THIRD SEMESTER

BL301: MICROBIOLOGY LABORATORY
CREDITS -2

BL301: MICROBIOLOGY LABORATORY CREDITS — 2

Laboratory Handout

Instructor — Dr. Harapriya Mohapatra

Projections:

- Total no. of classes@ 2/week = 7 classes (microbiology needs to have at least 2 consecutive classes in a week in order to make the students have a hands on experience in microbiological technique. Total = 13 weeks for 2 laboratory courses of 2 credits each)
- Total no. of students = 14

Exp.No.	Name of experiments	Tentative schedule
	Orientation & General laboratory safety Guidlines	1st week
1.	Culture media preparation, control of microbial growth disinfection & sterilization.	1st week
2.	Enrichment and isolation and characterization of pure culture of selective and differential media 2.1 – spread plate technique 2.2 – streak plate method	2nd week
3.	Microscopic examination of fresh culture with different staining procedure 3.1 – gram positive and gram negative staining 3.2 – Endospore staining	3rd week

4.	Culture dependent analysis of microbial communities 4.1 – use of differential, selective and enriched media 4.2 – determination of extra cellular enzymatic activities of microorganisms by (a) starch hydriolysis (b) lipid hydrolysis (c) casein hydrolysis (d) Gelatin hydrolysis 4.3 – use of representatives biochemical test 4.3.1- TSI 4.3.2 – IMViC test 4.3.3 – Nitrate reduction test 4.3.4 – Urease, Catalase and Oxidae test	4th &5th week
5.	Culture independent test analysis of microbial communities by 16srDNA sequencing method	6th week
6.	Identification of genus of unknown bacterial cultures	6th week
7.	Antibiotic susceptibility testing: 7.1 – Disk diffusion 7.2 – MIC by tube dilution	7th week

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Aim 3.2: To utilize differential staining for visualization of bacterial spore.

Principle/Theory: tinder unfavorable environmental conditions certain strains of bacteria undergo sprorogenesis and give rise to endospore. Unlike the vegetative cells the spore cells due to their impervious coat does not accept primary stains easily by common procedures. The primary stain used for spore staining is malachite green and for its penetration moist heat is applied with replenishin[g] the stain to prevent its evaporation. '1 he deco lionizing agent used here is water, As the spore once accepts malachite green it cannot he &colorized with water, instead the vegetative components which are stained with low affinity get decolorized. The counter stain used is safranin which colors the vegetative cells.

Material & Reagents required: End spore forming bacterial culture, Malachite green (5% solution in water), Bunsen burner, hot plate, water beaker, inoculating loop, staining tray , tissue paper, glass slides, and microscopes.

Procedure:

1. Prepare bacterial smear; air dry and heat fix them.
2. Flood smears with malachite green and place on top of a beaker of water sitting on a warm hot plate, allowing preparation to steam for 2-3 minutes. *Caution:* Do not allow stain to evaporate, replenish stain as needed. Prevent the stain from boiling by adjusting the temperature of the hot plate.
3. Remove slides from hot plate, cool and gently wash under running tap water.
4. Counter stain with safranin for 30 seconds
5. Wash with tap water.
6. Blot dry with tissue/blotting paper and observe under oil immersion.

Observation:-

(i) Make drawing of representative micros 'pie field

(ii) Describe the location of the endopsore within the vegetative cell as being central, sub terminal or terminal

(iii) Indicate color of the spore and vegetative cell on each preparation.

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Experiment 4: Culture dependent analysis of microbial communities

Aim 4.1: Enrichment and isolation and characterization of pure culture. Use of selective and differential media.

Objective: (i) to understand the use & function of specialized media for selection and differentiation of microorganisms,
(ii) To observe how an enriched media can also function both as a selective and differential medium,

Principle:

Special purpose media are used for specific purposes such as isolation of bacteria from mixed population, differentiation among closely related groups and for characterization and identification of bacteria by their abilities to produce chemical changes in different media,

Selective media are used to select specific group of bacteria, they incorporate .chemical substances tnat inhibit growth of one type of bacteria while permitting growth of another thus facilitating isolation. The selective media we would be using. Is 7.5% Sodium chloride. agar 'N nich allows only halophillie organisms to grow inhibiting others.

Differential media incorporate chemical compounds that following incubation produce characteristic change in appearance of bacterial growth and or the medium surrounding the colonies. These are used to distinguish morphologically & biochemically related groups of organisms. We would be working with 2 different media

(i) MacConkey agar which contains crystal violet that inhibits growth of gram positive organism and allows growth of gram negative bacteria. lneorporaiion of lactose, bile salts and pH indicator neutral red permits differentiation of enteric bacteria on the basis of their ability to ferment lactose. Coliform bacilli such as *Ecoli* produce acid as a result of lactose fermentation. When this occurs the colonies colonies become red as the acid precipitates bile salts followed by absorption of neutral red.

(ii) Fosin Methylene Blue agar which utilizes lactose and the dyes eosin and methylene blue to permit differentiation between enteric .lactose fermenters and non-fermenters as well. As identification of colon bacillus *E. colt* that produces blue-black colonies with a metallic green sheen caused by large quantities of acid produced *wh*ich precipitates the dyes onto growth's surface.

Enriched media are supplemented with highly nutritious material such as blood, serum or yeast extract to facilitate growth of Fastidious Organisms. We would be using blood agar.

Media preparation: per designated student one plate each of the following media

7.5% sodium chloride agar (g/L) Beef extract 3.0 Peptone 5.0 Sodium chloride 7.5 Agar 20 pH 7.0	MacConkey agar Dehydrated ready to use media will be prepared as per the manufacture's instruction.
Eosin Methyl Blue Agar Dehydrated ready to use media will be prepared per the manufacturer's instruction	Blood agar (g/L) Beef heart infusion 500 Tryptose 10.0 Sodium chloride 5.0 Agar 20 pH 7.3 Dissolve the above ingredients and autoclave. Cool the sterile blood agar base to 45^0C, aseptically add 50 ml of sterile defibrinated blood, and mix thoroughly avoiding accumulation of air bubbles. Dispense in appropriate vessels.

Procedure:

1. Prepare media and sterilize it as per the directions given.
2. Divide bottom of each plate into 4 quadrants label with name of the organism.
3. Using sterile inoculation needle inoculate the plates as directed with one culture in each quadrant.
4. Incubate the plates overnight & record your observations.

Observation:

1. Note the amount of growth along the line of inoculation as follows: 0 = none, 1+ scant and 2+ = moderate to abundant.
2. Appearance of the growth: coloration and transparency.
3. Note the change in appearance of the medium surrounding growth: coloration, transparency etc.

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L a b o r a t o r y H a n d o u t

Instructor — Dr. Harapriya Mohapatra

Aim 4.2: Determination of extracellular enzymatic activities of microorganisms by (a) Starch hydrolysis (b) Lipid hydrolysis (iii) Casein hydrolysis (iv) Gelatin hydrolysis.

Principle:

(a) Starch hydrolysis: Its degradation requires an extracellular enzyme amylase which breaks down glycosidic bonds hydrolysing the starch to dextrins, that are finally hydrolyzed to maltose and then to glucose by the C117}- 111C maltase. The starch agar plates are nutrient agar media supplemented with soluble starch upon which the microorganism is inoculated. Following incubation, the plate is flooded with iodine that imparts blue-black color to ihe medium indicating presence of starch. A clear zone indicates zone of hydrolysis and a positive result.

(b) Lipid hydrolysis: Degradation of lipids such as triglycerides is accomplished by extracellular enzyme lipase. Which cleaves ester bonds to form glycerol and fatty acids? The nutrient agar medium is supplemented with tributyrin that forms an emulsion; producing an opaque medium. Following inoculation and incubation, a positive organism develops a zone of lipolysis demonstrated by a clear area surrounding the bacterial growth.

(c) Casein hydrolysis: Casein ia a major component of milk- protein. Its degradation involves breaking down of pol.) pepetide bonds, in a process called proteolysis, which is mediated by enzyme protease. The medium contains nutrient agar supplemented with milk powder that gives medium its color and opacity. Lipon inoculation and incubation a positive culture will exhibit a clear zone of proteolysis due to hydrol^{y}sis of easeill to noncolloidal amino acids.

(d) Gelatin hydrolysis: Liquefaction of gelatin; is accomplished *by* enzyme gelatinase. Below temperature of 25°C gelatin will remain in gel state and above 25°C will remain in sol state. Upon degradation even at low iemperatures it will remain in sol state. Nutrient agar medium supplemented with 12% gelatin in deep tubes is inoculated and incubated for 48 hours. Then the cultures are refrigerated at 4°C. For 30 minutes. A liquefied culture represents a positive reaction and rapid gelatin hydrolysis. Following incubation for longer period say 5 days & repeating the above process, a liquelaction denotes slow gelatin hydrolysis.

Material, Reagents and methods: Starch, lipid and casein agar plates (1 00m I/group); gelatin deep tubes (251111/group): Gram's iodine solution, bacterial cultures

Preparation of Starch tf,ar[l].

Peptone	5.0 g/l
Beef extract	3.0 g/l,
Soluble starch	2.0 ulL
Agar	20 g/L
pH	7.0 ± 0.2

Preparation of milk-agar:

peptone	5.0 g/l
skim milk powder	100 g/l
agar	20 g/l
pH	7.2

Preparation of Tributyrin agar:

Peptone	5.0 g/L
Beef extract	3.0 g/l
Tributyrin	10 g/I
Agar	20 g/L
pH	7.7

Preparation of Nutrient gelatin:

peptone	5.0 g/1
Beef extract	3.0 g/I
Gelatin	120 g/1
pH	6.8

Procedure:

1. Prepare starch agar, tributyrin agar and milk agar plates. 2 plates per medium divide the bottom of each petri dish into 2 sections.
2. Using sterile technique make a single line streak inoculation of each test organism on agar surface of its appropriately labeled section.
3. Using sterile technique inoculate each experimental organism in its appropriately labeled gelatin deep tube by means of stab inoculation.
4. Incubate all plates in an inverted position for 24 to 48 hours at 37°C. Incubate the gelatin deep tube cultures for 48 hours.

Note: (i) Dissolve peptone, beef extract (adjust pH) & add agar while heating cool to 90°C, add tributyrin, emulsify in a blender).
(ii) Autoclave at 1 10°C for 15 minutes (121b for 15 minutes) to prevent chairing of media.

Observation:

(i) After incubation flood the starch agar plate, with Gram's iodine solution, allow the iodine to remain in contact with medium for 1-2 minutes & pour off the excess. Examine the cultures for the presence or absence of blue-black color surrounding the growth of each test organism.
(ii) Examine the tributyrin agar plate cultures for the presence or absence of a clear area or

zone of lipolysis surrounding the growth of each of the organisms.

(iii) Examine the milk agar cultures for the presence or-absence of a clear area or zone of proteolysis surrounding the growth of each of the bacterial test organisms.

(iv) Place all gelatin deep tube cultures into refrigerator at 4°C for 30 minutes. Examine all the cultures to determine whether the medium is solid or liquid. Based on the observation record gelatin hydrolysis ability and rate of hydrolysis of different organisms.

Bacterial strain	**Starch hydrolysis**		Tributyrin hydrolysis		**Casein hydrolysis**		Gelatin liquefaction (+) or (-)		
	Appearance of medium	Result	Appearance of medium	Result	Appearance of medium	Result	2 days	7 days	Slow or rapid

Aim 4.3.2: To utilize IMViC test (Indole Methyl red, Voges-Proskauer and Citrate utilization) to distinguish between different groups of Enterobacteriaceae.

Principle: Principles of all the four tests are as follows:

Indole Production test: The SIM agar medium contains amino acid tryptophan. Tryptophan is hydrolyzed to indole by enzyme tryptophanase by selected microorganisms, which serves as a biochemical marker. The presence of indole is detectable by adding Kovacs reagent that produces cherry red reagent layer indicating a positive reaction. This color is produced by Kovac's reagent that is composed of p-dimethyaminobenzaldehyde, butanol and I ICI. Indole is 'extracted from the medium into the reagent layer by the acidi Fled butyl alcohol component and forms complex with p¬dimethyaminobenz.aldehyde, yielding cherry red color.
SIM agar may also be used to detect motile of microorganisms. Motility is recognized when culture growth (turbidity) is not restricted to the line of inoculation. Growth of non-motile organisms is confined to the line of inoculation_
SIM agar is also used to detect HiS production. Ferrous ammo sulfate in the medium combines with forming an insoluble black FeS that is seen along ih of stab inoculation, indicating positive reaction for I LS production.

(ii) **Methyl red test:** This is helpful in differentiating particularly between E. coli and E. aerogenes. The end product of glucose oxidation serves as the differentiating marker. Both these organisms initially produce organic acid and products during the early incubation period. The low acidic p11 4.0 is stabilized and maintained by E. coil at the end of incubation. But, during later incubation E. aerogenes enzymatically converts these acids tt, non-acidic end products such as 23-butanediol and acetoin (acetylmethylcarbinol) resulting in an elevated pH 6.0 (approx.). Methyl red indicator in acidic pH turns red indicating a positiyc test while at higher pH turns yellow indicating a negative test.

(iii)**Voges-Proskauer test:** Determines the capability of microorganisms to produce non-acidic or neutral end products, such as acetylmethylcarbinol, from organic acids that result from glucose metabolism. The Barrett’s reagent used in the test consists of alcoholic a-napthol and 40% potassium hydroxide solution. -The acetNilmethy icarbinol in presence of catalyst a-napthol and guanidine group of peptone from the medium, form a diacetyl compound which gives the pink coloration, 15 minutes after the addition of Barritt's reagent. This indicates a positive reaction.

(iv) **Citrate utilization test:** The ability of microorganisms to utilize citrate as carbon source depends upon presence of enzyme citrate permease that facilitates transport of citrate into the cell. It is a major product of Kreb's cycle. The enzyme citrase degrades citric acid to oxaloacetic acid and acetate; these are finally converted to pyruvic acid and CO2, due to which the medium becomes alkaline (CO, combines with sodium and water to form sodium bicarbonate). Indicator bromothymol blue under alkaline conditions changes from green to deep prussian blue accompanied by growth of organisms on surface of slants. This reaction indicates a citrate positive colony.

Material, Reagents and methods: Per group 35mL of media, dispensed into tubes fit) 5ml tube = 7 tubes., 7 110.5 of clean sterile autoclaved test tubes, test tube rack, inoculating needle, Kovac's reagent, Methyl red indicator, Baritt's reagents A & B.

(i) For indole production test: SIM agar deep tubes		(ii) Methyl red and VP test: MR-VP broth	
Peptone	30.0g/L	Peptone	7.0g/L
Ferrous ammonium sulphate	3.0 g/L	Dextrose	5.0 g/L
Sodium thiosulphate	0.2 g/L	Potassium phosphate	5.2 g/L
Agar	3.0 g/L	Adjust pH to 6.9	
Adjust pH to 7.3		Autoclave at 110^0C for 15 min. Following inoculation, aliquots (2.5 ml) has to taken out into set of clean, dry, autoclaved tubes for VP test & rest proceeded for MR test	
Autoclave at 110^0C for 15 min.			

(iii) Citrate utilization test: Simmon's citrate agar

Ammonium dihydrogen phosphate	1.0g/L
Dipottasium phosphate	1.0 g/L
Sodium chloride	5.0 g/L
Magnesium sulphate	0.2 g/L
Adjust pH to 7.3	
Agar	3.0 g/L
Bromothymol blue	0.08 g/L.

Procedure:

1. Using sterile technique inoculate each experimental organism into its appropriately labeled tube as directed. The last tube will serve as control.

2. Incubate for IS to 24 hours at 37°C.

Observation:

(i) For indole production test:

a. Observe for the motility which iSH-ecognized when culture growth of flagellated organisms is not restricted to the line of iiuiIation. Growth of non-motile organisms is confined to the line of inoculation.

b. SIM medium contains peptone & sodium thiosulfate as sulfur substrates resulting in the production of 1-1,S gas. Ferrous ammonium sulfate in the medium serves as an indicator

by combining with the gas forming an insoluble black ferrous sulfide precipitate seen along the line of the stab.

c. Add 10 drops of Kovac's reagent to all deep tubes & agitate the cultures gently. Examine the colour of the reagent layer in each culture. Determine and record whether or not each organism was capable of hydrolyzing tryptophan.

Bacterial species	**Motility (+) or (-)**	**H_2S production (+) or (-)**	**Color of reagent laver**	**Tryptophan hydrolysis (+) or (-)**

(ii) Methyl red and VP test:

a. Transfer approximately 2.5m1 of each culture into labeled empty test-tube and set these tubes aside for VP test.

b. For MR test Add 5 drops of methyl red indicator to the remaining aliquot of each culture. Examine the color of these cultures & record whether each of the cultures as capable of fermenting glucose with the production & maintenance of high concentration of acid.

c. For VP test to the separated aliquots add l 0 drops of Barritt's reagent A and shake the culture. Immediately add 10 drops of Barritt's reagent B and shake, Reshake every 3-4 minutes. Examine the color of the cultures after addition of Barritt's reagent. Based on your observation determine whether or not each organism was capable of fermenting glucose with ultimate production of acetylmethylcarbinol.

Bacterial species	**Methyl red test**		**Voges-Proskauer test**	
	Color of medium	(+) or (-)	Color of medium	(+) or (-)

(iii) Citrate utilization test:

Examine all agar slant cultures for the presence or absence of growth and coloration of the medium. Based on your observation record whether or not each organism was capable of using citrate as its sole source of carbon.

Bacterial species	**Presence or Absence of growth (+) or (-)**	**Color of medium**	Citrate utilization **(+) or (-)**

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Laboratory Handout

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Aim 4.3.3: To determine the ability of some microorganisms to reduce nitrates (NO3-) to nitrites (NO2-) or beyond nitrite stage (Nitrate reduction test)

Principle: The reduction of nitrates can occur under aerobic or anaerobic conditions. In these anaerobic organisms the cell uses inorganic substances such as NO, to supply 02 that is subsequently utilized as a final H acceptor during energy formation. The biochemical transformation is as follows:

$NO_3 + 2H^- + 2e-$ …… N02- + H_20

Some organisms further can enzymatically convert nitrates to NH_3* or N_3
Following incubation of the cultures an organism's ability to reduce nitrates to nitrites is determined by addition of 2 reagents:

Solution A - sulfanilic acid followed by Solution 13 - a -napthylamine, which will produce an immediate cherry red compound due to formation of sulfobenzene azo-a-napthy lamine.

NO_3 ..... nitrate reductase NO2 — (red color on addition of Solutions A & B)

No change in color suggests 2 things: (i) nitrates were not reduced or (ii) the organism had the potential to rapidly convert nitrates to NEI3- or 1\17. To determine whether or not nitrates were reduced past the nitrite Stage small amount of Zn powder is added to the colorless solution already containing Solutions A &.. B. Z11 reduces N0 to NO-)-. If a red color develops then it signifies that nitrates were not reduced to nitrites, indicating a negative nitrate reduction test. If no color change has occurred it signifies that nitrates in the medium were reduced beyond nitrites to ammonia or nitrogen gas, indicating a positive nitrate reduction test.

Material, Reagents and methods: Nitrate broth per group 25mL of media dispensed into tubes @ 3mL/tube = approx. 7 tubes; Solution A (Sulfonilic acid), Solution B (α —napthylamine) and Zn powder, Bunsen burner, inoculating loop, te.4 tube rack. Nitrate broth composition (g/L):

Peptone — 5.0

Beef extract 3.0

Potassium nitrate — 0.5%

pH 7.2

Procedure

1. Using sterile technique inoculate each experimental organism into its appropriately labeled tube as directed. The last tube will serve as control.

2. Incubate lot. 24 to 48 hours at 37°C.

Observations:

- Add 5 drops of Solution A and then 5 drops of Solution B to ad nitrate broth cultures. Observe and record whether or not red coloration has developed.

- Add a pinch of Zn powder to the cultures which did not show any red coloration. Observe the color change if any.

- Based on your observations determine and record whether or not each organism was capable of nitrate reduction and to which extent.

Bacterial Species	**Red coloration with soln. A & B** (+) **or (-)**	**Red coloration with Zn** (+) **or (-)**	**Nitrite reductions** (+) **or (-)**	**End products**

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Aim 4.3.4: Detection of enzymes urease, catalase and cytochrome osidase activity in groups of bacteria

Principle: Urease enzyme produced by some inicro-organisms attacks the N2 and C bond in amide compounds such as urea and forms alkaline end product NH3_
The presence of urease is detectable when organisms are grown in urea broth medium containing pH indicator phenol red. As the substrate is split into its products, the alkaline environment created due to NH3 is detected as phenol red turns deep pink. This indicates a positive reaction. This is especially useful in identification of *Proteus vulgaris.*

During aerobic respiration microorganisms produce H_2O_2 which in some cases is an extremely toxic compound. Organisms capable of producing catalase rapidly degrade H_2O_2 to H_2O and O_2.

Catalase production can be determined by adding the substrate H_2O_2 to an appropriately incubated slant culture. If catalase is present bubbles of O_2 will be observed indicating a positive reaction.
Cytochrome oxidase catalyzes the oxidation of reduced cytochrome by O_2 resulting in the formation of H_2O or H_2O_2. The ability of bacteria to produce cytochrome oxidase can be determined by addition of test reagent p-aminodimethylaniline oxalate to colonies grown on a plate medium. This light pink reagent serves as an artificial substrate donating electrons and thereby becoming oxidized to a blackish compound in presence of oxidase and free O_2. Following addition of the test reagent the development of purple and then dark coloration is indicative of Cytochrome oxidase production and represents a positive test. No color change or light pink coloration indicates a negative test.

Material, Reagents and methods:

For urease test: Urea broth -21tubes @ 3ml/tube, add filter sterilized urea broth concentrate @ 10 % to sterilized distilled water: Bunsen burner, inoculating loop, test tube rack, methyl red solution,For catalase test: Fresh culture slants, 3% F170, solution, Bunsen burner, inoculating loop, test tube rack, For oxidase test: Fresh culture slants, p-aminodimethylaniline oxalate, Bunsen burner, inoculating loop, test tube rack.

Procedure: <u>For urease test:</u>

- Using sterile technique inoculate each experimental organism into its appropriately labeled tube as directed. The last tube will serve as control,
- Incubate for 24 to 48 hours at 37°C.

For catalase test:

- Using sterile technique, inoculate each experimental organism into its appropriately labeled tube by means of a streak inoculation. The last tube will serve as control.

For oxidase test:

- Prepare appropriate media plate for inoculation by dividing bottom of the plates into appropriate number of sectors.
- Inoculate the test organisms into each sector by a single line streak inoculation.
- Incubate for 24 to 48 hours at 37°C.

Observations:

For urease test: Examine the urea broth cultures for color & record your observations.

Bacterial species	Color of medium	Urea hydrolysis (+ or -)

For catalase test: Allow 3-4 drops of 3% 11202 to flow over the entire surface of **each** slant culture. Examine for the presence or absence of bubbling or foaming. Record your observations.

Bacterial species	Presence or Absence of bubbling	Catalase production (+ or -)

For oxidase test: Add 2 or 3 drop § of p-aminodimethylanffine oxalate to the surface of growth each test organism. Observe the color change from pink to deep purple within 5-10 seconds for +ve reaction. Record whether the colonies are oxidase +ve or —ve

Bacterial species	Color of colonies	Oxidase production (+ or -)

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Experiment 5: Culture independent analysis of microbial communities by 16srDNIA sequencing method

Principle: The culture independent method utilizes PCR to amplify the DNA encoding 16srRNA gene and then subsequently sequence the purified amplification product to obtain the sequence.

The DNA sequence thus, obtained is compared for identity using BLAST tool for identifying the species till the genus level.

Material, Reagents & equipments used: Test-tubes, 0.2m1 PCR tubes, 1.5ml MCTs 1-200u1 1 - 1000u I tips, autopippttes, scalpel blades, gloves, tissue papers. Ice-bucket, 96 well optical clear plates for sequencing machine.

I X PCR buffer, forward & reverse primers for target gene, cINIP (2.5m4), MgC17 (25uM), Taq polymerase, template DNA lysate, nuclease free water, ready reaction mix with dNTP's 8,7, co-factors, sequencing PCR dilution buffer. Sterile MQ water, Hi-Di formamide, gel band PCR purification kits, ethanol (70%) freshly prepared

PCR machine, water bath & dry bath, electrophoretic gel apparatus, Gel-Doc system & UV-transilluminator, DNA genetic analyzer.
Procedure:

1. PCR reaction is set up to amplify the gene of interest as per the protocol given by the instructor.
2. Following it the bands are separated by agarose gel electrophoresis using horizontal DNA gel electrophoretic apparatus
3. The PCR bands are eluted using PCR gel elution kit.
4. Sequencing PCR is set up using the purified PCR DNA as template and the 16srRNA as forward and reverse primers.
5. After this the sequencing PCR reaction mixture is cleaned up using standard protocol as per the instructions given by the instructor,
6. The purified amplified products are loaded onto the 3130XL Genetic analyzer and run as per the standard protocol.
7. Following completion of the run the sequence obtained is taken for alignment with the NCBT database and local alignment is done using BLAST tool. The genus shown highest identity is selected.

Note: For complete confirmation of say a new genus/species in addition to I 6srDNA sequencing other analytical methods such as fatty acid profile. DNA-DNA hybridization etc. also needs to be done.
Observations:
Copy & paste your sequence data. Note & write down your BLAST results along with alignment details.

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Experiment 6: Identification of the genus of unknown bacterial cultures

Principle: Utilizing the diagnostic biochemical tests such as done in experiments 1 to 4 and using the Bergey's manual the organism's arc assigned into different genus/groups. The results thus obtained is corroborated with the sequencing (expt. 5) data to finally assign a given isolate to the genus level.

Procedure:
Using identification flow charts as in Bergey's manual of determinative Bacteriology, assign the unknown bacterial isolates upto the genus level.
Co-relate your observations with those reported.

Observations:

Tabulate your observations as follows:

Biochemical test	Test Results	
	Isolate no.1	Isolate no.2

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Experiment 7: Determining the susceptibility pattern of the given bacterial isolate

Aim 7.1: Determining antibiotic susceptibility pattern of a bacterial isolate by disk diffusion method.

Principle: Antibiotic resistance is the acquired or inherent ability of a bacterial isolate to resists effects of the therapeutic agent. This is validated by creating a lawn of bacterial culture on a non-selective culture medium such as Muller-Hinton broth/agar. Followed by inoculating a standardized antibiotic disk above the lawn. Following overnight incubation the antibiotics loaded on the disk diffuses into the agar medium and affects the bacterial growth. If the strain is resistance towards the given antibiotics, then its growth will not be inhibited and it will not show a zone of growth inhibition surrounding the disk. Lithe strain is sensitive towards the antibiotics then a zone of inhibition would be observed around the disk. As per the CLSI standards which are followed universally, specific diameters of zone or inhibition has been allotted against resistance, intermediate or sensitive for a particular potency of the antibiotic loaded On the disk.
Materials and reagents required: Muller-Hint-on broth, Muller-Hinton agar plates, blunt ended forceps, different antibiotic disks, sterile cotton swab, burner, ethanol (70%).

Procedure

1. Raise a log phase of bacterial culture in Muller-Hinton broth.
2. Using sterile cotton swab make a lawn of the culture on Muller-Hinton agar plate.
3. On top of the lawn place carefully an antibiotic disk carefully so as not to dig into the agar or allow the disk to jump over the plate. Using a sterile blunt ended forceps slightly press the disk against agar, to make it stay.
4. Incubate the plates overnight in inverted position inside an incubator at 37°C for growth and observe the plates the following day.

Observations:

Tabulate our observations as follows:

Antibiotic used	Abbreviation	Potency	Diameter of clearance	Resistance/ Intermediate/ Sensitive

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Aim 7.2: Determining the Minimum Inhibitory Concentration (MEC) of a given antibiotic towards a bacterial isolate.

Principle: The MIC is defined as the concentration at which the bacterial ceases to grow. The MIC level of a resistant isolate is important criteria for determining the level/extent of resistance exhibited by the bacteria for a particular antimicrobial agent. This in turn will help determine whether a particular antibiotic can still be effectively used at higher concentrations as a therapeutic agent.

Materials and reagents required: Muller-Hinton broth, different antibiotic stock solution, sterile 1- 200u1 and 1 00-1 000u1 sterile rips, test-tubes, burner, ethanol (70%), sterile 0.22micron syringe filters, 5m1 disposable syringes, 0.22micron filter membranes for syringe litters. autopippettes, sterile saline.

Procedure:

1. A pure culture of a single microorganism is grown in Muller-Hinton broth, or other broth as appropriate.

2. The culture is standardized using standard microbiological techniques to have a concentration of very near I million cells per milliliter. The more standard the microbial culture, the more reproducible the test results.

3. The antimicrobial agent is diluted a number of times, 1:1, through sterile diluents (usually Mueller- Hinton broth).

4. After the antimicrobial agent has been diluted, a volume of the standardized inoculums equal to the volume of the diluted antimicrobial agent is added to each dilution vessel, bringing the microbial concentration to approximately 500,000 cells per milliliter.

5. The inoculated, serially diluted antimicrobial agent is incubated at an appropriate temperature for the test organism for a pre-set period, usually .18 hours. The more standard the incubation period, the more reproducible the test results.

6. After incubation, the series of dilution Vessels is observed for microbial growth, usually indicated by turbidity and/or a pellet of microorganisms in the bottom of the vessel. The last tube in the dilution series that does not demonstrate growth corresponds with the minimum inhibitory concentration (M1C) of the antimicrobial agent.

Observations:

Observe the series of dilution for microbial growth and note the antibiotic concentration of the tube that does not show any bacterial growth.

STANDARD OPERATING PROCEDURE FOR MICROBIOLOGY LAB

1. Handling Microscopic slide: Holding the slide through its side, clean commercially available microscopic slides to remove finger marks, dust particles.

2. Handling culture vessels: Organize all experimental culture media & sterile vessels at the beginning of each experiment. Label culture vessels with non-water soluble marker directly below the cap of the culture tube. Label should always contain name of the test organism, name of the medium dilution if nay, date and your initials. Use leak proof containers in sealed plastic bags for transportation of the sample.
In case of petri plates organism name should be written on the bottom periphery to prevent obstruction for viewing g the results.

3. Handling media & reheating media: While weighing media for preparation make sure you switch off the ceiling fans, use a clean, dry spatula & use a clean butter paper or any other material for dispensing the dehydrated materials. After use close tight the respective bottles with their lid. Make sure NOT to SWA P the covers. Add required amount of water, cover with plug put in the autoclave for sterilization. Make sure there is sufficient water in the autoclave before switching it on. Take out the media from the autoclave only when the pressure in the autoclave has come down and materials/glassware's cool enough to handle. Always use gloves. DO "NO' swirl vigorously hot agar/solidified media. The plug might pop-up due to steam pressure and spills the hot media on hand.

4. Handling inoculation loops & needles: r is essential that you incinerate entire wire to ensure absolute sterilization. Briefly also pass the shaft through the flame to remove dust or possible contaminants. Cool the inoculation loop b slowly and gently tapping the inner surface of culture tube or petri dish cover before taking out inoculums.
While transferring from an agar culture touch only a single area of growth, NEVER drag the loop or needle over entire surface. DO NOT dig into the solid medium.
In case of broth culture, DO NOT lap the tubes vigorously as this may lead to frothing and denaturation of protein s in the medium.

5. Using Laminar Air Flows/Biosafety cabinets: Perform all work on isolates in Laminar Airflows or Biosafety safety cabinets. DO NOT open culture plate/tubes outside the cabinets. Clean the hoods/cabinets before and after use with 70% ethyl alcohol. DO NOT leave used tips, swabs, loops unsterilized inside the cabinet. Sterilize and dispose of them aseptically immediately after you have finished working. DO NOT crowd up the cabinet with items which are not in regular use such as test-tubes, unopened petridishes. Beakers with tips, swabs etc.

6. Place test-tubes containing culture medium in racks for incubation. Always incubate petri-dishes in inverted position to prevent water condensation from dropping Ol1to surface of culture medium. Excess moisture serves as vehicle for producing content rather than discrete colonies.

7. Meticulously record all observed data, wherever necessary illustrate your observations/techniques by drawing and label the diagram.

8. At the end of the session disposes off contaminated materials such as swabs, disposable pipettes and paper towels in autoclavable bags/biohazard receptacles.
9. Neatly place all the reagents, supplies and chemicals t their original locations. Place all capped test tube cultures and closed petri-dishes in designated incubation/disposal area as appropriate. Wipe the working table clean with disinfectant & wash your hand before leaving the laboratory.

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Laboratory Handout

Instructor -Dr. Harapriya Mohapatra

Experiment 1: CULTURE media preparation, Control of microbial growth disinfection & sterilization

Aim: To get to know basic laboratory techniques involved in microbiology such as:

(i) Media preparation (ii) sterilization (iii) disinfection

Theory/Principle: A solution containing soluble low molecular weight substances often derived frol11 enzymatic degradation of complex nutrients that supports growth of microorganisms is called a culture medium. Culture medium may be liquid i.e. lacks a solidifying agent (called broth) or solid i.e. supplemented with a solidifying agent such as agar.

Agar serves as solidifying agent because it liquefies at 100°DC and solidifies at 40°C. A completely solid medium requires an agar concentration of 1.5-2%. Solidified medium help in isolation of discrete colonies of unadulterated species of cells -termed as pure culture, from a mixed microbial culture.
The solidified culture media when in gel state can be placed in test-tubes which subsequently are allowed to harden in slanted position referred to as slants or hardened in upright position called deep tubes or poured into petri dishes producing agar plates. In addition to nutritional need it is essential to maintain the cultures at appropriate environmental conditions such as pH, temperature and gaseous requirements.

Diagrams

In order to successfully ark with only a single microbial species all the equipments needs to be sterilized. Sterilization refers to the process by which an article, surface or medium is freed from all living microorganisms either in vegetative or spore state, whereas, disinfection means destruction or removal of all pathogenic organisms. Various physical and chemical agents can be used for the purposes. Most commonly used methods include dry heating -flaming, incineration, hot air; moist heating boiling, steam under pressure; filtration -bacterial membranes; radiation -UV, y-irradiation. Most common chemicals used include -ethyl alcohol, formaldehyde, gluteraldehyde, halogens such as I2, surface active agents as found ill wetting agents, detergents and emulsifiers. In day to day laboratory practices UV irradiation, flaming & steam under pressure are commonly used methods of physical sterilization while chemical agents used include ethyl alcohol, formaldehyde etc. Steam under pressure is carried out in specialized equipment called as 'autoclave' which essentially works on the principles used in pressure cooked in our homes, For efficient sterilization it is recommended to use a temperature of 121°C (15lb pressure) for 15 minutes.
Material, Reagents and methods: Per group/student: Test-tubes -16no.s; Petri dishes -5 no's; Marker pen I no's; Pipette for dispensing 5m.1 -1 no's Conical flask 250ml -2 no's, 100 ml-l no's; Test-tube rack -2 no's; Peptone, Beef extract, 0.0 I N NaOH I 0.0 IN HCI for pH adjustment, Agar-agar.

Procedure:

- Per group/student will be preparing nutrient broth tubes -5 no's @ 3ml/tube, nutrient agar deep tubes -.5 110.S & Nutrient agar slants -5 no's @ 3ml/tube, and plates -5 no's @ 18-20 ml/plate

- Accordingly calculate the quantity of each type of media to be prepared.

- Preparation of Nutrient Broth: In a 100mi conical flask prepare 25ml of nutrient broth using the following composition (g/L):

    - Peptone - 5.0
    - Beef extract - 3.0

- Using 0.01N NlaOH/ HCl adjust pH to 7.0.

- In another 250ml conical flask prepare 125ml Nutrient broth, adjust H and then add agar-agar @ 2%.
- Dispense the media into respective tubes but not into petri plates. Keep the media for the petri plates in the flask only.
- Plug the tubes with non-absorbent cotton, wrap with brown paper and put them in a basket, keep ready for autoclaving.
- Set the autoclave at required temperature and sterilize the media.
- Remove from the autoclave after cooling it down, keep the nutrient agar tubes in slanting position 8t 30-45° angle for slant preparation , keep others upright standing for deep tube, pour the rest of the media into petri-dishes inside laminar flow for plate preparation.
- Keep the media overnight for solidification & checking for contamination.

Observation:
- Observe and draw the slant deep to be and plate formation 0f the media
- See if there is any turbidity in the liquid media or any colonies on the solid media.

Conclusion:

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Laboratory Handout

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Expedment 2: isolation and culture and characterization of pure culture; enumeration & use of selective and differential media.

Aim 2.1 Isolation of single colony by streak plate method and determining cultural characteristics of organisms,

Theory'/Principle: Isolation of discrete colonies requires that the number of organisms in the inoculull1s be reduced, The resulting diminishing population size ensures that after

inoculation, individual cells wil.1 be sufficiently far apart on the surface of agar medium to allow the separation of different species/colonies present. Streak plate technique involves spreading a loopful of culture over the surface of an agar plate. The four-way quadrant streaking technique is most commonly used the purpose. (a) A loopful of inoculums is placed on the surface of agar (area I), subsequently the I OR is flamed, cooled by touching the unused part of agar surface close to the periphery of the plate, dragged rapidly over area I (b) reflamed, petri dish turned 90°C, loop touched to the comer of the culture area I, dragged several times in area 2 (c) & (d) the process repeated two more times as depicted in the figure.

When grown on a variety of media, microorganisms exhibit differences in macroscopic appearance of their growth. These characteristics aid in separating the micro organisms ms into taxonomic groups and (Ire determined by culturing them on nutrient agar, slants, broths and plates.

(i) Nutrient agar slants: (a) Abundance of growth: Amount of growth is designated as none, slight, moderate or large; (b) Pigmentation: Many of the chromogenic microorganisms produce either intracellular pigment responsible for coloration of the colonies while others may produce diffusible extracellular soluble pigments. Majority of the microorganisms however appear white or gray. (c) Optical diffusive characteristics: Colonies may be opaque, translucent or transparent; (d) Form: Appearance of colony on agar plates.

(ii) Nutrient agar plates: (a) Form (b) Margin (c) Elevation
(iii) Nutrient broth cultures: (a) Uniform fin e turbidity -finely dispersed growth throughout;
(b) Flocculent -flaky aggregates dispersed throughout; (c) pellicle -thick, padlike growth on surface; (d) sediment -concentration of growth at the bottom of broth culture may be granular, flanky or flocculent
Material, Reagents and methods: Per groups/student: nutrient agar slants tubes -2 no's each; Petri dishes -2 no's; nutrient broth -2 no's. Marker pen I no.; Test-tube rack -I no's; Bunsen burner, laminar' air hood, revived cultures, inoculating needles.

Procedure:

1. Using sterile transfer technique, inoculate each of the appropriately labeled media as follows:

    (a) Nutrient agar plates: With a sterile loop prepare a streak plate inoculation of the cultures provided for isolation of discrete colonies. Inoculate the plates in inverted position.

    (b) On nutrient agar slants make a single line streak of the culture provided, drawing the needle frol11 the centre to the outer edge

(c) On nutrient broth using a sterile loop inoculate .each organism into a tube of nutrient broth. Shake the loop well to dislodge the inoculul11s.

2. Incubate the cultures at 37°C for 24 hours & make your observation.

Observation:

(a) From nutrient agar plates draw distribution of colonies & their morphology
(b) Draw the distribution of growth on the slant surface
(c) Observe and draw the growth pattern in nutrient broth.

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Aim 2.2 Isolation of single colony by spread plate method and determining bacterial cell count by CFU method.

Principle: This technique requires spreading and separating out individual bacterial cells from a mixed population using previously diluted bacteria I cultures (this can be a mixed population or single population). In case of a mixed population the technique helps isolate distinct colonies while in a single culture would enable indirect determination of the bacterial cells in a given population,

The dilution technique followed for spread plate is mainly the 10-fold di1ution technique depicted in the figure. The number' of bacterial cells in the parent is given by:
Number of bacteria I cells/ml = number of colonies on plate x reciprocal of dilution of sample used

Materials & reagents required: Bent glass rod, beaker, turn table, 95% ethyl alcohol, nutrient agar plate, auto pipette, 1ml tips. Phosphate buffer saline (sterile), test-tubes, Bunsen burner, bacterial inoculums.

Procedure:

1. Place bent glass rod into a beaker to which sufficient amount of 95% EtOH is added.
2. With a sterile pipette onto agar plate place 50 µl of diluted culture mixture.
3. Remove glass rod from ethyl alcohol, flame, cool for 10-15 seconds, remove the petri dish cover and spread the mixture with moving glass rod to and fro over the plate.
4. Observation: Draw the colonies as they appear on agar plate

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Experiment 3: Microscopic examination of fresh culture with differential staining producers
Aim 3.1: To differentiate the given bacterial strains into two principal groups of bacteria: Gram negative and Gram positive

Principle/Theory): Differential staining requires the use of at least three chemical reagents that are applied to a heat fixed smear. fi rs t is the primary stain which colors all cells. Second the decolorizing agent which depending upon the chemical composition of the cell mayor may not re 1 e t he primary stains from the entire cell. Final one is the counter stain which gets absorbed only if. The primary stain is washed out. In addition sometimes certain chemical compounds

may be used which increases the cells affinity for a stain. In Gram staining procedure Crystal violet is used as the primary stain, Gram's Iodine is used as a mordant which forms a complex with crystal violet and intensifies the color. The decolorizing agent is 95% ethyl alcohol and the counter stain is safranin.

Material and reagents required: 24 hours old bacterial cultures, crystal violet, Gram's iodine, 95% ethyl alcohol and safranin, Bunsen burner, inoculating loop, staining tray, tissue paper, glass slides, and microscopes.

Procedure:

1. Using sterile technique prepare smear of each of the organisms given on the glass slides. Allow it to air dry & then heat fix.
2. Gently flood smears with crystal violet and let stand 1 minute.
3. Gently wash off the excess dye with tap water.
4. Gently flood smears with Gram's iodine morden and let stand for 1 minute.
5. Gently wash with tap water
6. Decolorize with 95% ethyl alcohol Caution: Care has to be taken so a~ not to over decolorize the primary stain. Add reagent drop by drop until alcohol runs almost clear, showing only a blue tinge.
7. Gently wash with tap water.
8. Counter stain with safranin for 45 seconds.
9. Gently wash with tap water.
10. Blot dry with tissue/blotting paper and observe under oil immersion

Observation:

(ii) Make drawing of a representative microscopic field

(iii) Describe cells according to morphology and arrangement

(iv) (iii) Describe the color of the stained cells