

Title: Prokaryote Species Identification by Peptide Isolation and Selective Medias
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Abstract

It is vital that high-achieving high school students are exposed to modern ideas and methodologies in the biological sciences. In order for students to better understand microorganisms, proteins, and modern biotechnology they will complete a multistep laboratory (including protein extraction, gel electrophoresis, mass spectrometry [outsourced] and data analysis) that will walk them through the identification of proteins in an assigned microorganism. In addition to this, they will use this collected data and classical laboratory techniques to help determine the genus and species of their organisms. By completing this laboratory, students will be exposed to numerous techniques, ideas, methodologies, and technologies that will better prepare them for their future education.

Rational

It has been a long running idea that students working in a laboratory setting will foster learning (Downing, 1917). Although high-achieving students in Advanced Placement Biology courses have to complete twelve mandatory laboratories, it has been suggested that these “cookbook” laboratory activities are not necessarily beneficial to student learning, but instead the activities need to have aspects of planning and ownership by the student to better understand concepts (Vance, 1952). The laboratory activity that is being proposed takes this into account and allows students to “make discoveries” with minimal direction (with the exceptions being new techniques not known to the student) and focuses on active exploration and discovery which has been shown to engage students in sciences courses (American Association for the Advancement of Science, 1990; National Research Council, 1996).

While it has been shown that it is important to allow for student ownership, it is also important to make sure that the content of the laboratory activity aligns with the requirements of the course. According to the College Board (2010), the Advanced Placement Biology course must include information on the following content: organic molecules in organisms, prokaryotic cells, molecular genetics, diversity of organisms, and the use of technology to study DNA/proteins. The laboratory activity that is proposed will explore all of these concepts with a heavy focus on prokaryotes and organic molecules that can be studied with modern technology. Students will cover a broad range of techniques and methodologies for this activity including the use of micropipettes, the extraction of proteins from living organisms, using gel electrophoresis to separate proteins, the digestion of proteins to peptides, the use of mass spectrometry, and the interpretation of these results in order to identify chosen proteins. Although there is a very broad scope to this activity, it has been shown that knowledge is most usable when ideas are linked into large, conceptual networks (Marx et al., 1997). There have been many laboratory activities in schools that will focus on one part mentioned above, such as gel electrophoresis. However, when the proposed activity is completed, it can be suggested that students will form a conceptual network of not only what gel electrophoresis does, but why scientists use it. Additionally, because students will be choosing the peptides that they want to identify, it allows then a certain amount of ownership to the project.

It has been shown that students will actively construct ideas when working on authentic tasks where they have to apply ideas (Collins, Brown, & Newman, 1989; Newman, Griffin, & Cole 1989; Resnick, 1987). It is believed by this author that this activity allows students to work on an authentic task that will require student planning and a degree of ownership.

Description of Teaching Unit

The activity that is being proposed is a modified version of the activity “Lesson 5: Proteomics- Protein extraction, separation and identification” (Chen, 2010). Although the basis of the activity will be the same, the organisms will be different. Additionally, students will use the acquired data to help determine the genus and species of organism that was assigned to them. Students will work in groups of two or three because it has been shown small groups better allow them to construct scientific concepts because they are able to share ideas and concepts (Roth, 1989; Wheatley, 1991). A large block of time (2-3 weeks) will be required for this activity and is suggested to follow the completion of the Advanced Placement Biology exam.

Students will be given a single sample of freshly grown organism (several species of prokaryote will be used) however they will not know the genus or species. The organisms that will be used meet the following two requirements: safe for student use at the high school level and the genes have been previously sequenced and published. The laboratory will begin with the protein extraction from their organisms by using Thermo Scientific B-PER Bacterial Protein Extraction Reagents. Once the proteins have been extracted, they will be loaded into a polyacrylamide gel and ran through electrophoresis. Staining with comassie blue will follow.

The next step that students will complete is the in-gel digestion of proteins. Methodology for this procedure will be the same as used in “Lesson 5: Proteomics- Protein extraction, separation and identification” (Chen, 2010). Once proteins have been digested and prepared for MALDI-TOF MS, they will be shipped to Dr. Sixue Chen’s laboratory at the University of Florida for protein identification. After processing, the data should return to the students in a format that can be inputted into both Moverz Bioinformatics Software and Matrix Science MASCOT Peptide Mass Fingerprint which are both available online. Students will analyze this data and try to determine what gene and organism the peptide came from. The use of these online resources is not only invaluable to help in the identification of genes, but also supplements the education of the student (Owston, 1997).

Once students narrow down their results they will continue the activity by completing classical laboratory techniques in order to determine what genus and species their organism is. These techniques include (but are not limited to) gram staining, shape determination by the use of digital microscopes, motility, oxidase, catalase, oxygen requirements, carbohydrate fermentation, MR-VP, starch hydrolysis, and casein hydrolysis tests. The only limiting factor to what tests can be completed by the student are time and what media is available at the given time. These highly visual tests are promote student learning because more students than ever are visual learners (Papert, 1993).

Although the extent of learned outcomes can be difficult to predict ahead of time, the expected student outcomes from this laboratory activity are as follows:

- How to use aseptic technique
- How to accurately use micropipettes
- The basics of protein extraction
- How to setup and run gel electrophoresis
- What it means to digest a protein
- How mass spectrometry works and is used
- How to process data from mass spectrometry using online sources
- How to use different biochemical tests to determine genus and species of a given organism
- How to keep a scientific journal
- How to defend their results when being questioned

Data Collection Techniques

Although there are many different types of data collection and assessment, it has been shown that students must be able to think critically, problem solve, communicate in writing, and collaborate in order to be successful (Uchida, 1996). Because of this, the assessment will have multiple parts to it. Student's final grades will be determined by their laboratory journal and the oral defense of their work. The following is expected to be in the journals: methodology for every test completed (it will be permitted to reference the instruction packet for the methodology in it, but any deviations from the given methodology must be recorded), results from every test, and conclusions that can be drawn from every test. Pictures of student work (from the digital microscopes, scanners, digital cameras) will be permitted and encouraged. The journal will be due when they orally defend their work. The oral defense will be composed of the instructor questioning students about their methodology for conducting the tests and why they came to the conclusion they came to (what the genus and species their organism is). The final grade will NOT depend on if their final conclusion is correct about the genus and species, but instead will be based on a good defense of their work and justification for their conclusion. However, if their methodology is good then they should come to a correct conclusion.

It is believed by the author that this assessment method fulfills the aforementioned requirements of successful science students proposed by Uchida (1996). The laboratory activity has a heavy focus on critical thinking and problem solving because the students are using data that they collected to identify an "unknown" organism. The laboratory journals that will be kept allow for communication in writing to be fulfilled. These journals will be inspected, commented on, and then returned to the students. Because students will be working in small groups, there will be a heavy focus on collaboration in order to draw their conclusions.

ICORE Elements Included

Many different elements of the ICORE (2010) program have been included in this laboratory. Students will be working with micropipettes, gel electrophoresis, protein digestion, and data analysis of mass spectrometry data. All of these concepts have been focused on during some part of the program. Additionally, students will be collaborating with Dr. Chen's laboratory with their mass spectrometry data. "Lesson 5: Proteomics- Protein extraction, separation and identification" (Chen, 2010) will also be put to use in a modified version appropriate for protein extraction from prokaryotes. This laboratory activity was used and completed successfully during the ICORE program (2010). Some of the equipment required for this activity to be completed will be borrowed from the University of Florida.

Literature Cited

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Budget and Budget Justification

Equipment included with the ICORE "Protein Electrophoresis Kit" is not included in the budget. Other equipment needed for the laboratory activity but is not listed is already owned by the school.

ITEM	COST	JUSTIFICATION
Protein Extraction Reagents	\$259	Used in order to extract proteins from prokaryotes
Extra Micropipette Tips	\$40	Used in case student needs extra
ACN	\$100	Used in protein digestion
Trypsin	\$50	Used in protein digestion
Acetonitrile with 0.1% Formic Acid	\$90	Used in protein digestion
Prokaryotes	\$100	Used for protein extraction
TOTAL	\$639	

Items needed from the University of Florida ICORE program are listed below.

ITEM
Protein Electrophoresis Kit
Pipetting Locker
Microtube Racks
Minicentrifuges
Vortexes

Lesson Plan
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Title: Prokaryote Species Identification by Peptide Isolation and Selective Medias

Target Students: High-level biology students who have completed, or are completing, AP, IB, or AICE Biology.

Overview: This is an extensive project that allows students to walk through the steps required to extract proteins from a microorganism, separate them based on size, digest them in preparation for analysis by mass spectrometry, and then analyze data to determine what gene(s) the peptide(s) came from in order to identify the organism. Students will then also complete a series of other biochemical tests to confirm/establish their findings.

Timeframe Required: This is a very scalable laboratory activity. The following is a breakdown of recommended timeframes for each part of the lab.

- Fundamentals of micropipetting (if not already known)- 1 Day
- Protein extraction- 1 Day
- Gel electrophoresis (and staining)- 2 Days
- Protein Digestion- 1-2 Days
- Send for analysis by mass spectrometry- 1-2 Weeks (?)
- Gram staining and inspection by microscopy- 1-2 Days
- Biochemical testing of prokaryotes- 1-3 weeks (depending on how many different types of media are made available). Begin these tests when samples are sent out for analysis by mass spectrometry.
- Analysis of mass spectrometry results- 1-2 Days

Goal(s): The goal of this laboratory activity is to allow students to see that specific, researchable proteins are present in all organisms. In addition to this, it will allow students to understand characteristics of prokaryotic cells including structure (gram + and gram -) and cell metabolism (with the biochemical testing). Students will also be expected to keep a full scientific journal and defend their work which is meant to mimic what will be expected of them in the future if they continue in the sciences.

Standards:

Advanced Placement Biology standards met: organic molecules in organisms, prokaryotic cells, molecular genetics, diversity of organisms, and the use of technology to study DNA/proteins. This activity will explore all of these concepts with a heavy focus on prokaryotes and organic molecules that can be studied with modern technology.

AICE standards met: describe the structure of a prokaryotic cell and compare and contrast the structure of prokaryotic cells with eukaryotic cells; describe the structure of an amino acid and the formation and breakage of a peptide bond; explain the meaning of the terms primary structure, secondary structure, tertiary structure and quaternary structure of proteins and describe the types of bonding (hydrogen, ionic, disulfide and hydrophobic interactions) that hold the molecule in shape; state that a gene is a sequence of nucleotides as part of a DNA molecule, which codes for a polypeptide; and outline the principles of electrophoresis.

Procedures:

Day 1- Fundamentals of micropipetting (if needed)- students practice using all different sizes of micropipette in order to become comfortable with using them. Students can dispense different amounts of liquid into wells.

Students should be provided with the following:

- Micropipettes (any and all sizes)
- Micropipette tips (any and all sizes)
- Well plates

Day 2- Protein extraction. Students will receive their prokaryotes and extract protein samples from them.

Students should be provided with the following:

- Plated sample of a common prokaryote (such as *E. coli*)
- Inoculation loop
- Sterilizer/flame
- Eppendorf tube(s)
- Mini- pestles
- 4 ml of B-PER Reagent (protein extraction fluid)
- Centrifuge (only one needed for class)
- Pipettes/tips

Instructions:

- Using aseptic technique, take a loop full of the assigned prokaryote and put it into an eppendorf tube with a small amount of distilled water.
- Pellet bacterial cells by centrifugation at $5,000 \times g$ for 10 minutes.
- Add 4 ml of B-PER Reagent per gram of cell pellet. Pipette the suspension up and down until it is homogeneous.
- Incubate 10-15 minutes at room temperature.
- Centrifuge lysate at $15,000 \times g$ for 5 minutes to separate soluble proteins from the insoluble proteins.

Day 3- Protein separation and staining. Students will separate the extracted proteins by gel electrophoresis and stain with comassie blue.

Students should be provided with the following:

- 5 ul Molecular weight marker
- Polyacrylimide gel (have multiple groups use same gel)
- De-ionized water
- Comassie blue stain (or equivalent)
- Pipettes/tips

Instructions:

- Load 25 ul of supernatant from the bacterial cells into a well
- Load 5 ul molecular weight marker into a well
- Run gel electrophoresis until complete
- Remove gels and rinse with de-ionized water, repeat
- Stain with 20 ml comassie blue stain, let sit for hour
- De-stain with distilled water and store in 2% NaCl solution

Day 4- Protein Digestion- Students will pick a protein on the gel, remove it, and then extract the peptides from it.

Students should be provided with the following:

- Razor blade/scalpel
- 2, 1.5 ml Microcentrifuge tubes
- 200 ul 100% ACN
- Pipettes/tips
- 35 ul trypsin
- 80 ul 80% acetonitrile/ 0.1% formic acid
- 55 C water bath (one per class)
- (micro)Centrifuge (one per class)
- Vortex (one per class)

Instructions:

- Cut a band of interest from the gel into 1mm squares and then place into 1.5 ml microcentrifuge tube
- Add 200ul of 100% CAN. Vortex for 5 minutes to dry gel
- Remove all liquid from the tube
- Add 35 ul of trypsin solution to the gel. Let sit for 5 minutes.
- Place in hot water bath for 10 minutes
- Add 40 ul of 80% acetonitrile/ 0.1% formic acid then vortex for 5 minutes
- Spin briefly, remove all liquid to a new 1.5 ml microcentrifuge tube
- Add an additional 40 ul of 80% acetonitrile/ 0.1% formic acid then vortex for 5 minutes
- Spin briefly, remove all liquid to a new 1.5 ml microcentrifuge tube
- Let all liquid evaporate out of tube
- Mail completed samples to Dr. Chin at the University of Florida for mass spectrometry analysis

Days 5 through 20 (modify the amount of tests to fit your time frame)- Biochemical tests (all methodology included in the appendix) including, but not limited to:

- Gram staining
- Acid fast staining
- Motility Test
- Oxygen requirement test
- Gelatin liquefaction test
- Carbohydrate fermentation tests
- MR-VP tests
- Catalase test
- Oxidase test
- Nitrate reductase test
- Starch hydrolysis test
- Casein hydrolysis test
- Fat hydrolysis test
- Tryptophan/Indole test
- Urease test
- Hydrogen sulfide production test
- Citrate utilization test
- Phenylalanine deamination test
- Litmus milk test
- Lysine Decarb Test

Day “x”- Analysis of mass spectrometry results. Students will use the data from Dr. Chin’s laboratory to determine what peptides they contained.

Students should be provided with the following:

- Computer with Internet access
- Results from Dr. Chin's laboratory

Instructions:

- Students need to go to <http://www.moleculardetective.org/>, go to "fingerprint library" and download the Moverz Bioinformatics Software. Once downloaded, analyze data and get peptide fingerprint for journal purposes
- Students will then need to go to <http://www.moleculardetective.org/> and click on "Protein Search Engine". Input the data provided by Dr. Chin's laboratory. Student will then analyze the data to figure out what protein/gene/organism their peptide belongs to.

Day "y"- Compilation of all data to determine what genus and species their microorganism is. Students will compile all collected data from mass spectrometry and their own biochemical tests and begin a literature search. Recommended books include *Bergey's Manual of Determinative Bacteriology*. Journals will be completed and students will complete an oral defense of their results.

Assessment: Student's final grades will be determined by their laboratory journal and the oral defense of their work. The following is expected to be in the journals: methodology for every test completed (it will be permitted to reference the instruction packet for the methodology in it, but any deviations from the given methodology must be recorded), results from every test, and conclusions that can be drawn from every test. Pictures of student work (from the digital microscopes, scanners, digital cameras) will be permitted and encouraged. The journal will be due when they orally defend their work. The oral defense will be composed of the instructor questioning students about their methodology for conducting the tests and why they came to the conclusion they came to (what the genus and species their organism is). The final grade will NOT depend on if their final conclusion is correct about the genus and species, but instead will be based on a good defense of their work and justification for their conclusion.

Appendix:

Methodology for recommended biochemical tests

Gram stain:

Heat fix: Light the Bunsen burner. Pass the slide (with the bacteria mounted on it) through the interface between the blue flame and the yellow flame - this is the hottest region of the flame - 5 times. Be careful doing this because it will get hot very quickly so do not burn yourself. The slide should sit in this region for no more than a second, as if it gets too hot the bacterial will rupture, and you will not get a good stain.

- Place your slide on a slide holder or a rack. Flood (cover completely) the entire slide with crystal violet. Let the crystal violet stand for about 60 seconds. When the time has elapsed, wash your slide for 5 seconds with the water bottle. The specimen should appear blue-violet when observed with the naked eye.

- Now, flood your slide with the iodine solution. Let it stand about a minute as well. When time has expired, rinse the slide with water for 5 seconds and immediately proceed to step three. At this point, the specimen should still be blue-violet.

- This step involves addition of the decolorizer, ethanol. To be safe, add the ethanol dropwise until the blue-violet color is no longer emitted from your specimen. As in the previous steps, rinse with the water for 5 seconds.

- The final step involves applying the counterstain, saffranin. Flood the slide with the dye as you did in steps 1 and 2. Let this stand for about a minute to allow the bacteria to incorporate the saffranin. Gram positive cells will incorporate little or no counterstain and will remain blue-violet in appearance. Gram negative bacteria, however, take on a pink color and are easily distinguishable from the Gram positives. Again, rinse with water for 5 seconds to remove any excess of dye.

Acid Fast Stain:

Bacteria with an acid-fast cell wall (high lipid content) resist decolorization with acid-alcohol and stain red, the color of the initial stain, carbol fuchsin. All other bacteria will be decolorized and stain blue, the color of the counterstain methylene blue. The acid-fast stain is an especially important test for the genus *Mycobacterium*.

PROCEDURE (Ziehl-Neelsen Method)

- Cover the smear with a piece of blotting paper and flood with carbol fuchsin.
- Steam for 5 minutes by passing the slide through the flame of a gas burner.
- Allow the slide to cool and wash with water.
- Add the acid-alcohol decolorizing slowly dropwise until the dye no longer runs off from the smear.
- Rinse with water.
- Counterstain with methylene blue for 1 minute.
- Wash with water, blot dry, and observe using oil immersion microscopy.

Acid-fast bacteria will appear red; non-acid-fast will appear blue.

Motility Test (Motility Medium):

*This is not a biochemical test but must be determined for the unknowns!

A. Reason: Used to determine if a bacteria is motile

B. Procedure:

- Use a needle to inoculate by making a single stab about two thirds down and then pull the needle up the same path.
- Incubate for 24-48 hours

C. Interpretation:

- Motile: the tube will appear cloudy and usually the organism will spread over the top of the media.
- Non-Motile: the organism will grow along the streak line only; the media will not be cloudy.

Oxygen Requirements (Fluid Thioglycollate Medium)

A. Reason: Used to determine oxygen requirements. The media contains glucose, cystine, and sodium thioglycollate to lower the oxidation-reduction potential. The oxygen tension is high at the surface of the media (allowing the media to grow) and decreases toward the bottom of the media (for anaerobic growth). Resazurin (a dye) causes the media to turn pink in the presence of oxygen.

B. Procedure:

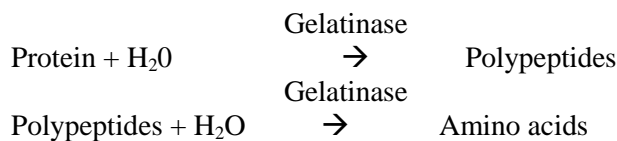
- Boil and cool media with the screw cap loose
- Inoculate media with the organism using a wire loop. **DO NOT SHAKE THE MEDIA.**
- Incubate at optimum temperature for 24 hours.

C. Interpretation:

- Aerobe- Growth at the top of the media
- Facultative- Growth throughout the media
- Anaerobe- Growth at the bottom of the media

Gelatin Liquefaction Test (Nutrient Gelatin):

A. Reason: Used to determine the ability of an organism to produce enzyme gelatinase, which liquefies gelatin. Gelatinase breaks down large proteins into smaller components, which can then enter the organism and be metabolized.



B. Procedure:

- Stab gelatin with organism using a straight wire
- Incubate at optimum temperature for 24-48 hours
- Place tubes in ice water bath for at least 30 minutes

C. Interpretation:

- Positive- Gelatin is liquefied

- Negative- Gelatin is solid (Note- continue incubation of negative tubes for another 4 to 5 days to see if gelatinase is produced slowly)

Carbohydrate Fermentation (Glucose, lactose, mannitol, maltose, sucrose)

A. Reason: Used to determine the ability of an organism to ferment a specific carbohydrate with or without the production of gas.

-Phenol Red is used as an indicator in the media. At a neutral pH, the media is red; at a pH of less than 7, the media is yellow. Fermentation of the carbohydrate produces acid, causing the media to change from red to yellow.

- The inverted tube in the broth, called a Durham tube, captures some of the gas the organism produces, allowing production to be seen (if it ferments, gas will be produced).

B. Procedure:

- Inoculate each media with the organism
- Incubate at the optimum temperature for 24-48 hours

C. Interpretation:

- Positive- Media turns yellow (fermentation has occurred) and gas produced
- Negative- Media remains red (no fermentation). Continue incubation of negative tubes for up to 2 weeks to detect slow fermenters.

Methyl Red Test and Voges-Proskauer Test (MR-VP Broth):

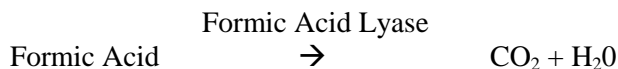
Methyl Red Test-

A. Reason:

-Used to determine the ability of an organism to produce mixed acid end products from glucose fermentations.

- Some organisms produce large amounts of various acids (lactic, acetic, succinic, formic) plus H₂ and CO₂. The large amounts of acids lower the pH to lower than 5.0.

- These organisms also produce great amounts of gas due to the presence of the enzyme formic hydrogen lyase.



Voges- Proskauer Test

A. Reason

- Used to determine the ability of an organism to produce acetoin; 2,3 butanediol; and ethanol which causes less lowering of the pH than the methyl red positive organisms.

- VP test detects the presence of acetoin, which is a precursor to 2,3 butanediol.

B. Procedure for BOTH:

- Inoculate two MR-VP broths with the organism.
- Incubate at optimal temperature for 3-5 days
- Add 3-4 drops of Methyl Red reagent to one tube
- Interpretation for methyl red test-

- Positive- Red color develops
- Negative- Yellow color develops
- Pipette 1 ml of culture from the other MR-VP tube into a small screw cap test tube.
- To the extracted 1 ml of culture, add 18 drops of Barritt's Solution A (alphanaphthol) and 18 drops of Barritt's Solution B (KOH).
- Agitate vigorously for 1-2 minutes. Let stand for 1-2 hours.

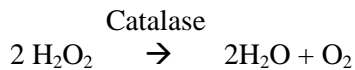
Interpretation for VP test-

- Positive- Wine red (burgundy) color develops
- Negative- Brown color develops

Catalase Test (Nutrient Agar slant):

A. Reason: Used to test for the presence of enzyme catalase.

- Hydrogen peroxide (H₂O₂) is formed as an end product of the aerobic breakdown of sugars. When H₂O₂ accumulates, it becomes toxic to the organism. Catalase decomposes H₂O₂ and enables the organism to survive. Only obligate anaerobes lack this enzyme.



B. Procedure:

- Streak nutrient agar slant with the organism
- Incubate at optimum temperature for 24-48 hours
- Place a few drops of 3% H₂O₂ on the slant culture

C. Interpretation:

- Positive- Bubbling (O₂ gas is liberated from the H₂O₂)
- Negative- No bubbling

Oxidase Test (tryptic soy agar plate):

A. Reason: Used to determine the presence of oxidase enzyme.

- Aerobic organisms obtain their energy by respiration, which is responsible for the oxidation of various substrates through the cytochrome oxidase systems (ETC). Obligate aerobes have this enzyme.

B. Procedure:

- Make an isolation streak of the organism on the TSA plate
- Incubate at optimum temperature for 24-48 hours
- Add several drops of oxidase test reagent directly to organism; let stand for 10-15 minutes.

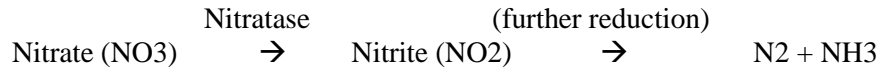
C. Interpretation:

- Positive- Organisms change color to a dark red/black
- Negative- No color change

Nitrate Reductase Test (Nitrate Broth):

A. Reason: Used to determine the ability of an organism to reduce nitrate (NO₃) to nitrite (NO₂) or nitrogen gas (N₂) by the production of the enzyme nitratase.

- The reduction of nitrate to nitrite or nitrogen gas takes place under anaerobic conditions in which an organism derives its oxygen from nitrate.



B. Procedure:

- Boil and cool media with screw cap loose
- Inoculate nitrate broth with the organism
- Incubate at the optimum temperature for 24-48 hours
- Add 5 drops of Nitrate Reagent A (sulfanilic acid) and 5 drops of Nitrate Reagent B (dimethyl alpha naphthalamine) to the tube

C. Interpretation:

- Positive- Red color; nitrate reduced to nitrite; test is complete
- Negative- No color change; do confirmation test by adding a small pinch of zinc powder
 - Interpretation of Confirmation Test-
 - Positive- No color change; organism reduced nitrate completely to ammonia and nitrogen gas.
 - Negative- Red color; nitrate reduced by zinc, not the organism (confirms negative test)

Starch Hydrolysis (Starch Agar Plate):

A. Reason: Used to determine the ability of an organism to hydrolyze (break down) starch

- The enzyme amylase breaks starch down into components more easily metabolized by the organism.



B. Procedure:

- Make a single streak of the organism on a starch agar plate
- Incubate at optimal temperature for 24-48 hours
- Drop a small amount of IKI (Gram's Iodine) onto the plate and rotate the plate gently. (Iodine is an indicator of starch; in the presence of starch the iodine will turn blue/black)

C. Interpretation:

- Positive- A zone of clearing appears adjacent to the streak line.
- Negative- No clearing; only a blue/black area surrounding the streak line.

Casein Hydrolysis (Skim milk agar):

A. Reason: Used to determine the ability of an organism to produce the enzyme caseinase, which hydrolyzes (breaks down) casein (a white protein in milk) to more soluble products.

B. Procedure:

- Make a single streak of the organism on a skim milk agar plate.
- Incubate at the optimum temperature for 24-48 hours

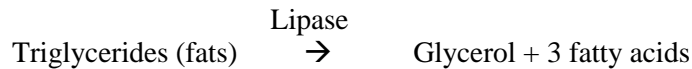
C. Interpretation:

- Positive- A zone of clearing occurs along the streak line
- Negative- No zone of clearing
(note- compare results with the Litmus milk test)

Fat Hydrolysis (Spirit Blue Agar Plate):

A. Reason: Used to determine the ability of an organism to produce the enzyme lipase which hydrolyzes fat.

- Lipase splits fats into glycerol and fatty acids that can be used for anabolism or energy production.



- The media contains vegetable oil that, when hydrolyzes, lowers pH.

B. Procedure:

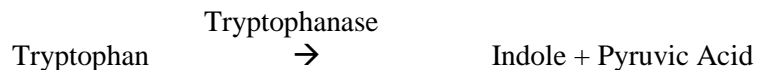
- Make a single streak of the organism on a spirit blue agar plate.
- Incubate at optimum temperature for 24-48 hours.

C. Interpretation:

- Positive- A dark precipitate forms along the streak line or oil droplets are depleted in this area if the pH is not lowered sufficiently (hold the plate up to the light)
- Negative- No change in medium

Tryptophan Hydrolysis/ "Indole Test" (Tryptone Broth):

A. Reason: Used to determine the ability of an organism to split indole from the amino acid tryptophan using the enzyme tryptophanase.



B. Procedure:

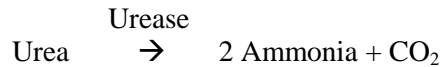
- Incubate broth (at optimum temperature) with your organism for 24-48 hours.
- Add 10-12 drops of Kovacs Reagent.

C. Interpretation:

- Positive- Red layer forms on surface of the media
- Negative- Yellow layer forms on the surface of the media

Urease Test (Urea Broth):

A. Reason: Used to determine the ability of an organism to split urea to form ammonia (an alkaline end product) by the action of the enzyme urease. Media also contains the pH indicator phenol red, which turns an intense pink at alkaline pH.



B. Procedure:

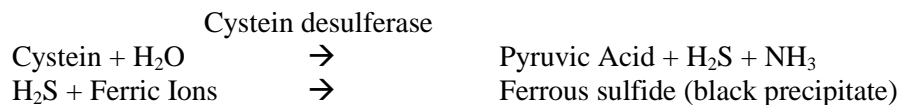
- Inoculate urea broth with the organism
- Incubate at optimum temperature for 24-48 hours

C. Interpretation:

- Positive- Intense pink/red color
 - Negative- No color change
- (note- Continue incubation of negative tubes for a total of 7 days to check for slow urease producers)

Hydrogen Sulfide Production (Kligler's Iron Agar):

A. Reason: Used to determine the ability of an organism to produce H₂S (Hydrogen sulfide)



- The media also contains glucose, lactose, and phenol red as a pH indicator to show fermentation of these sugars. Gaps, cracks, or bubbles in the agar indicate gas production.

B. Procedure:

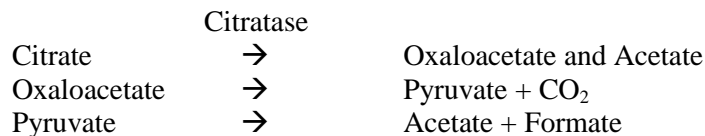
- Stab KIA with straight wire inoculum of organism
- Incubate at optimum temperature for 24-48 hours

C. Interpretation:

- Positive- Black precipitate along stab line
- Negative- No precipitate

Citrate Utilization (Simmons Citrate Agar):

A. Reason: Used to determine if an organism is capable of using citrate as the sole source of carbon with production of the enzyme citratase.



- The media contains sodium citrate as the carbon source, and ammonium salts as the nitrogen source, with bromothymol blue as the pH indicator. An organism that uses citrate breaks down the ammonium salts to ammonia, which creates an alkaline pH.

B. Procedure:

- Stab and streak Simmons citrate agar slant with the organism.
- Incubate at the optimum temperature for 24-48 hours.

C. Interpretation:

- Positive- Alkaline pH causes media to change from green to Prussian blue
- Negative- No color change

Phenylalanine Deamination (Phenylalanine Agar):

A. Reason: Used to determine the ability of an organism to deaminate the amino acid phenylalanine resulting in the production of phenylpyruvic acid and ammonia. This reaction is catalyzed by the enzyme phenylalanase.



B. Procedure:

- Streak phenylalanine agar slant with the organism.
- Incubate at optimum temperature for 24-48 hours
- Place 5-10 drops of 10% Ferric Chloride on the slant culture. Use a loop to mix the organism into the solution.

C. Interpretation:

- Positive- A deep green color appears within 1-5 minutes
- Negative- An amber color develops

Litmus Milk Test (Litmus Milk Tube):

A. Reason: Used to differentiate organisms in skim milk agar according to metabolic properties:

- Lactose fermentation
- Reduction of litmus
- Clot formation
- Peptonization (digestion)

- Litmus is used as a pH and oxidation- reduction (E_h) indicator. In un-inoculated milk, litmus will be a purple/blue (pH 6.8). In acidic solution (pH 4.5) litmus will be pink, and in alkaline solution (pH 8.3) litmus will be blue.

- Lactose fermentation- If the organism can ferment lactose, an acidic condition occurs and the media will be pink.



- If the organism cannot ferment lactose, it may act on nitrogenous substances in the milk to release ammonia and the media will be blue.
- Reduction of litmus- Litmus is used as an E_h indicator. An organism capable of reducing litmus will cause the media to turn white.
- Clot formation- Proteolytic enzymes (rennin, pepsin, or chymotrypsin) cause the hydrolysis of milk proteins, which result in the coagulation of milk.
- Peptonization (digestion) - Hydrolysis of casein by caseinase causes the caseinogen precipitate (clot) to be converted to a clear liquid.

B. Procedure:

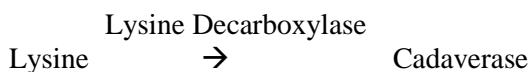
- Inoculate litmus milk media with the organism using a wire loop.
- Incubate at the optimum temperature for up to 5 days.

C. Interpretation:

- Your result will be one or more of the following
 - Pink- Acid reaction, lactose fermented (verify with Carbohydrate fermentation test if possible)
 - Purple/blue- No fermentation of lactose
 - Blue- Alkaline reaction, no fermentation of lactose; organism attacks nitrogenous substances.
 - White- Reduction of litmus
 - Clearing of the media- peptonization (verify with Casein hydrolysis test)
 - Clot/Curd- Milk protein coagulation

Lysine Decarboxylase Test (Lysine Decarboxylase Broth):

A. Reason: Used to determine the ability of an organism to decarboxylate the amino acid lysine, resulting in the production of alkaline end- product cadaverine, by producing the enzyme lysine decarboxylase.



- The media contains lysine, glucose (as a substance for fermentation), and the pH indicator Brom Cresol Purple (purple at alkaline pH; yellow at acid pH)
- The enzyme requires an acidic pH (below 5) for activation. IF the organism is capable of glucose fermentation (check with carbohydrate broth if available), AND has the enzyme lysine decarboxylase, the following events occur.
 - Microbe ferments glucose, producing low pH; indicator turns yellow
 - Lysine decarboxylase is activated
 - Cadaverine is formed, pH rises and the media returns to its original purple color.

B. Procedure:

- Inoculate broth with the organism using a wire loop.
- Incubate at optimum temperature for 24-48 hours.

C. Interpretation:

- Positive- Purple (Verify organism ferments glucose by checking the carbohydrate fermentation test, if the organism does NOT ferment glucose then a purple color is a NEGATIVE)
- Negative- Yellow