultures of *Pseudomonas aeruginosa* and *Staphylococcus aureus* (24- to 48-hour).
- Thioglycollate broth culture of *Clostridium sporogenes* or *C. butyricum* (48- to 72-hour).

Procedure

1. Place the brain-heart infusion (BHI) agar deeps into a boiling water bath.
2. After the BHI agar melts, allow the water to boil for at least five more minutes. Turn the heat off, and place a thermometer in the bath. Monitor the temperature of the bath.
3. When the temperature reaches 45°C, remove the tubes from the bath. **Be careful not to shake, stir or otherwise disturb the agar.**
4. Inoculate each culture into a separate agar deep by carefully stabbing with an inoculating needle (Figure 10.2). Avoid shaking, stirring or getting bubbles into the medium. Immediately after inoculation, place the deeps upright in an ice-water bath until they are completely solid.
5. Incubate the deeps at 37°C. Check the growth at 24, 48 and 72 hours.
6. After incubation, note the pattern of growth in each tube. Compare your results to Figure at the end of the exercise.

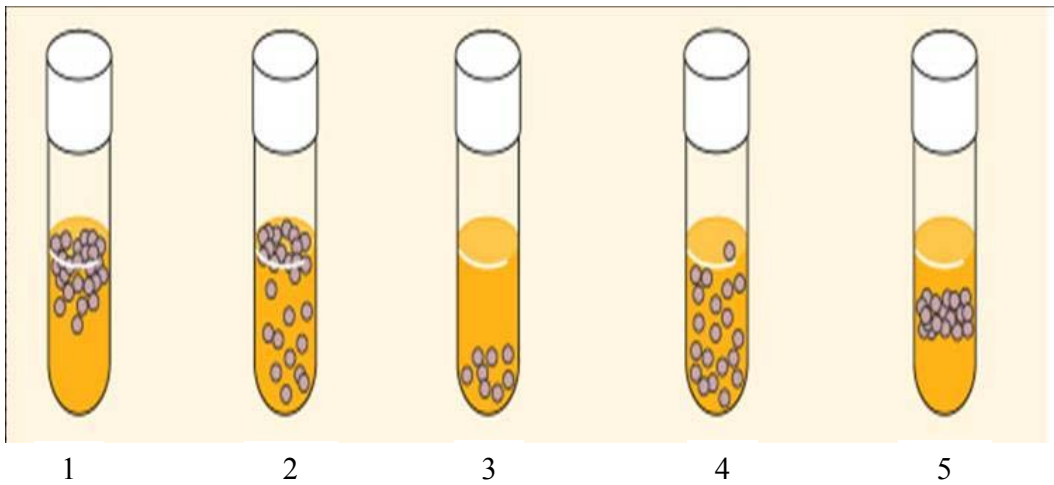


Figure (35): Growth patterns of bacterial metabolic types

1. Obligate aerobe--growth only at the surface.
2. Aerotolerant anaerobe--relatively low growth throughout.
3. Microaerophile--growth just below the surface.
4. Obligate anaerobe--growth only at the bottom.
5. Facultative anaerobe--growth throughout, often with heavier growth toward the top.

Answer the following questions:

1. Why should you heat thioglycollate broth if it is not freshly made?
2. Which environment would a microaerophilic bacterium like the best?

Exercise 20: Anaerobic Bacteria

Bacteria are often categorized according to their growth responses to atmospheric oxygen. This may vary from species that can grow only in the presence of oxygen to those that can grow only in the absence of oxygen.

The strict or obligate aerobes must have oxygen to metabolize. The strict or obligate anaerobes must reside in oxygen-free environments in order to survive. Their metabolism does not use molecular oxygen and they cannot protect themselves from the toxic byproducts that oxygen forms within the cell. Thus in the presence of even trace amounts of oxygen these cells will die.

Facultative anaerobes can grow in either the presence or absence of oxygen. They have two forms of metabolism, one is aerobic and the other is anaerobic. They use oxygen if it is available and switch to the anaerobic pathways if it is not.

Aerotolerant anaerobes are indifferent to the presence of oxygen. They only have anaerobic metabolism, but can remove the toxic byproducts of oxygen. Thus these anaerobes can survive in oxygen even though they don't use it for energy production.

Microaerophiles require oxygen but at reduced levels below the 20% in the atmosphere. Higher levels of oxygen may inhibit enzymes critical for growth or may be toxic to the cell.

Fluid thioglycollate broth is a reducing medium, that is, it contains compounds that react with molecular oxygen keeping the free levels low. It also contains the indicator dye resazurin which turns pink in the presence of oxygen. Since oxygen is present at the surface of the medium, the upper layer is usually pink whereas the dye is colorless in the remainder of the tube. Agar is also included in this medium to give it a semisolid consistency. This prevents the movement of inoculated organisms. Fluid thioglycollate broth has something for every microbe. Strict aerobes will grow only at the top of the tube, strict anaerobes only at the bottom, facultative and aerotolerant anaerobes throughout the tube, and microaerophiles somewhat below the surface.

Anaerobic jars such as the Gas Pak are vessels in which an anaerobic environment is generated after inoculated media are sealed into the chamber. Anaerobiasis is achieved by adding water to commercially available gas generator envelopes that are placed in the jar just prior to sealing. Chemicals in the envelope produce hydrogen gas and carbon dioxide. The hydrogen combines with free oxygen in the chamber to produce water. The carbon dioxide is required for the growth of certain organisms. A methylene blue indicator strip is usually placed in the jar. It turns colorless when the oxygen has been removed.

The candle jar is used to create microaerophilic conditions. It is a large screw-capped container into which the medium is placed along with a candle. The candle is lit and the jar is sealed. The candle will burn and reduce the oxygen concentration.

MATERIALS

Cultures (in broth)

- Clostridium sporogenes* (strict anaerobe)
- E. coli* (facultative anaerobe)
- B. megaterium* (strict aerobe)
- N. sicca* (microaerophile)

Procedure

FLUID THIOGLYCOLLATE

1. Check to make certain that no more than 20% of the upper portion of the medium is pink.
2. Inoculate the tube to the bottom and gently rotate between the palms of your hands to disperse the organism. Do not shake or oxygen will be added to the medium.
3. Incubate the tubes for 48 hours.
4. Record the pattern of growth.



Figure(36) : fluid thioglycollate

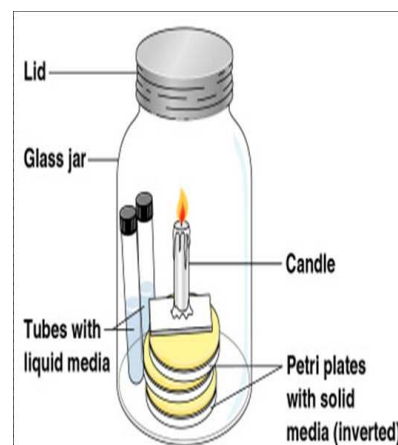
ANAEROBIC JAR

1. Streak the organism on the appropriate agar medium.
2. Place the plate in the anaerobic jar. When all plates are in the container, the indicator strip (it should be blue) and the gas generator envelope are added. Water is added with a pipette. The jar is sealed and placed in an incubator for 48 hours. The indicator should be checked to make certain that all oxygen has been removed.
3. A second plate should be placed in the incubator as an aerobic control.
4. Observe for growth.



CANDLE JAR

1. Streak the organism on the appropriate agar medium
2. Place the plate in the glass candle jar. When all plates are in the jar, the instructor will place a candle in the jar, light the candle and close the lid. When the candle has stopped burning, place the jar into an incubator for 48 hrs.
3. Observe for growth.



Exercise 21: **The serial dilution method of bacteria enumeration**

Introduction

Because of their very small size, counting the number of bacteria in a sample can be difficult at best. Although direct counts are possible with a microscope, they require a lot of time and expertise. An easier method is to spread bacteria over a wide area (i.e. nutrient agar plate) and count the number of colonies that grow. If the bacteria are spread out enough, each bacterial cell in the original sample should produce a single colony. Usually, bacterial samples must be diluted considerably to obtain reasonable counts.

This method has some drawbacks, however. Injured bacteria may not always form colonies. Also, since there is no single medium which supports the growth of all types of bacteria, some bacteria may be left out of any given counting procedure. Therefore, bacterial counts by these methods are usually expressed as colony forming units per milliliter (CFU/mL). Since some types of bacteria may not be detected, the expression "bacteria/mL" may not always be accurate.

Materials

Each student/team:

- Small sterile glass vials.
- Sterile rubber bands.
- 5 9 mL Dilution blanks of sterile water (screw tops).
- 5 Sterile Petri dishes.
- 5 Molten nutrient agar deeps, cooled to 45°C.
- 10 1 mL graduated pipettes.
- 1 Test tube rack.
- 1 Pipette bulb or pump.

Lab equipment:

- Hot water bath at 45°C.
- Thermometer

Procedure

1. Using sterile technique, transfer 1 mL saliva to the first dilution blank. Mix the bottle by inverting it 20 times. Label the bottle " 10^{-1} ."
2. Using a fresh pipette, transfer 1 mL from the first blank to the second blank. Mix as before. Label the second bottle " 10^{-2} ."

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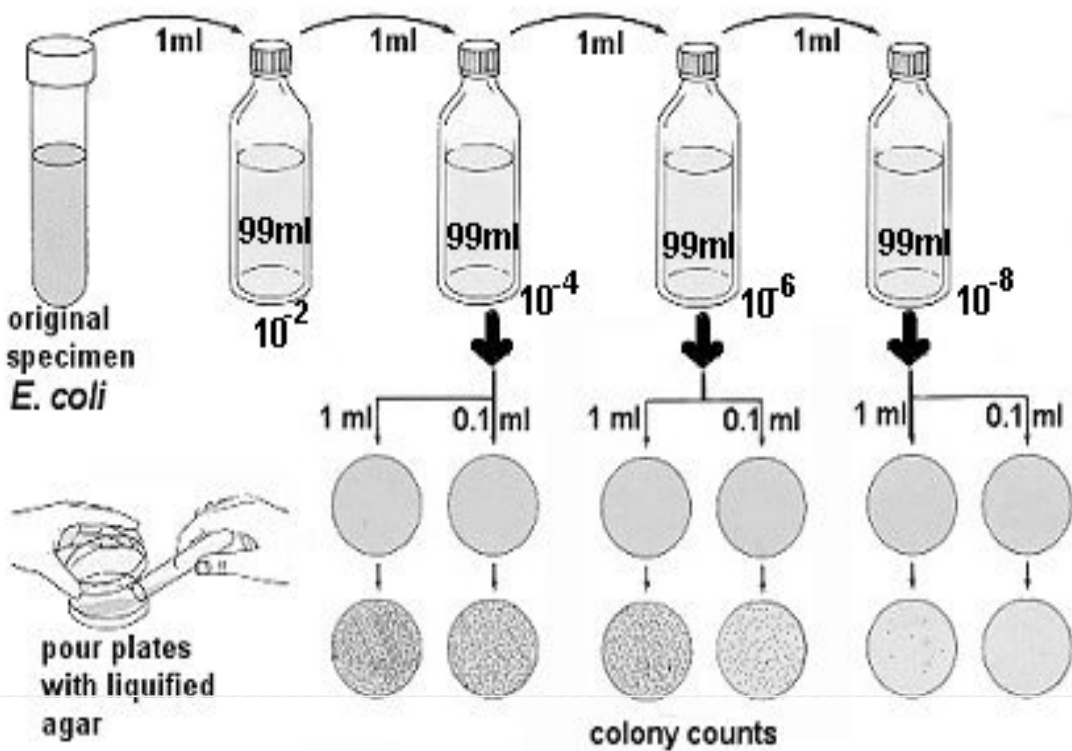
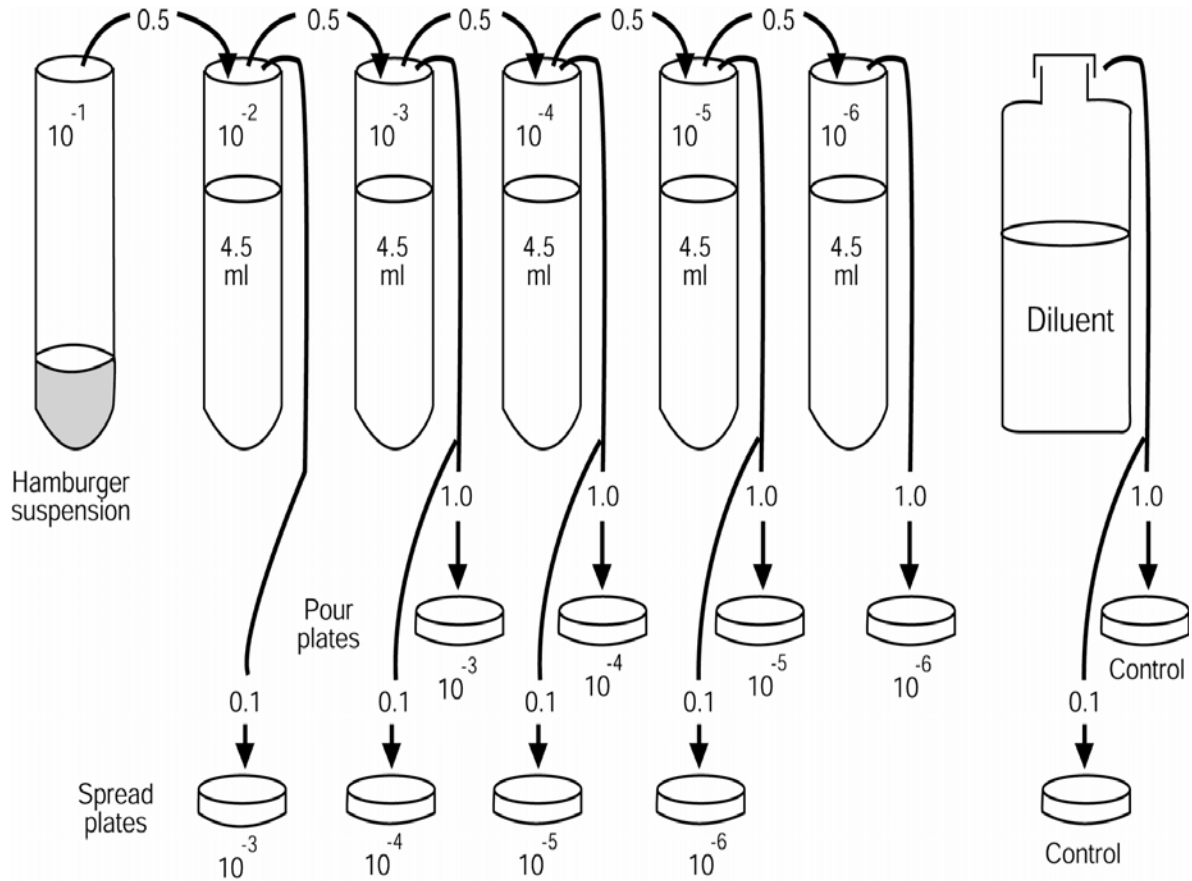
3. Using a fresh pipette, transfer 1 mL from the second blank to the third blank. Mix as before. Label the third blank " 10^{-3} ."
4. Using a fresh pipette, transfer 1 mL from the third blank to the fourth blank. Mix as before. Label the fourth blank " 10^{-4} ."
5. Using a fresh pipette, transfer 1 mL from the fourth blank to the fifth blank. Mix as before. Label the fifth blank " 10^{-5} ."
6. Label the Petri dishes: 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} , respectively.
7. Using the measurements shown in Figure 12.1, transfer liquid from the dilution blanks to the Petri dishes. Use a separate pipette for each blank, not for each plate (i.e. if more than one plate uses liquid from a single blank, a single pipette may be used for that blank).
8. One at a time, add a tube of molten nutrient agar to each Petri dish. After adding the agar, gently swirl the dishes in pattern for 30 seconds to mix the bacteria with the agar.
9. After the agar has thoroughly solidified, incubate the plates at 37°C for 24 to 48 hours.
10. Count the number of colonies on a plate that has between 30 and 200 colonies. Any plate which has more than 200 colonies is designated as "too numerous to count" (TNTC). Plates with fewer than 30 colonies do not have enough individuals to be statistically acceptable.
11. To compute the number of CFU/mL, use the formula:
$$c = \frac{n}{sd}$$

c = concentration, CFU/mL
n = number of colonies
d = dilution blank factor
s = volume transferred to plate.
12. Record your results as CFU/mL of saliva.

Petri Dish	Dilution Blank	Transfer Volume
10^{-2}	10^{-2}	1.0 mL
10^{-3}	10^{-3}	1.0 mL

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10^{-4}	10^{-4}	1.0 mL
10^{-5}	10^{-5}	1.0 mL
10^{-6}	10^{-5}	0.1 mL



CALCULATIONS

Practice your understanding of dilutions with the following problems:

1. You perform a serial dilution.

First dilution - pipette 1 ml of culture into 9 ml of water.

Second dilution - pipette 1 ml of first dilution into another 9 ml of water.

Third dilution - pipette 1ml of the second dilution into 99 ml of water.

Diagram this procedure. What is the total dilution?

2. You perform a serial dilution starting with a culture containing 1,000,000 organisms/ml.

First dilution - pipette 1 ml of culture into 99 ml of water.

Second dilution - pipette 1 ml of first dilution into another 99 ml of water.

Third dilution - pipette 1ml of the second dilution into 9 ml of water.

What is the total dilution at each step? What is the number of organisms/ml at each step?

3. In the previous question, you prepare pour plates from the second dilution by pipetting either 0.1 ml or 1 ml samples. How many organisms would you expect on these plates?

4. You have a culture containing 10^9 organisms/ml and wish to dilute it to 10^2 (100) organisms/ml. Design a serial dilution.

5. You have performed the following serial dilutions: 1:100, 1:100, 1:10. Diagram this dilution scheme and include the overall dilution in each tube.

From each dilution, you then prepare pour plates using either 0.1 ml or 1 ml samples. After incubation, you count the plates and get the following results:

What was the number of organisms/ml in the initial culture?

Final dilution	No of colonies 1 ml	No. of colonies 0.1 ml
1:100	TNTC	TNTC
1:10,000	TNTC	63
1:100,000	59	7

Exercise 22: Bacteria generation Time

Introduction

Bacterial population can be estimated by measuring the optical density or turbidity of a suspension. When several measurements are made over time, the growth rate of the bacteria can be calculated. Bacteria multiply by binary fission; one bacterial cell divides to form two cells. Optical density is directly proportional to the number of cells. When the bacterial population doubles, the optical density of the bacterial suspension also doubles. The average time required for one bacterium to divide under optimum conditions is called the generation time.

In this experiment, students will measure optical density with a spectrophotometer. The spectrophotometer measures the amount of light that is absorbed by a sample (in this case, a bacterial suspension). As the number of cells within a given volume increases, the amount of light transmitted through the sample decreases. Therefore, light absorption is positively correlated with cell concentration.

Materials

Each student/team:

- 2 Spectrophotometer sample tubes (cuvettes), sterile.
- 2 Nutrient broth tubes.
- 1 1 mL pipette, sterile.
- 1 400 mL beaker

Lab supplies:

- Nutrient broth cultures of *E. coli* (6- to 18-hours old). If possible, cultures should be transferred to fresh broth 2-3 hours before the lab starts.
- Spectrophotometers (Spectronic 20 or equivalent).
- 37°C water.

Procedure

1. Mix the *E. coli* culture well. Transfer 1 mL of the culture to one of the nutrient broth tubes. Mix the tube gently to evenly suspend the bacteria.
2. Set the wavelength control on the spectrophotometer to 750 nm.

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3. With no tube in the sample holder, set the spectrophotometer meter needle to infinity (not all spectrophotometers have this adjustment; check with the instructor for specific details concerning each spectrophotometer). Note that most spectrophotometers have two scales- transmission and absorbance. Use the absorbance scale for this experiment.
 4. Aseptically transfer the contents of the second nutrient broth tube to one of the spectrophotometer tubes. Fill the spectrophotometer tube approximately three-quarters full. This will be used as a blank, against which the bacterial population will be compared.
 5. Insert the blank into the spectrophotometer, and set the meter needle to zero.
 6. Pour the contents of the first nutrient broth tube (the one with the *E. coli*) into the other spectrophotometer tube.
 7. Insert the spectrophotometer tube into the sample holder and read the absorbance scale. Record this as the absorbance for time 0.
 8. Remove the spectrophotometer tube and place it in the beaker of 37°C water in an incubator. Check the absorbance of the bacteria every 10 minutes for the next hour. Keep the tube in the warm water at all times except when the optical density is being measured. The spectrophotometer should be reset to 0 with the blank tube before each measurement.
1. Plot the results of the experiment on the graph on the following page. Draw a straight, "best fit" line through the data points. Use the graph to estimate the generation time of the bacteria. (How much time was required to double the absorbance?)

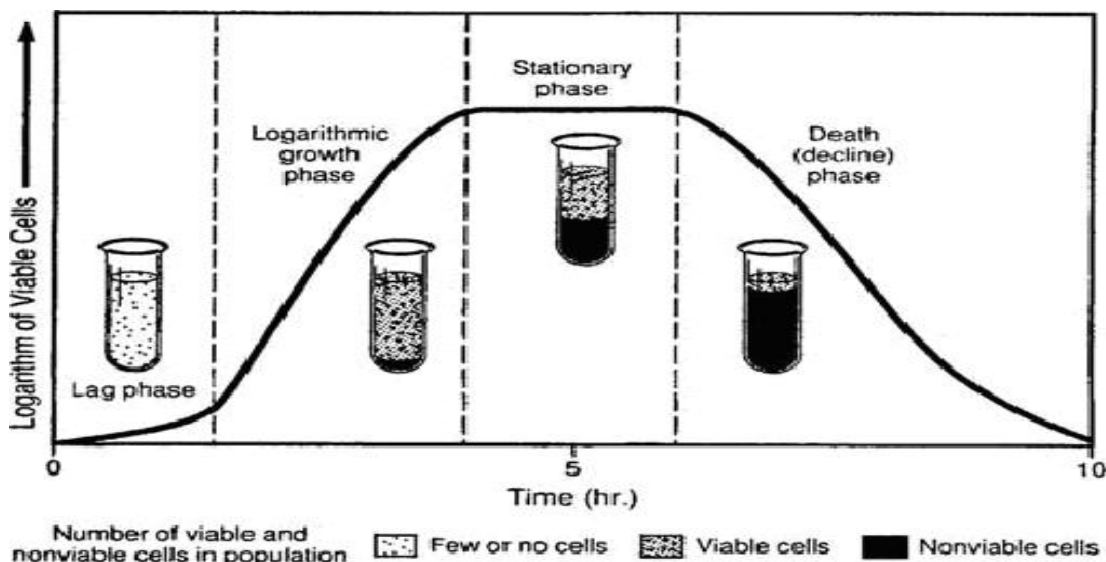


Figure (37): Growth curve

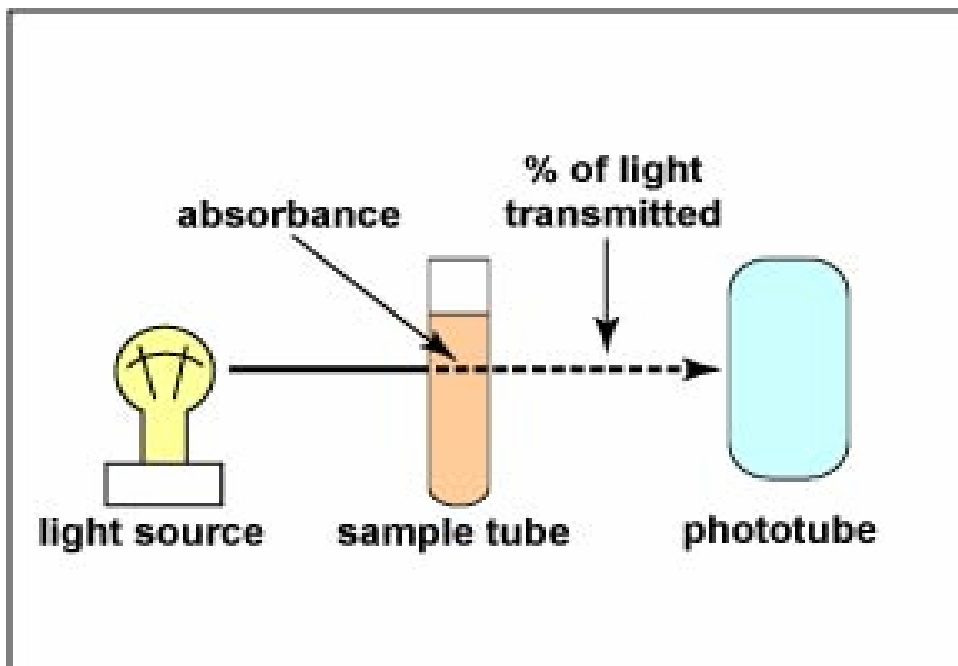
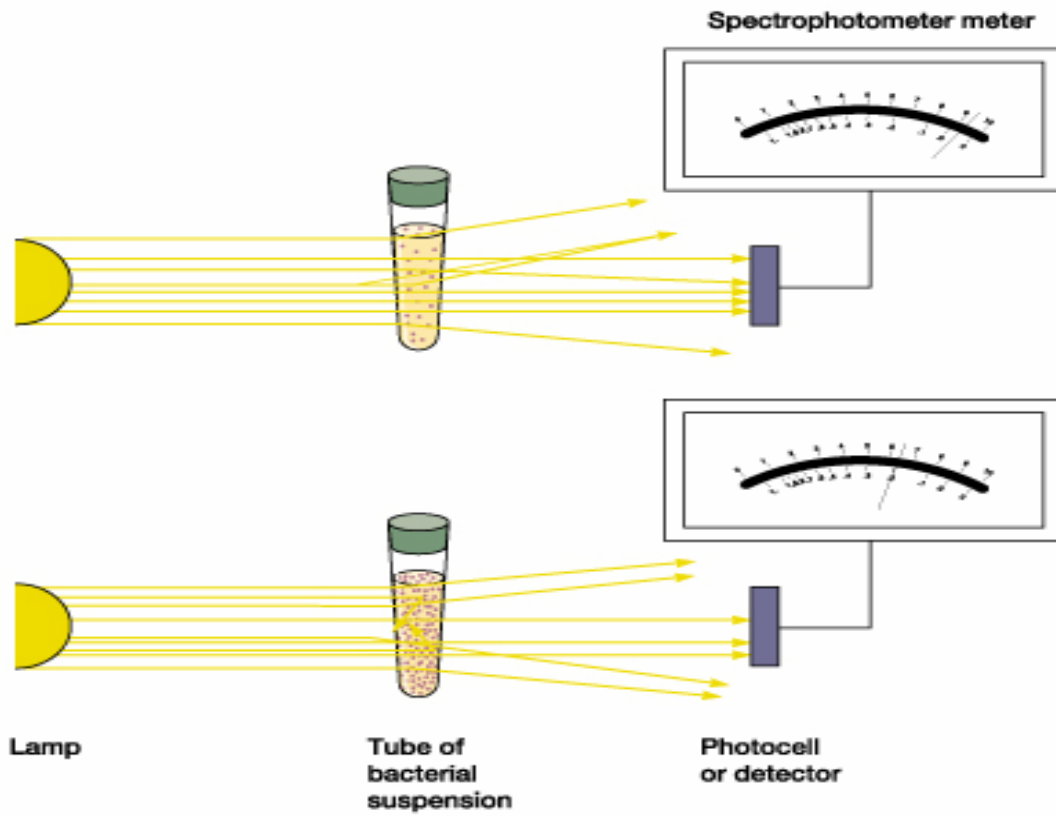


Figure (38):

Microbial control agents

Introduction

Even though some students have trouble getting bacteria to grow, usually the problem is just the opposite. Bacteria and other microorganisms are found almost everywhere. Usually, microbial controls are used to avoid contamination of pure cultures, prevent infection, or treat existing diseases.

A **microbicidal** effect kills microorganisms. A **microbistatic effect** prevents the reproduction of microorganisms. **Antiseptics** are chemicals used on living tissues to inhibit the growth of microorganisms. **Disinfectants** are chemicals used on nonliving surfaces to inhibit the growth of microorganisms. **Chemotherapeutic agents (antibiotics)** are chemicals used to destroy or inhibit the growth of microorganisms in living tissues.

Ionizing radiation (gamma, x-ray) kills microorganisms by causing the loss of electrons from molecules, thus ionizing them. The ionized molecules may lose critical chemical and physical structures, or react with other molecules, making the biochemicals useless. Ionization of water molecules in cells can produce highly toxic hydrogen peroxide (H₂O₂), which also damages cell components. Ultraviolet light does not have enough energy to cause ionization. UV causes damage to nucleic acids by inducing covalent bonds between adjacent thymine bases, resulting in **thymine dimerization**. The thymine dimers change the structure of DNA, preventing DNA replication and RNA transcription.

Chemical agents of microbial control act by changing critical cell chemicals, or interfering with cellular processes. Many antibiotics are structurally similar to chemicals processed by enzyme systems. They work by competing with normal chemicals for enzyme active sites. Other antibiotics interfere with protein synthesis at some point in the process.

Some Concept:

Selective toxicity: kills harmful microbes without damaging the host.

Broad-spectrum: Drugs that are effective against a variety of both gram-positive and gram-negative bacteria .

Narrow-spectrum: Drugs that are effective against gram-positive bacteria, just gram negative bacteria, or only a few species

Combination of drugs:

Synergism : action of two antibiotics greater

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Antagonism : action of drug is reduced; less effective

Minimum Inhibitory Concentration (MIC): The lowest concentration of chemotherapeutic agent capable of preventing growth of the test organism.

Minimum Bactericidal Concentration (MBC). MBC is the lowest concentration of the chemotherapeutic agent that results in no growth (turbidity) of the subcultures

Five Modes of Antimicrobial Activity:

1. Injury to Plasma Membrane (polymixin B).
2. Inhibition of Cell Wall Synthesis (penicillins, bacitracin).
3. Inhibition of Protein Synthesis (translation).
4. Inhibition of Nucleic Acid replication & transcription.
5. Inhibition of essential metabolites.

Materials

Each student/team:

8 nutrient agar plates.
1 thumb forceps (tweezers).
1 bent glass rod.
2 sterile 1 mL pipettes.
Alcohol for sterilization.
Metric ruler.



Lab supplies:

Antibiotic sensitivity disks.
Sterile paper disks.
Assorted antiseptics.
Ultraviolet lamps.
Nutrient broth cultures of *Escherichia coli* and *Bacillus cereus* (both 24- to 48-hour).

Procedure

1. Using a pipette and proper sterile technique, transfer 0.5 mL of *E. coli* suspension to each of four nutrient agar plates. Sterilize the bent glass rod by dipping it in alcohol and igniting it. Repeat this two more times. Allow the glass rod to cool in the air (**do not blow on the rod to cool it**). Use the glass rod to evenly spread the bacteria around the plates.
2. Using the other four plates, repeat step 1 with *B. cereus*.
3. Uncover one of the *E. coli* plates. Place it under the UV lamp for three minutes. Replace the lid on the plate, and incubate it for 24-48 hours at 37°C. Repeat this process with one of the *B. cereus* plates.
4. Dispense a set of antibiotic sensitivity disks onto an *E. coli* plate and a *B. cereus* plate. Consult the instructor for directions on using the disk dispenser. Use a sterile loop or inoculating needle to lightly press each disk against the agar. Do not push the disks down into the agar. Incubate the plates for 24-48 hours at 37°C.
5. Sterilize the forceps in the same manner as the bent glass rod. Use the forceps to dip a sterile paper disk into an antiseptic. Place the antiseptic disk onto an *E. coli* plate. Repeat this with at least two other antiseptics on the same plate. Apply one untreated paper disk as a control. Treat a *B. cereus* plate in the same manner. Incubate the plates for 24-48 hours at 37°C.
6. The remaining two plates will be used as controls. Incubate them with the treated plates.
7. After incubation, record your observations of each plate. Measure the diameters of the clear zones (in mm) around the antibiotic and antiseptic sensitivity disks. Note the differences in the numbers of colonies between the UV treated plates and the control plates.

Exercise 23: Bacterial Isolation and Identification

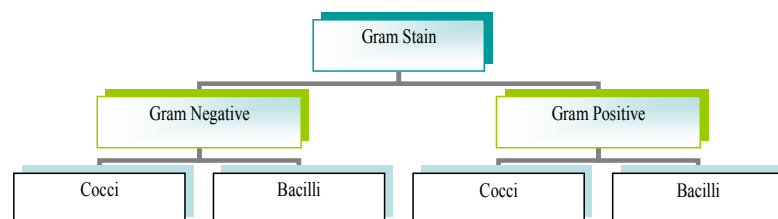
A. Gram positive coccus identification

Introduction

The previous laboratory exercises were intended to provide the basics of microbiological techniques. In this exercise and in subsequent exercises, those techniques will be put to practical use in the identification of microorganisms.

Staphylococcus, *Enterococcus* and *Lactococcus* are Gram positive, facultatively anaerobic cocci. Many species of *Lactococcus* and *Enterococcus* were once members of *Streptococcus* (another genus of Gram positive cocci). One of the primary differences between the genera is that *Staphylococcus* possesses the enzyme, **catalase**, while *Enterococcus* and *Lactococcus* do not. *Enterococcus* and some members of *Staphylococcus* are capable of growing in media containing 6.5% (w/v) sodium chloride (NaCl), while *Lactococcus spp.* do not tolerate more than 0.5% NaCl. These will be the principal characteristics used to differentiate the genera in this exercise. In addition, *Staphylococcus* usually forms much larger colonies than the other two genera. Other biochemical tests are used to identify individual species within each genus.

Although many biochemical tests are required to identify all of the species within each genus, we will only examine a few tests for each. *Lactococcus* and *Enterococcus* can be differentiated by whether or not they grow in the presence of 6.5% NaCl. Noting how they ferment mannitol and trehalose under anaerobic conditions can identify three species of *Staphylococcus*. To test for carbohydrate fermentation, we will use oxidative-fermentative (O-F) medium. It is similar to phenol red media, but turns from green to yellow if the particular carbohydrate is used. O-F medium can be used to test both oxidative and fermentative metabolism. If fermentation needs to be tested, the medium is overlaid with a layer of vaspar. Vaspar is a 50/50 mixture of mineral oil and paraffin that is used to seal media from oxygen, thus producing an anaerobic environment.



Materials

Each student/team:

- 2 O-F D-mannitol deeps.
- 2 O-F D-trehalose deeps.
- 2 O-F sucrose deeps.
- 2 6.5% NaCl nutrient broth tubes.

Lab supplies:

- 3% H₂O₂.
- Glass slides.
- Tryptose agar slants of *Enterococcus (Streptococcus) faecalis*, *Lactococcus (Streptococcus) lactis* (24- to 48-hour).
- Tryptose agar slants of **two** of the following: *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Staphylococcus saprophyticus* (24- to 48-hour).

Note: cultures should be labeled with numbers only. Allow students to attempt to identify each culture.

Procedure

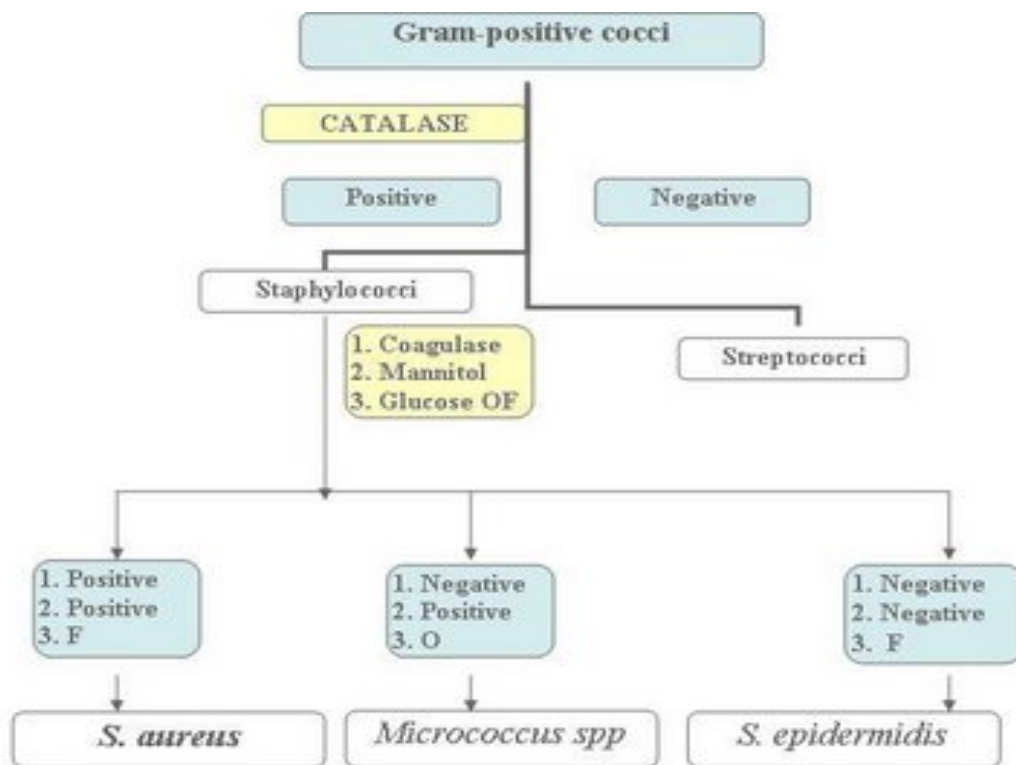
1. Using a loop, apply a heavy inoculum of each culture to a separate glass slide. Apply a drop of hydrogen peroxide (H₂O₂) to each inoculum. Note whether each culture produces bubbles. Bubble production is a positive indication of catalase.
2. Using an inoculating needle and proper sterile technique, inoculate catalase positive cultures into separate deeps of O-F mannitol and O-F trehalose (do not use the samples from the catalase test). Each deep should be stabbed four times in different places.
3. Incubate the deeps at 37°C for 24 to 48 hours. After incubation, check the tubes for the presence of acid (yellow color).
4. Using a loop and proper sterile technique, inoculate the catalase negative cultures into separate tubes of 6.5% NaCl nutrient broth (do not use the samples from the catalase test). Incubate the tubes at 37°C for 24-48 hours.
5. After incubation, examine the tubes for growth. *Enterococcus spp.* grow in 6.5% NaCl; *Lactococcus spp.* do not. Refer to chart to identify the organisms.



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Test	<i>S. aureus</i>	<i>S. epidermidis</i>	<i>S. saprophyticus</i>	<i>Enterococcus faecalis</i>	<i>Lactococcus lactis</i>
Catalase	+	+	+	-	-
Acid aerobically from D-mannitol	+	-	+/-	n/a	n/a
Acid aerobically from D-trehalose	+	-	+	n/a	n/a
Acid aerobically from sucrose	+	+	+	n/a	n/a
Growth in 6.5% NaCl	n/a	n/a	n/a	+	-

Identification key for selected cocci. "+" = positive result, "-" = negative result, "+/-" = variable result, "n/a" = not applicable



Exercise 23: B. *Pseudomonas* identification

Introduction

The genus *Pseudomonas* consists of Gram negative, non-fermentative, straight or slightly curved rods. Most species are motile. Some species may use nitrate as a final electron acceptor. Some species are facultative chemolithotrophs, using H₂ or CO as energy sources.

The genus *Pseudomonas* contains over sixty species, and a large battery of tests is necessary to identify all of them. We will concentrate on only three tests and four species. These tests should already be familiar: gelatin hydrolysis, starch hydrolysis, and carbohydrate use (trehalose). To test for trehalose utilization, we will use oxidative-fermentative (O-F) medium. It is similar to phenol red media, but turns from green to yellow if the particular carbohydrate is used.

Although *Pseudomonas* is nonfermentative, O-F media can be used to test both oxidative from fermentative metabolism. If fermentation needs to be tested, the medium is overlaid with a layer of vaspar. In this exercise, the vaspar is not used.

Many species of *Pseudomonas* produce fluorescent and/or non-fluorescent pigments under specific conditions. While we will not specifically test for these pigments, pigments show up in some of the commonly used media.

Materials

Each student/team:

- 3 Nutrient gelatin deeps.
- 3 Starch agar plates.
- 3 Trehalose O-F deeps.

Lab supplies:

Nutrient agar slants of **three** of the following: *Pseudomonas aeruginosa*, *P. fluorescens*, *P. putida*, *P. saccharophila* (all 24- to 48-hour).

Note: cultures should be labeled with numbers only. Allow students to attempt to identify each culture.

Procedure

1. Using an inoculating needle and proper sterile technique, inoculate separate O-F trehalose deeps with each of the organisms.
2. using an inoculating needle, inoculate separate nutrient gelatin deeps with each organism.

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- Using a loop, inoculate separate starch agar plates with a single streak of each organism.
- Incubate all of the media at 20-25°C (**room temperature**) for 24-48 hours.
- Chill the nutrient gelatin to 4°C. Check for gelatin hydrolysis.
- Flood the starch agar plate with IKI solution or Gram's iodine. Check for clear zones that indicate starch hydrolysis

Organism	Gelatin Hydrolysis	Starch Hydrolysis	Acid aerobically from D-Trehalose	Fluorescent Pigment	Non-fluorescent Pigment
<i>Pseudomonas aeruginosa</i>	+/-	+/-	-	+	+
<i>Pseudomonas fluorescens</i>	+	-	+	-	+
<i>Pseudomonas putida</i>	-	-	-	+	+
<i>Pseudomonas saccharophila</i>	+	+	+	-	-

Identification key for selected species of *Pseudomonas*. "+" = positive result, "-" = negative result, "+/-" = variable result.

Exercise 23: C. Enterobacteriaceae identification

Introduction

All members of the family Enterobacteriaceae are Gram negative, non-acid-fast, non-spore-forming rods that ferment glucose. The organisms are also facultatively anaerobic, oxidase negative and reduce nitrate. Most members are inhabitants of the gastrointestinal tract, though many live in other environments as well. Some are pathogenic.

Identification of genera within this family is often difficult so labs usually identify isolates directly to the species level. Since the family Enterobacteriaceae contains at least 115 species, a large battery of tests must be used. This exercise will use only a small subset of the normal battery of tests to identify a few species of bacteria.

This exercise introduces some new media. **Triple sugar iron (TSI)** agar is used to test for the production of hydrogen sulfide (H_2S) by some bacteria. The medium contains glucose, lactose and sucrose; phenol red as a pH indicator; and ferrous sulfates ($FeSO_4$). The $FeSO_4$ reacts with H_2S to form a black precipitate. TSI agar is also used to screen for carbohydrate utilization. Yellow color only on the slant indicates glucose fermentation. Yellow throughout the medium indicates lactose and/or sucrose fermentation. Bubbles in the agar indicate gas production. **Simmons citrate agar** is used to test for the ability of a bacterium to utilize citrate (citric acid) as its sole carbon source. When citrate is utilized, the agar turns from a green color to a bright blue color.

Materials

Each student/team:

- 3 Phenol red glucose broth tubes with Durham tubes.
- 3 Phenol red lactose broth tubes.
- 3 Triple sugar iron agar slants.
- 3 Simmons citrate agar slants.



Lab supplies:

Nutrient agar slants of **three** of the following: *Citrobacter freundii*, *Citrobacter intermedius*, *Escherichia coli*, *Salmonella typhimurium* (all 24- to 48-hour).

Procedure

1. Using a loop and proper sterile technique, inoculate separate phenol red glucose and phenol red lactose broth tubes with each of the cultures.
2. using a needle, inoculate separate TSI slants with each culture by first streaking the organisms across the surface of the slant, and then stabbing the needle to the butt of the slant. Do not flame the needle between the streak and the stab
3. using a loop, inoculate the surfaces of separate Simmons citrate agar slants with each culture.
4. Incubate all of the media at 37°C for 24-48 hours.
5. Record the results of the tests. Use the Figure to identify the organisms.

	Gas from glucose	Acid from lactose	Citrate utilization	H ₂ S production
<i>Escherichia coli</i>	+	+	-	-
<i>Citrobacter freundii</i>	+	+	+	+
<i>Citrobacter intermedium</i>	+	+	+	-
<i>Salmonella typhimurium</i>	+	-	+	+

Identification key for selected species of the family Enterobacteriaceae. "+" = positive result, "-" = negative result

Exercise 23: D. Identification of unknown bacteria.

Introduction

In this lab, the instructor will give each student an unlabelled culture. The student will be responsible for identifying the bacterium in question. The possible bacteria will be drawn from those used in previous exercises.

Materials

Cultures (broth) and supplies from previous exercises.
Nutrient agar slants.

Procedure

1. Perform a Gram stain on the unknown culture.
2. Inoculate the culture onto a nutrient agar or tryptose agar slant. Incubate it at 37°C for 24-48 hours.
3. Refer to Figure 39 to determine the next steps involved. If Figure 39 refers to methods involving the catalase test, use organisms from the slant in step 2.
4. If the Gram stain reveals a Gram negative rod, determine the organism's oxygen requirements by inoculating it into a BHI deep that has been melted and cooled).
5. After all tests have been completed, give the identification of the unknown to the instructor.

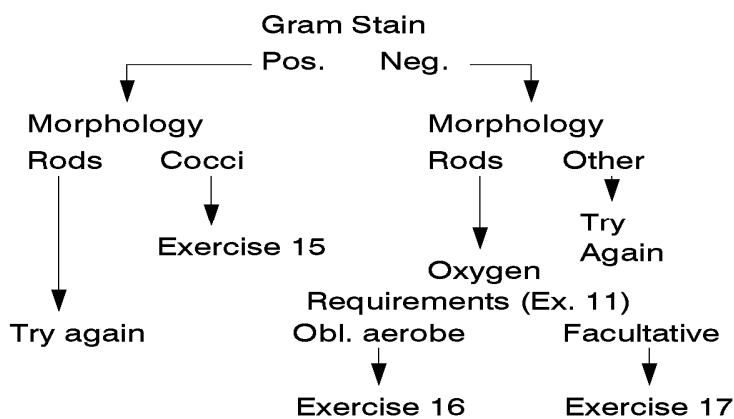
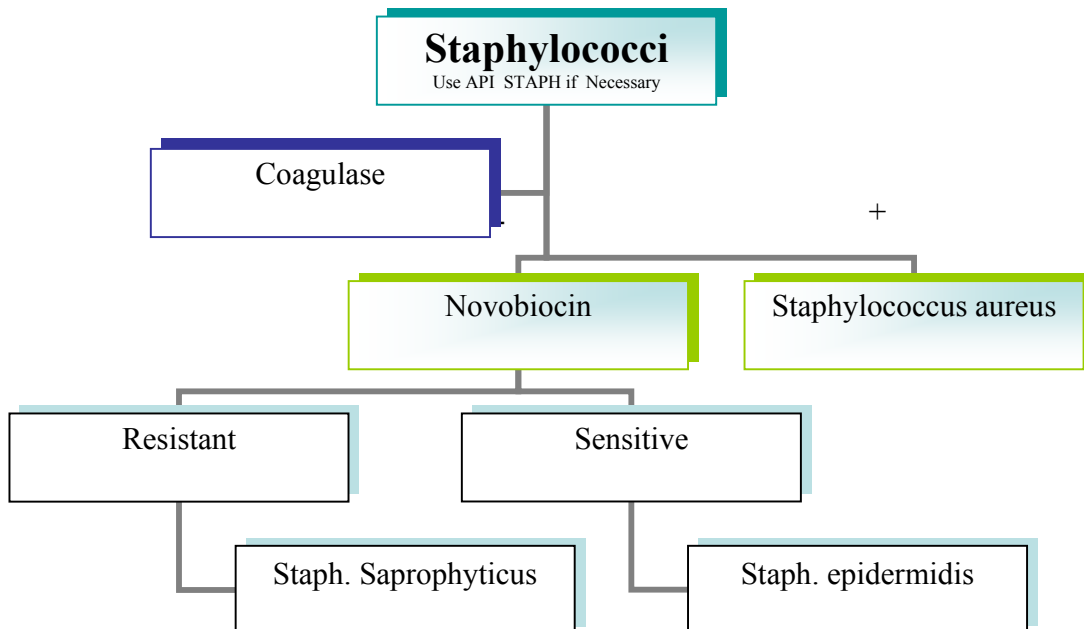
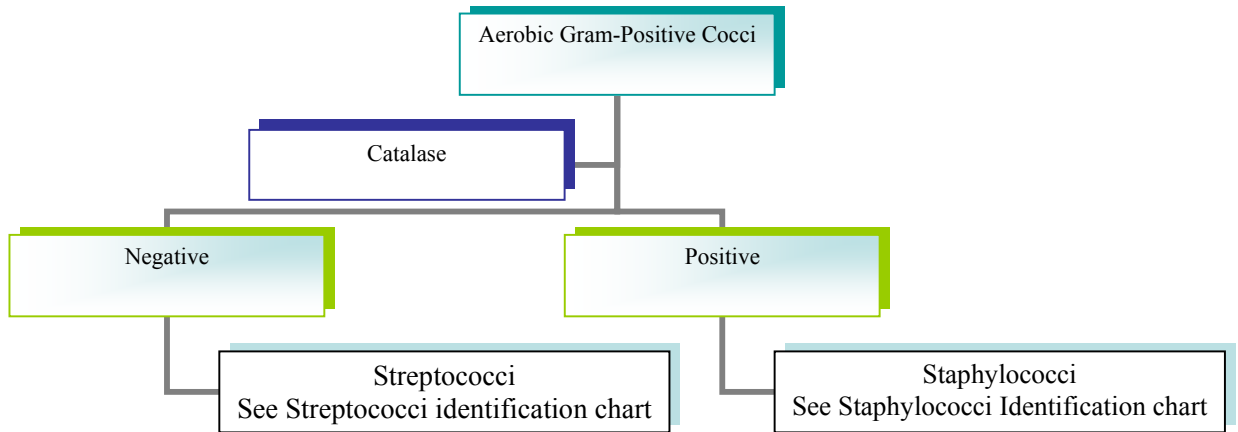
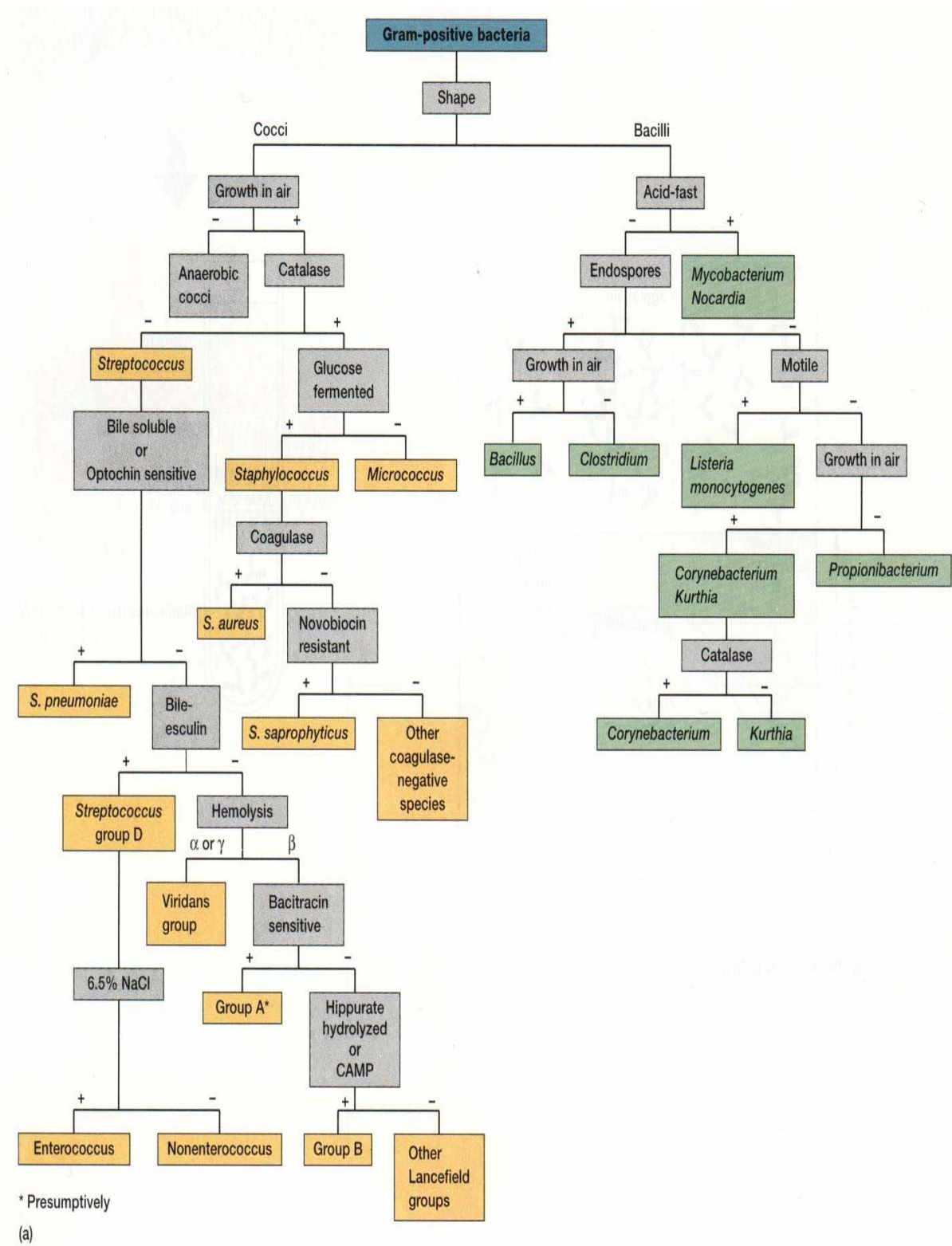


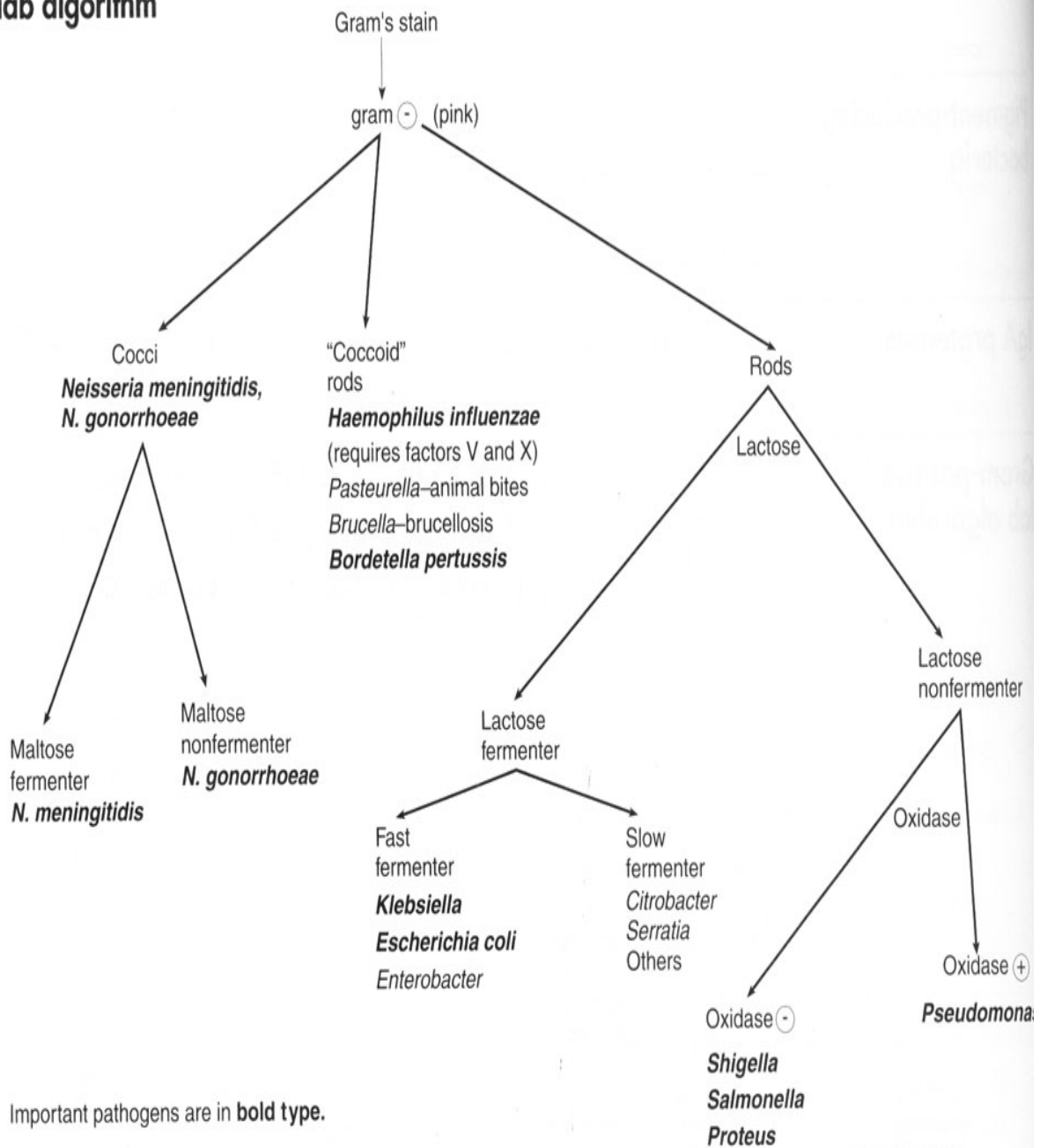
Figure (39): Key for unknown bacteria cultures





MICROBIOLOGY – CLINICAL BACTERIOLOGY (continued)

Gram-negative lab algorithm



Exercise 23: **E. Microbes in the atmosphere.**

Introduction

Throughout this lab manual, proper sterile technique has been repeatedly stressed because of the possibility of contamination by airborne and other microorganisms. In this exercise, we will attempt to isolate and characterize a few airborne microorganisms. BG-11 is a nutrient salts medium that is used to isolate photosynthetic microorganisms (cyanobacteria and algae). It contains virtually no organic carbon, but the sodium bicarbonate provides an inorganic carbon source.

Materials

Each student/team:

4 BG-11 (10 mM NaHCO₃) agar plates.

4 Nutrient agar plates.

Gram stains materials.

Procedure

1. Choose four locations from which bacteria will be sampled. These may be different rooms, locations within one room, or even outside of the building.
2. Label the plates with the sample location as well as students' names, dates, etc.
3. Put an **open** plate of each type at each location. Leave it there for 30 minutes.
4. Cover the nutrient agar plates and incubate them for 24-48 hours at 37°C.
5. Cover the BG-11 plates and wrap the edges with laboratory film (Parafilm). Incubate them at 20°-30°C in moderate light for 120 or more hours. A plant growth chamber or indirect sunlight may be used for incubation. The laboratory film prevents the agar from drying out during the long incubation time.
6. After incubation, perform a Gram stain for at least one isolated colony from each nutrient agar plate. Examine any green colonies on the BG-11 plates under the microscope. Also note the number and types of colonies on each plate.

Exercise 23: F. Microbes in the soil.

Introduction

Soils harbor a wide variety of microbial life. Most soil organisms are responsible for decomposition and nutrient cycling. Without these microorganisms, life on Earth would very quickly come to a halt. Normal soil microbial communities include single-celled bacteria, filamentous bacteria (actinomycetes), fungi, protozoa, cyanobacteria and algae.

In this lab, we will attempt to isolate soil bacteria, algae and fungi. This lab introduces two new types of media. Rose Bengal agar contains a bacterial inhibitor, and is selective for fungi. Starch casein agar promotes the growth of actinomycetes (filamentous bacteria). Actinomycetes and fungi often have similar outward appearances to the naked eye. However, the differences between the two are easily seen under the microscope. Fungi have larger cells with nuclei, while actinomycetes have smaller cells without nuclei.

Materials

Each student/team:

- 2 BG-11 (10 mM NaHCO₃) agar plates.
- 2 Nutrient agar plates.
- 2 Rose bengal agar plates.
- 2 Starch casein agar plates.
- 2 99 mL water blanks.
- 1 1 mL sterile pipette.
- 1 Pipette bulb or pump.

Laboratory supplies:

- Soil samples.
- Triple beam balances.
- Sterile tongue depressors or chemical spatulas.
- Sterile weighing paper.

Procedure

1. Using the triple beam balance, measure out approximately 0.5 g of a soil sample onto a piece of sterile paper.
2. Transfer the soil sample into one of the sterile water blanks. Shake the soil suspension for one minute.

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3. Using the sterile pipette, transfer 1 mL from the soil suspension to the second dilution blank. Shake the second blank for one minute.
4. Using a loop and proper sterile technique, inoculate samples from the first blank onto one of each of the plates. Streak for isolation as shown in Figure 20.1.
5. Repeat the process in step 4 with the second blank and the remaining plates.
6. Incubate the plates at 25°C (room temperature) for 48-72 hours. Photosynthetic organisms may take longer to appear on the BG-11 plates. Be sure to provide them with moderate light.
7. After incubation, perform Gram stains on at least one isolate from each type of plate. Note the differences in size and morphology between single-celled bacteria, actinomycetes and fungi.

Selected website

<http://www.microbes.info/>
<http://science.nhmccd.edu/biol/microbio.html>
<http://www.kcom.edu/faculty/chamberlain/website/links.htm>
<http://www.sgm.ac.uk/links/>
<http://www.geocities.com/CapeCanaveral/3504/>
<http://www.microbiologyonline.org.uk/>
<http://www.microbiol.org/>
http://www.biology.duke.edu/bio103l/Bio103_Links.html
<http://www.google.com/Top/Science/Biology/Microbiology/Education/>
<http://www.bacteriamuseum.org/niches/wabacteria/bacteriologyL3.shtml>
<http://www.lib.uiowa.edu/hardin/md/micro.html>
<http://www.textbookofbacteriology.net/>
<http://www.medialabinc.net/clinical-laboratory-microbiology.asp>
<http://www.slic2.wsu.edu:82/hurlbert/micro101/pages/101hmpg.html>
<http://www.sp.uconn.edu/~terry/229su03/lectures.html>
<http://student.cbcemd.edu/~gkaiser/goshp.html>
<http://www.bact.wisc.edu/Bact330/Bact330Homepage>
<http://gsbs.utmb.edu/microbook/toc.htm>
<http://www.umsl.edu/~microbes/index.html>
<http://www2.hawaii.edu/~johnb/micro/>
<http://www.bact.wisc.edu/Bact303/Bact303mainpage>
<http://www.umsl.edu/~microbes/links-teachers.htm>

Appendix 1: Formula for microbiological media.

Most of the following media are available commercially as concentrates or dehydrated preparations.

When necessary, the pH of the media may be adjusted to the proper values with the addition of 0.1 N HCl or NaOH. For the best results, media pH should be within ± 0.1 of the listed value.

BG-11 freshwater cyanobacteria medium

	Stock g/L	Working mL/L	Working 2x mL/L	Final Conc.
NaNO ₃	150	10	20	17.6 mM
CaCl ₂ · 2 H ₂ O	86	1	2	200 μ M
FeNH ₄ -Citrate	12	1	2	
Na ₂ EDTA	1	1	2	
K ₂ HPO ₄	4	1	2	230 μ M
MgSO ₄ · 7 H ₂ O	75	1	2	304 μ M
Na ₂ CO ₃	20	1	2	190 μ M
Micro-nutrients	see below	1	2	
1 M HEPES or KH ₂ PO ₄	pH 8	20	40	

Micronutrient stock solution

	Stock, g/L	Stock conc., mM	Final conc., μ M
H ₃ BO ₃	2.86	46	46
MnCl ₂ · 4 H ₂ O	1.81	9.1	9.1
ZnSO ₄ · 7 H ₂ O	0.222	0.77	0.77
NaMoO ₄ · 2 H ₂ O	0.39	1.61	1.61
CuSO ₄ · 5 H ₂ O	0.079	0.316	0.316
Co(NO ₃) ₂ · 6 H ₂ O	0.0494	0.168	0.168

Make 1 L of each stock solution (micronutrients is one solution containing all of the micronutrient ingredients).

For *BG-11 broth*, add the appropriate amount of each stock solution listed under the **working** heading to 500 mL water. Add water to bring the final volume to 1000 mL. Autoclave at 121°C for 20 minutes.

For *BG-11 agar*, add the appropriate amount of each stock solution under the **working 2x** heading to 350 mL water. Add water to bring the final volume to 500 mL. In a separate container, dissolve 10 g agar in 500 mL water. Autoclave both containers at 121°C for 20 minutes. After cooling the liquids to 50-70°C, mix the two solutions and pour into plates or tubes.

If desired, 10 mL 1 M NaHCO₃ may be added to either preparation prior to autoclaving as an additional source of inorganic carbon.

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Brain-heart infusion agar

200	g	Calf brain infusion.
250	g	Beef heart infusion.
10	g	Proteose peptone.
2	g	Glucose.
5	g	NaCl.
2.5	g	Na ₂ HPO ₄ .
15	g	Agar.

Suspend the ingredients in 1000 mL cold distilled water. Heat to boiling to dissolve completely. Dispense into appropriate containers and autoclave for 15 minutes at 121°C. The medium should be pH 7.4 at room temperature.

Eosin-methylene blue (E.M.B.) agar (Levine)

10	g	Peptone.
10	g	Lactose.
2	g	K ₂ HPO ₄ .
15	g	Agar.
0.4	g	Eosin Y.
0.065	g	Methylene blue

Suspend the ingredients in 1000 mL cold distilled water. Heat to boiling to dissolve completely. Dispense into appropriate containers and autoclave for 15 minutes at 121°C. The medium should be pH 7.1 at room temperature.

MacConkey agar

17	g	Peptone.
3	g	Proteose peptone.
10	g	Lactose.
1.5	g	Bile salts.
5	g	NaCl.
13.5	g	Agar.
0.03	g	Neutral red.
0.001	g	Crystal violet

Suspend the ingredients in 1000 mL cold distilled water. Heat to boiling to dissolve completely. Dispense into appropriate containers and autoclave for 15 minutes at 121°C. The medium should be pH 7.1 at room temperature.

Mannitol salt agar

1	g	Beef extract.
10	g	Proteose peptone.
75	g	NaCl.
10	g	d-Mannitol.
15	g	Agar.
0.025	g	Phenol red.

Suspend the ingredients in 1000 mL cold distilled water. Heat to boiling to dissolve completely. Dispense into appropriate containers and autoclave for 15 minutes at 121°C. The medium should be pH 7.4 at room temperature.

Milk, skim

100	g	Skim milk powder.
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Suspend the ingredients in 1000 mL cold distilled water. Dispense into appropriate containers and autoclave for 15 minutes at 121°C. Be careful not to overheat the medium. The medium should be pH 6.4 at room temperature.

Nutrient agar

3	g	Beef extract.
5	g	Peptone.
15	g	Agar.

Suspend the ingredients in 1000 mL cold distilled water. Heat to boiling to dissolve completely. Dispense into appropriate containers and autoclave for 15 minutes at 121°C. The medium should be pH 6.8 at room temperature.

Nutrient broth

3	g	Beef extract.
5	g	Peptone.

Suspend the ingredients in 1000 mL cold distilled water. Dispense into appropriate containers and autoclave for 15 minutes at 121°C. The medium should be pH 6.8 at room temperature.

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Nutrient gelatin

3	g	Beef extract.
5	g	Peptone.
120	g	Gelatin.

Suspend the ingredients in 1000 mL cold distilled water. Heat to 50°C to dissolve completely. Dispense into appropriate containers and autoclave for 15 minutes at 121°C. The medium should be pH 6.8 at room temperature.

Oxidative-fermentative (O-F) test medium

2	g	Peptone or tryptone.
5	g	NaCl.
2.5	g	Agar.
0.3	g	K ₂ HPO ₄ .
0.03	g	Bromthymol blue.
100	m	10% carbohydrate solution
	L	(sterile).

Suspend the dry ingredients in 1000 mL cold distilled water. Heat to boiling to dissolve completely. Autoclave for 15 minutes at 121°C. After cooling the medium to 55°C, aseptically add the carbohydrate solution of choice and mix completely. Note that the carbohydrate solution should be sterilized by filtration through a 0.2 µm membrane filter; heat sterilization may decompose some carbohydrates. Dispense 5 mL aliquots into screw cap test tubes, and allow the medium to solidify. The medium should be pH 7.1 at room temperature. Tests for oxidative utilization are performed by inoculating (stabbing) the medium several times, and incubating for 24-48 hours. Tests for fermentative utilization are performed as above, but the tubes are overlaid with 1 cm of vaspar.

Phenol red agar base

1	g	Beef extract.
10	g	Proteose peptone.
5	g	NaCl.
15	g	Agar.
0.025	g	Phenol red.
10	g	Selected carbohydrate

Suspend the ingredients in 1000 mL cold distilled water. Heat to boiling to dissolve completely. Dispense into appropriate containers and autoclave for 15 minutes at 121°C. The medium should be pH 7.4 at room temperature.

Phenol red broth base

1	g	Beef extract.
10	g	Proteose peptone.
5	g	NaCl.
0.018	g	Phenol red.
5	g	Selected carbohydrate.

Suspend the ingredients in 1000 mL cold distilled water. Heat to boiling to dissolve completely. Dispense into appropriate containers and autoclave for 15 minutes at 121°C. The medium should be pH 7.4 at room temperature.

Rose bengal agar

10	g	Glucose.
5	g	Peptone.
1	g	KH ₂ PO ₄ .
0.5	g	MgSO ₄ ·7H ₂ O
0.03	g	Rose bengal.
20	g	Agar.

Suspend the ingredients in 1000 mL cold distilled water. Heat to boiling to dissolve completely. Dispense into appropriate containers and autoclave for 15 minutes at 121°C. Cool to 48°C, then add 1 mL of 30% (w/v) streptomycin solution.

Simmons citrate agar

0.2	g	MgSO ₄ .
1	g	NH ₄ H ₂ PO ₄ .
1	g	K ₂ HPO ₄ .
2	g	Sodium citrate.
5	g	NaCl.
15	g	Agar.
0.08	g	Bromthymol blue.

Suspend the ingredients in 1000 mL cold distilled water. Heat to boiling to dissolve completely. Dispense into appropriate containers and autoclave for 15 minutes at 121°C. The medium should be pH 6.8 at room temperature.

Starch agar

15	g	Proteose peptone.
10	g	Starch
5	g	NaCl.
3	g	Na ₂ HPO ₄ .
20	g	Agar.

Suspend the ingredients in 1000 mL cold distilled water. Heat to boiling to dissolve completely. Dispense into appropriate containers and autoclave for 15 minutes at 121°C. The medium should be pH 7.3 at room temperature.

Starch-casein agar

10	g	Starch.
0.3	g	Casein (vitamin free).
2	g	KNO ₃ .
2	g	NaCl.
2	g	K ₂ HPO ₄ .
0.05	g	MgSO ₄ ·7H ₂ O.
0.02	g	CaCl ₂ .
0.01	g	FeSO ₄ ·7H ₂ O.
15	g	Agar.

Suspend the ingredients in 1000 mL cold distilled water. Heat to boiling to dissolve completely. Dispense into appropriate containers and autoclave for 15 minutes at 121°C. The medium should be pH 7.2 at room temperature.

Thioglycollate broth

5	g	Yeast extract.
15	g	Casitone.
1	g	Glucose.
2.5	g	NaCl.
0.05	g	l-Cysteine.
0.3	mL	Thioglycollic acid.

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Suspend the ingredients in 1000 mL cold distilled water. Heat to boiling to dissolve completely. Dispense into appropriate containers and autoclave for 18-20 minutes at 121°C. The medium should be pH 7.1 at room temperature.

Triple sugar iron agar

3	g	Beef extract.
3	g	Yeast extract.
15	g	Peptone.
5	g	Proteose peptone.
10	g	Lactose.
10	g	Sucrose.
1	g	Glucose.
0.2	g	FeSO ₄ .
5	g	NaCl.
0.3	g	Na ₂ SSO ₃ .
12	g	Agar.
0.024	g	Phenol red.

Suspend the ingredients in 1000 mL cold distilled water. Heat to boiling to dissolve completely. Dispense into appropriate containers and autoclave for 15 minutes at 121°C. The medium should be pH 7.4 at room temperature.

Tryptose agar

20	g	Tryptose.
1	g	Glucose.
5	g	NaCl.
15	g	Agar.
0.005	g	Thiamine hydrochloride.

Suspend the ingredients in 1000 mL cold distilled water. Heat to boiling to dissolve completely. Dispense into appropriate containers and autoclave for 15 minutes at 121°C. The medium should be pH 7.2 at room temperature.

Appendix 2: Bacterial cultures Stated in this manual.

Bacillus cereus
Bacillus subtilis
Citrobacter freundii
Citrobacter intermedius
Clostridium butyricum
Clostridium sporogenes
Enterococcus (Streptococcus) faecalis
Escherichia coli
Lactococcus (Streptococcus) lactis
Mycobacterium smegmatis
Pseudomonas aeruginosa
Pseudomonas fluorescens
Pseudomonas putida
Pseudomonas saccharophila
Salmonella typhimurium
Staphylococcus aureus
Staphylococcus epidermidis
Staphylococcus saprophyticus
Streptococcus (Enterococcus) faecalis
Streptococcus (Lactococcus) lactis

Appendix 3: Formulation for stains and reagents.

Acid alcohol

3	mL	Concentrated HCl.
97	mL	Ethanol (95%).

Add HCl to ethanol.

Carbolfuchsin stain

0.3	g	Basic fuchsin.
5	mL	Phenol (melted at 56°C).
10	mL	Ethanol (95%).
95	mL	Distilled water.

Dissolve basic fuchsin in the ethanol. Mix phenol with water, and then mix the two solutions together. Allow to set at 37°C for 24 hours. Filter through coarse paper before using.

Carbolfuchsin stain with Tergitol

Make carbolfuchsin stain as above, then add four drops of Tergitol #7 (Sigma Chemical Co.).

Crystal violet stain, stock solution

20	g	Crystal violet.
100	mL	Ethanol (95%).

Dissolve the crystal violet in the ethanol. Filter through coarse paper.

Crystal violet stain, working solution

1	mL	Crystal violet stock solution.
9	mL	Distilled water.
40	mL	Oxalate stock solution.

Mix crystal violet stock solution with water. Add to oxalate stock solution.

Gram's iodine solution

1	g	I ₂ crystals.
2	g	KI.
5	mL	Distilled water.
240	mL	Distilled water.
60	mL	NaHCO ₃ 5% aqueous solution.

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Dissolve I₂ and KI in 5 mL water. Add remaining water, followed by the nacho solution. Store in an amber glass bottle.

Malachite green stain

5	g	Malachite green.
100	mL	Distilled water.

Dissolve malachite green in water.

Methylene blue stain

0.3	g	Methylene blue.
30	mL	Ethanol (95%).
100	mL	Distilled water.

Dissolve methylene blue in ethanol. Add distilled water.

Nigrosin stain

10	g	Nigrosin.
100	mL	Distilled water.
0.5	mL	Formalin.

Mix nigrosin with distilled water. Heat in a boiling water bath for 30 minutes. Add formalin. Filter twice through fine paper.

Oxalate, stock solution

1	g	Ammonium oxalate.
100	mL	Distilled water.

Dissolve ammonium oxalate in water.

Safranin stain, stock solution

2.5	g	Safranin O.
100	mL	Ethanol (95%).

Dissolve safranin in ethanol.

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Safranin stain, working solution

1	mL	Safranin stock solution.
9	mL	Distilled water.

Mix ingredients together.

Appendix 4: Commons in Microbiology Lab.

Each student or student team should have the following:

Stain set:

- Acid alcohol
- Carbolfuchsin
- Carbolfuchsin with Tergitol
- Crystal violet
- Ethanol (95%)
- Gram's iodine
- Malachite green
- Nigrosin
- Safranin.

Staining tray.

Clothespin or commercial slide holder.

Test tube rack.

Inoculating loop.

Inoculating needle.

Bent glass spreading rod ("hockey stick").

Thumb forceps (tweezers).

Technical grade ethanol in wide mouth glass jar (for sterilization).

Bunsen burner or alcohol lamp.

Hot plate.

Lens paper.

Tissue paper.

Compound microscope with oil immersion objective lens.

Immersion oil.

Hydrogen peroxide (3%).

400-600 mL Beakers (2)