Preparation of culture media

Microbiology I

13.1.21

Culture media:

- A growth or culture medium is a gel or liquid substance designed to support the growth of micro organisms or cells.
- There are different types of media used for different types of micro organisms or cells.
- The most commonly used growth media for micro organisms are nutrient broths and agar plates.
- Some fastidious organisms need specialized media for there growth.

Agar

- Used for preparing solid medium.
- Obtained from seaweeds.
- No nutritive value.
- Not affected by the growth of Bacteria.
- Melt at $98^{o}C$ and sets at $42^{o}C$.
- Approx. 2% agar is employed in solid medium.

TYPES OF CULTURE MEDIA

- Based on their consistency

a) Solid medium
b) Liquid medium
c) Semisolid medium

- Based on constituents

a) Simple medium
b) Complex medium
c) Synthetic and defined medium
d) Special media

Types of culture media (cont.)

- Based on oxygen requirements

a) Aerobic media
b) Anaerobic media

- Special media

a) Enrichment media
b) Enriched media
c) Selective media
d) Indicator media
e) Differential media
f) Sugar media
g) Transport media
h) Media for bio-chemical reaction

Complex Media

- Media other than basal media.
- They have added ingredients.
- They provide special nutrients.

Synthetic or Defined Media

- Media prepared from pure chemical substances and its exact composition is known. (E.g. peptone water - 1% peptone + 0.5% NaCl in water)

Nutrient broth

- It is a general purpose medium
- used for cultivating a broad variety of microorganisms
- with non-specific nutritional requirements.
- **Contents**:
- Peptone
- yeast extract
- Sodium chloride

Nutrient Broth

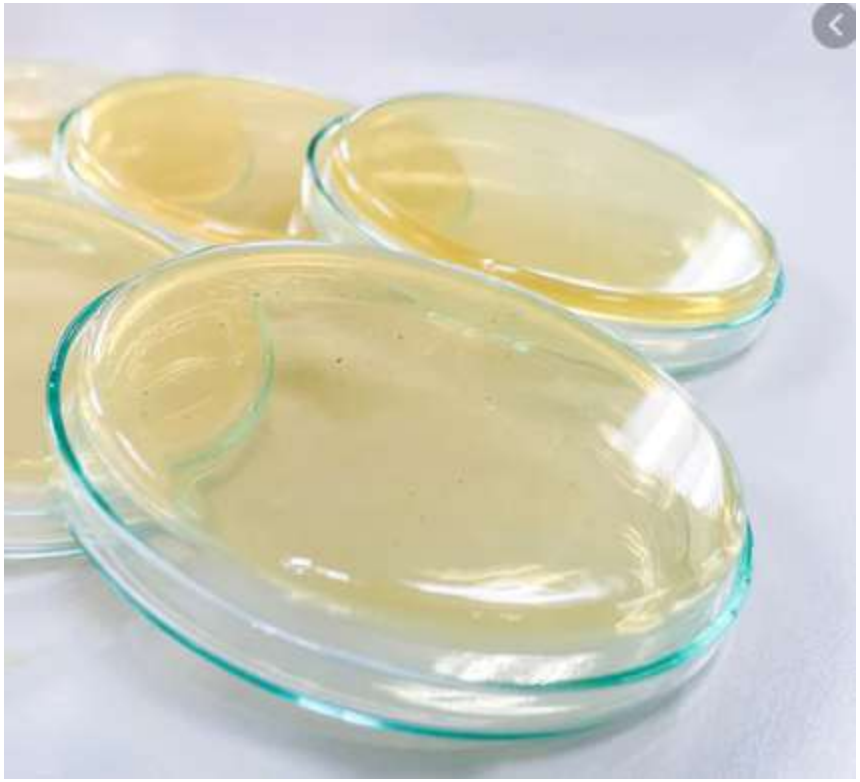
Nutrient broth

- Peptone and yeast extract:
  - nitrogenous compounds,
  - vitamin B complex,
  - amino acids
  - essential growth nutrients.
- Sodium chloride: maintain the osmotic balance
- Glucose: carbohydrate source.

Nutrient agar :

Nutrient broth medium is supplemented with agar

- It contains all the elements that most bacteria need for growth.
- It is a undefined and non-selective medium.



Nutrient Agar

Composition of Nutrient Broth and Nutrient Agar:

- **Nutrient Broth**
- Peptic Digest of animal tissue - 5 g
- Sodium chloride - 5 g
- Beef Extract - 1.5 g
- Yeast Extract - 1.5g
- Distilled Water - 1000 ml

- **Nutrient Agar**
- Peptic Digest of animal tissue- 5 g
- Sodium chloride - 5 g
- Beef Extract - 1.5 g
- Yeast Extract - 1.5g
- Agar - 15g
- Distilled Water - 1000 ml

- **Preparation of Nutrient Broth: 100ml**
- Weigh 1.3 gram of nutrient broth powder and put it in a conical flask with a cotton plug.
- Add 100 ml of distilled water to the flask
- Dissolve the nutrient broth powder in the water by swirling.
- Autoclave it at 15 psi pressure, 121°C in autoclave.

Preparation of Nutrient Agar (Solid medium): 100ml

It is usually used at a concentration of 2.8 gm in every 100 ml distilled water.

i. Prepare as instructed by the manufacturer.
ii. Sterilize by autoclaving at 121°C for 15 minutes.
iii. Pour aseptically to agar plate.
iv. Date the medium and give it a batch number.
v. Store in a cool dark place.

Note:

Shelf life is up to 2 years.

pH of medium should be within the range of 7.2 to 7.6

Uses

- It is used for culturing almost every micro organism.
- Non-fastidious organisms grows well on nutrient agar.
- It is used for routine culturing in microbiology lab.
- In the early 1900's it is suggested for water testing.

- Precautions:
- Always use the distilled water for making the media.
- All the glassware should be properly sterilized.

Blood agar

- It is an enriched and differential media.
- It is used to grow fastidious organisms.
- It contains defibrinated mammalian blood (usually sheep or horse blood).

Note:

Sheep blood may contain inhibitors to *Heamophilus Influenzae.*

Expired, citerated, donor blood should not be used because this may contain substance inhibitory to the growth of some pathogens (e.g. β *Hemolytic Streptococci*).

CONTENTS

- Blood agar base
  - Enzymatic Digest of Casein ..... 15 g
  - Enzymatic Digest of Animal Tissue ..... 4 g
  - Yeast Extract ..... 2 g
  - Corn Starch ..... 1 g
  - Sodium Chloride ..... 5 g
  - Agar ..... 14 g
- Columbia agar
- Tryptone soya agar

PREPARATION

i. Prepare as instructed by the manufacturer.
ii. Sterilize by autoclaving at 121^{o}C for 15 minutes.
iii. Transfer to a water bath at 50^{o}C.
iv. When the agar is cooled to 50^{o}C, add the blood aseptically and mix gently.
v. Pour aseptically 15 ml in the sterile petri dish.
vi. Date the medium and give it a batch number.
vii. Store plates at 2^{o}C - 8^{o}C preferably in sealed plastic bags to prevent loss of moisture.

Note:

Shelf life up to 4 weeks.

pH ranges from 7.2 to 7.6 at room temperature.

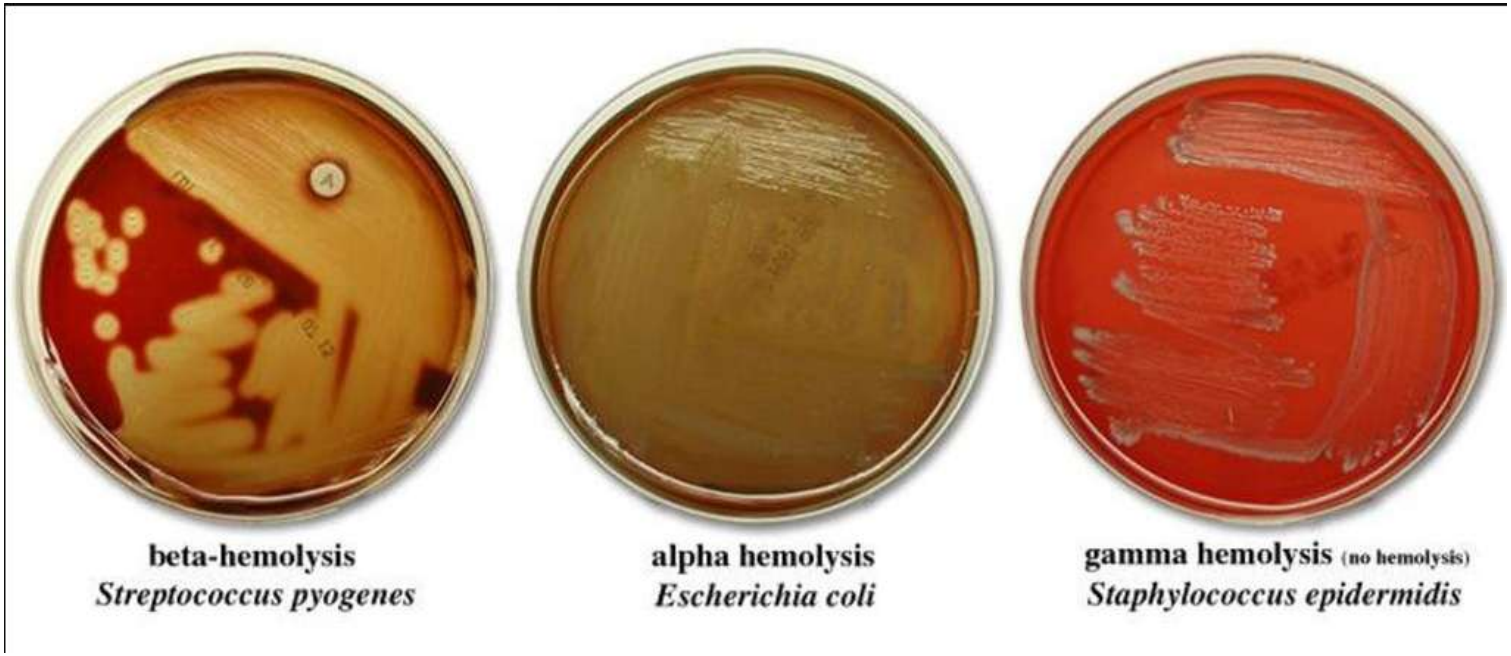
Uses

- It is used for the growth of fastidious organisms.
- It is a differential medium based on the hemolytic reaction.

There are 2 types of hemolytic reactions shown by the organisms o blood agar plate.

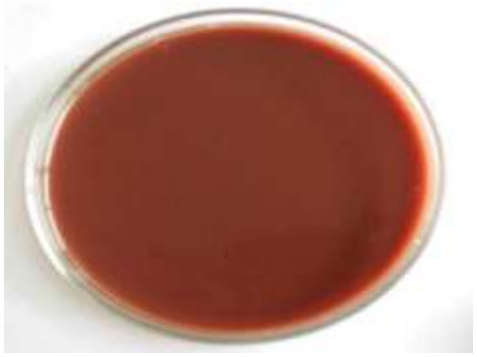
- α-Hemolysis
- β-Hemolysis

Hemolysis types



CHOCOLATE AGAR

- When blood agar is heated, the red cells are lysed and medium becomes brown in color. It is referred to as chocolate agar and supplies the factors required for the growth *Haemophilus influenzae*.
- It is also used to culture nutritionally demanding pathogens such as *Neisseria meningitides* and *Streptococcus pneumoniae*



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  - Yeast Extract ..... 2 g
  - Corn Starch ..... 1 g
  - Sodium Chloride ..... 5 g
  - Agar ..... 14 g
- Columbia agar
- Tryptone soya agar

PREPARATION

1. Prepare as describe for blood agar except after adding the blood, heat the medium in a 70ºc in water bath until it becomes brown in color. This takes about 10-15 minutes during which time the medium should be mixed gently several time.
2. Allow the medium to cool to about 45ºc, remix and pour in sterile petri dishes as describe for blood agar.

Note:

1. Care must be taken not to over heat or prolong the heating of the medium because this will cause it to become granular and unfit for use.
2. Date the medium and give it a batch number store the plates as describe for blood agar.
3. pH ranges from 7.1 to 7.5

PERFORMANCES

- Test the medium by inoculating it with *Haemophilus influenzae*. After overnight incubation in a candle jar at 35–37 ºC and record the growth.

Uses

To isolate *Haemophilus influenzae* & *Neisseria gonorrhoeae.*

MacConkey's Agar

- First solid differential media to be formulated and developed in 20th century by Alfred Theodore MacConkey
- Selective and differential medium for cultivation of coliform organisms.
- It is used for the isolation and differentiation of Gram-negative rods
- It is used for the isolation and differentiation of members of the *Enterobacteriaceae* family
  - *E. coli*
  - *Salmonella*
  - *Shigella*
  - *Klebsiella*

- Crystal violet and bile salt - inhibitory to the growth of Gram positive bacteria such as *S. aureus*
  - the selective nature of MacConkey agar.
- Neutral red is the pH indicator:
- red at a pH below 6.8 and is colorless at any pH greater than 6.8.
  - the differential nature of the media

Contents

Ingredients	Amount
Peptone (Pancreatic digest of gelatin)	17 gm
Proteose peptone (meat and casein)	3 gm
Lactose monohydrate	10 gm
Bile salts	1.5 gm
Sodium chloride	5 gm
Neutral red	0.03 gm
Crystal Violet	0.001 gm
Agar	13.5 gm

Preparation:

1. Suspend 49.53 grams of dehydrated medium in 1000 ml purified/distilled water.
2. Heat to boiling to dissolve the medium completely.
3. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.
4. Cool to 45-50°C.
5. Mix well before pouring into sterile Petri plates.

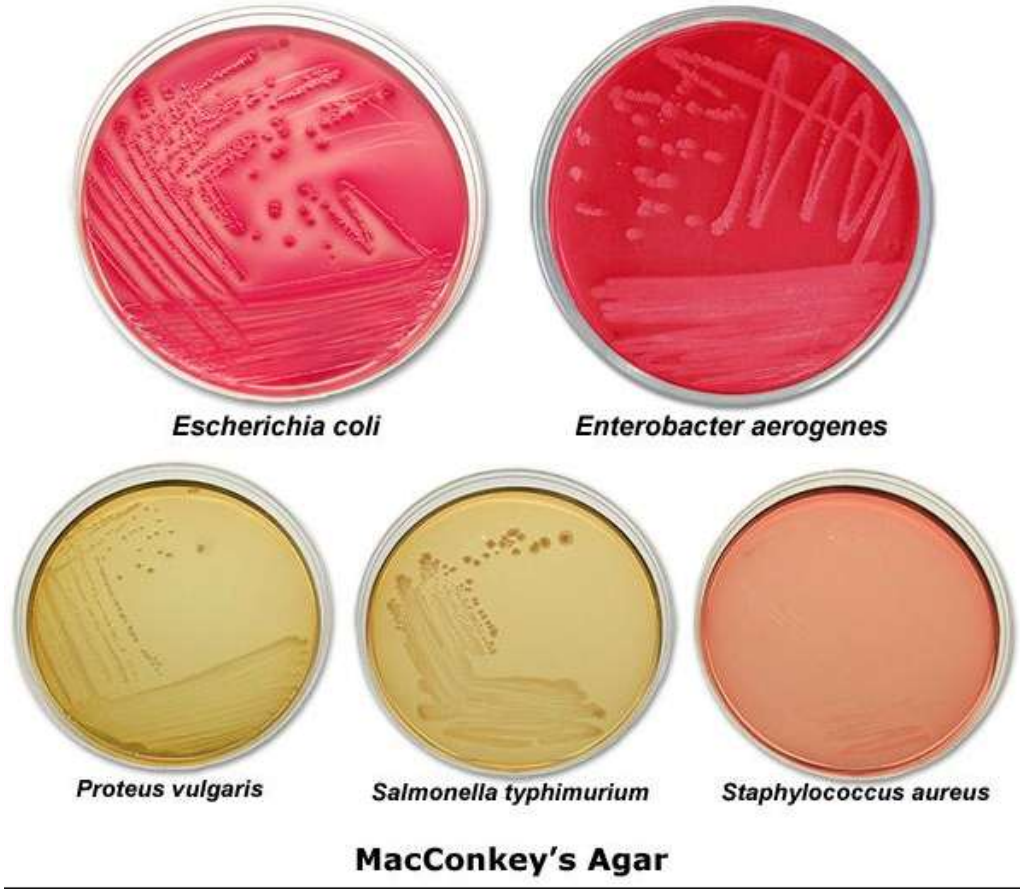
Uses:

- MacConkey agar is used for the isolation of gram-negative enteric bacteria.
- It is used in the differentiation of lactose fermenting from lactose non-fermenting gram-negative bacteria.
- It is used for the isolation of coli forms and intestinal pathogens in water, dairy products and biological specimens.

- Interpretation:
- **Lactose fermenters** - red/pink colony ex. *E. coli*
- **Non-lactose fermenters** - colourless colony ex. *Shigella* and *Salmonella*

Lactose Fermenting Colonies

non-Lactose Fermenting Colonies



Salmonella Shigella Agar (SSA)

- It is a selective and differential medium
- Used for isolation, cultivation and differentiation of gram-negative enteric microorganisms such as *Salmonella* spp. and some strains of *Shigella* spp.
- SS Agar is a modification of the Desoxycholate Citrate Agar.
- It is recommended for testing clinical specimens and food testing for the presence of *Salmonella* spp. and some *Shigella* spp.

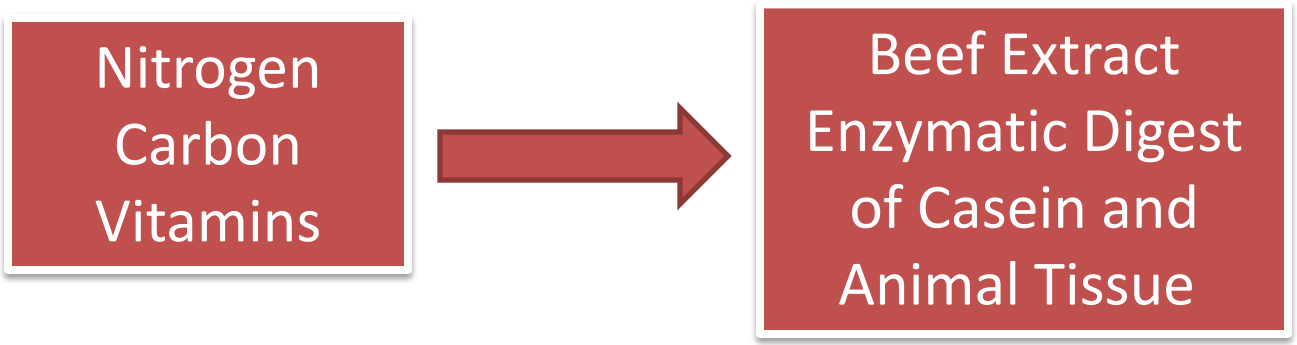
Composition of *Salmonella Shigella* Agar

Ingredients	**Gms / Litre**
Beef Extract	5.00
Enzymatic Digest of Casein	2.50
Enzymatic Digest of Animal Tissue	2.50
Lactose	10.00
Bile Salts	8.50
Sodium Citrate	8.50
Sodium Thiosulfate	8.50
Ferric Citrate	1.00
Brilliant Green	0.00033
Neutral Red	0.025
Agar	13.50

Distilled Water = 1000 ml

Principle of *Salmonella Shigella* Agar

- Bile Salts, Sodium Citrate and Brilliant Green inhibits :
  - gram-positive
  - coliform organisms
  - swarming *Proteus* spp.
- Allows growth of *Salmonella* spp.



- **Lactose:** carbohydrate
- **Thiosulfate** and **Ferric Citrate**:
  - detection of hydrogen sulfide
  - production of colonies with black centers.
- **Neutral red** pH indicator:
  - red in acidic pH
  - fermentation

Preparation of SS Agar

- Suspend 60.0 grams of SS Agar in 1000 ml distilled water.
- Heat to boiling to dissolve the medium completely.
- Do not autoclave.
- Mix well and pour into sterile Petri plates.

Result Interpretation on SS Agar

Organism	Lactose fermentation	H_2S production	Colony morphology
Salmonella	- ve	+ ve	Black center
Shigella	- ve	- ve	colorless
E. coli	+ ve	- ve	Pink
Enterobacter	+ ve	- ve	mucoid, pale, opaque cream to pink
Klebsiella	+ ve	- ve	

Escherichia coli

Salmonella

Shigella

Uses of SS Agar

- It is used as a selective and differential medium for the isolation of *Salmonella* and some *Shigella* species from clinical and non-clinical specimens.
- This medium is not recommended for the primary isolation of *Shigella*.
- It was also developed to aid in the differentiation of lactose and non-lactose-fermenters from clinical specimens, suspected foods, and other such samples.

Eosin Methylene Blue (EMB) Agar

- Differential medium
- Slightly inhibits the growth of Gram-positive bacteria
- Provides a color indicator
- Distinguishes lactose fermenters (*E. coli*) and non lactose fermenters (*Salmonella, Shigella*).

- EMB agar was originally devised by Holt-Harris and Teague and further modified by Levine.

- Combination of:

1. Levine formulae
   - peptic digest of animal tissue
   - phosphate
2. Holt-Harris and Teague
   - two carbohydrates

- The medium is important in medical laboratories to distinguish gram-negative pathogenic microbes in a short period of time.

Composition of EMB Agar

Ingredients	Gms/litre
Peptic digest of animal tissue	10.000
Dipotassium phosphate	2.000
Lactose	5.000
Sucrose	5.000
Eosin – Y	0.400
Methylene blue	0.065
Agar	13.500

Final pH (at 25°C): 7.2±0.2

Principle of EMB Agar

- Two dyes - eosin and methylene blue – absorbed at acidic pH
- Lactose fermenters:
  - Gram-negative
  - produce acid - lowers the pH
  - Dark colonies with a green metallic sheen - ***E. coli***
- Other lactose fermenters produce - larger, mucoid colonies, purple only in their center
- Non lactose fermenters:
  - Alkaline products - increase the pH
  - colorless colony

- Peptic digest of animal tissue:
  - source of carbon, nitrogen, and other essential growth nutrients.
- Lactose and sucrose :
  - fermentable carbohydrates
  - sources of energy
- Eosin-Y and methylene blue (dyes):
  - differential indicators (color)
- Phosphate: buffers the medium

Preparation of EMB Agar

- Suspend 35.96 grams in 1000 ml distilled water.
- Mix until the suspension is uniform.
- Heat to boiling to dissolve the medium completely.
- Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.
- **AVOID OVERHEATING.**
- Cool to 45-50°C and shake the medium in order to oxidize the methylene blue (i.e. to restore its blue color) and to suspend the flocculent precipitate.
- Pour into sterile Petri plates.

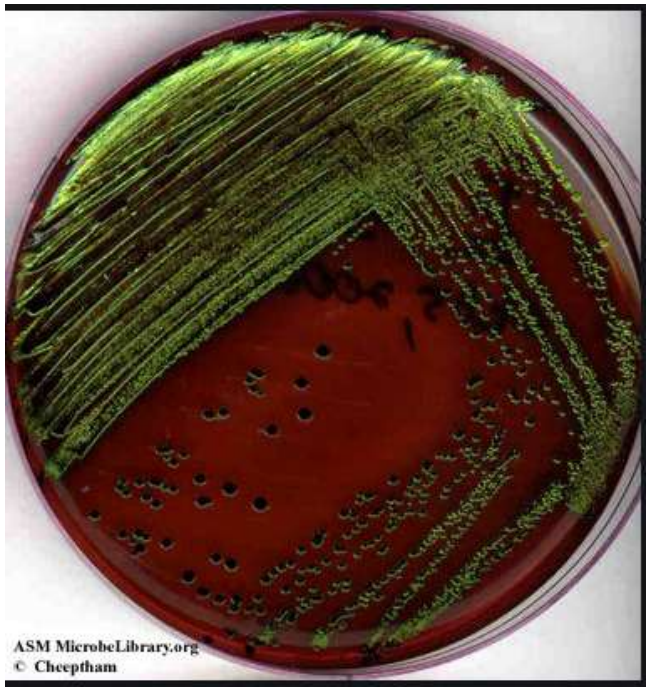
- Allow plates to warm to room temperature.
- The agar surface should be dry before inoculating.
- Inoculate and streak the specimen as soon as possible after collection.
- Incubate plates aerobically at 35-37°C for 18-24 hours and protect from light.
- Examine plates for colonial morphology.
- If negative after 24 hours, reincubate an additional 24 hours.

Result Interpretation on EMB Agar

Organisms	Growth
Escherichia coli	Blue-black bulls eye; may have green metallic sheen
Pseudomonas aeruginosa	Colorless
Enterobacter aerogenes	Good growth; pink, without sheen
Klebsiella pneumoniae	Pink, mucoid colonies
Proteus mirabilis	Luxuriant growth; colorless colonies
Salmonella typhimurium	Luxuriant growth; colorless colonies

Lactose fermenters on EMB Agar:

E. coli



Enterobacter aerogenes

Uses of EMB Agar

- Isolation and differentiation of gram negative enteric bacteria from clinical and nonclinical specimens.
- It also helps in the isolation and differentiation of lactose fermenting and non-lactose fermenting enteric bacilli.
- EMB media assists in visual distinction *Escherichia coli*, other nonpathogenic lactose-fermenting enteric gram-negative rods, and the *Salmonella* and *Shigella* genera.
- It is used in testing the quality of water, especially in determining if the water is contaminated by harmful microorganisms.
- It differentiates microorganisms in the colon-typhoid-dysentery group.

Limitation of EMB Agar

- Some strains of *E. coli* may fail to produce a characteristic green metallic sheen
- the green metallic sheen is not diagnostic for *E. coli*.
- Non-pathogenic, non-lactose-fermenting organisms will also grow on this medium.
- Additional biochemical tests must be performed in order to distinguish these organisms from pathogenic strains.

Mueller Hinton Agar (MHA)

- Developed by Mueller and Hinton in 1941
- Isolation of pathogenic *Neisseria* species.
- Antibiotic susceptibility testing of non-fastidious microorganism
  - checks resistance to some antibiotics
- Kirby-Bauer disk diffusion technique.
- 5% sheep blood + nicotinamide adenine dinucleotide added
  - susceptibility testing
  - *Streptococcus species* and *Campylobacter*.

Composition of MHA

Ingredients	Gram/Litre
Beef Extract	2 g
Acid Hydrolysate of Casein	17.5 g
Starch	1.5 g
Agar	17 g
Distilled Water	1000 ml
Final pH 7.3 ± 0.1 at 25ºC	

Principle of MHA

- **Beef Extract** and **Acid Hydrolysate of Casein:**
  - Nitrogen
  - vitamins
  - carbon
  - amino acids
  - sulphur
  - essential nutrients.
- **Starch:** absorb any toxic metabolites produced.
  - Starch hydrolysis yields dextrose
  - serves as a source of energy.
- **Agar:** solidifying agent.

- Suitable medium - testing the antibiotic susceptibility of microorganisms
  - **Sulfonamides**
  - **Trimethoprim**

  } antibiotics

- Inhibitors of antibiotics are present in the medium :
  - Para-aminobenzoic acid (PABA)
  - Thymidine
- **Para-aminobenzoic acid (PABA)** and its analogs
  - Inhibits Sulfonamide activity
- **Thymidine** reduces the activity of **trimethoprim**
  - smaller inhibition zones and
  - innerzonal colonies

- Mueller Hinton Agar ⟹ **PABA and thymine/thymidine –** minimum amount
  - reduces the inactivation of **sulfonamides and trimethoprim**
  - when the media is used for testing the susceptibility of bacterial isolates to these antibiotics.

Preparation of MHA

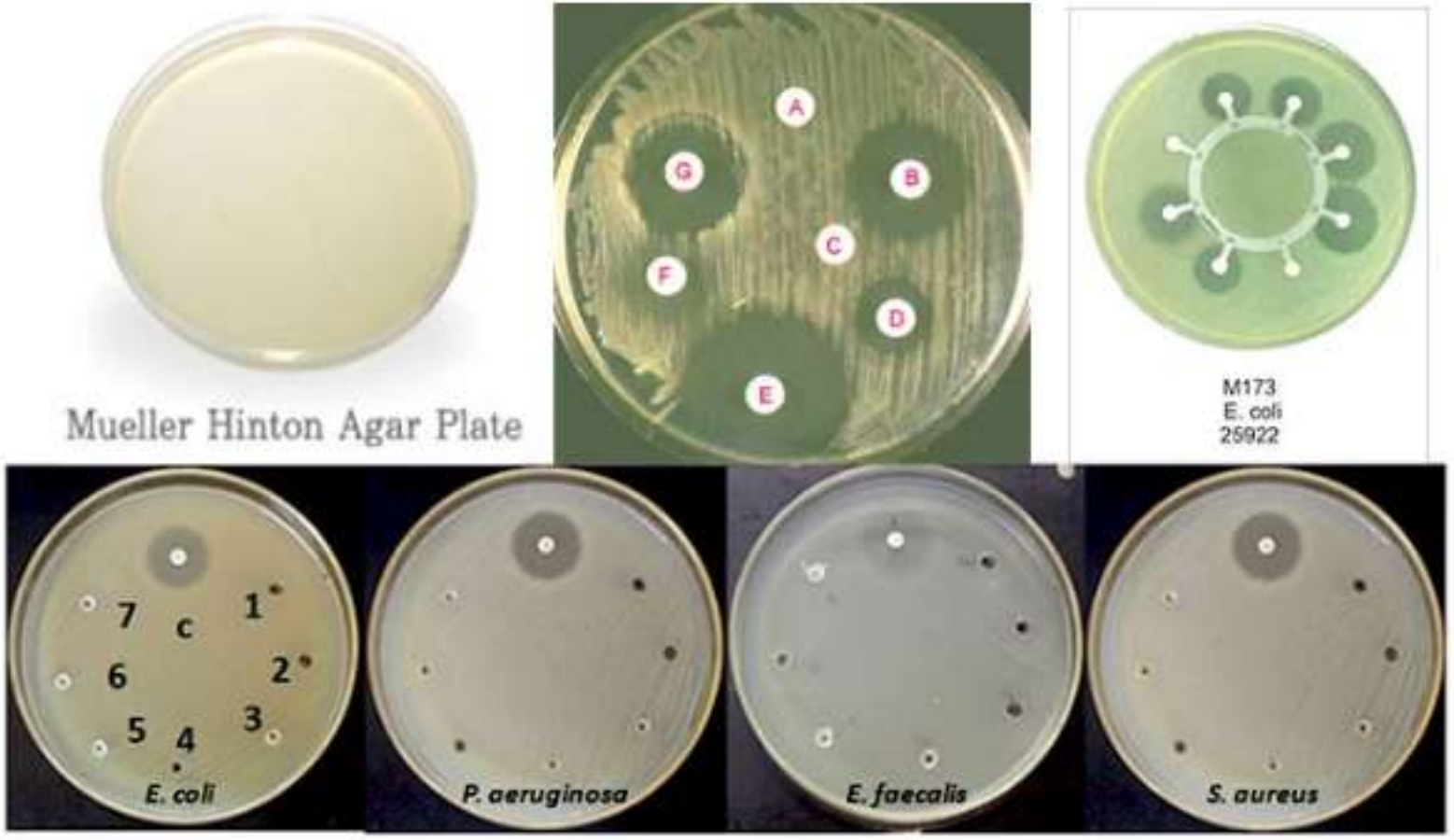
- Suspend 38 gm of the medium in one liter of distilled water.
- Heat with frequent agitation and boil for one minute to completely dissolve the medium.
- Autoclave at 121°C for 15 minutes. Cool to room temperature.
- Pour cooled Mueller Hinton Agar into sterile petri dishes on a level, horizontal surface to give uniform depth.
- Allow to cool to room temperature.
- Check for the final pH 7.3 ± 0.1 at 25°C.
- Store the plates at 2-8°C.

Uses of MHA

- The major use of Mueller Hinton Agar is for antimicrobial susceptibility testing.
- It has become the standard medium for
  - **Kirby Bauer method**
  - performance is specified by the NCCLS (National Committee for Clinical Laboratory Standards).
- It can be used to cultivate *Neisseria.*
- It is specified in FDA Bacteriological Analytical Manual
  - food testing
  - aerobic and facultative anaerobic bacteria.

- It contains starch which
  - absorb toxins released from bacteria
  - they cannot interfere with the antibiotics.
- It contains loose agar which
  - mediates the rate of diffusion of the antibiotics through the agar.
  - That leads to a truer zone of inhibition.
- MHA is low in antibiotic inhibitors (PABA and thymidine)

Antibiotics susceptibility test



Quality control of MHA

Positive controls:	**Expected results**
Escherichia coli ATCC® 25922	Good growth; pale straw coloured colonies
Pseudomonas aeruginosa ATCC® 27853	Good growth; straw coloured colonies
Staphylococcus aureus ATCC® 25923	Good growth; cream coloured colonies
Negative control:	
Un-Inoculated medium	No change

Limitations of MHA

- Numerous factors can affect results:
  - inoculum size
  - rate of growth
  - medium formulation
  - pH
- Strict adherence to protocol is required to ensure reliable results.

Limitations of MHA

- Drug inactivation may result from the prolonged incubation times required by slow growers.
- Variation in the concentration of divalent cations – Ca^{2+} and Mg^{2+} - affects results
  - aminoglycoside
  - tetracycline
  - colistin test with *P. aeruginosa* isolates.

Lowenstein-Jensen (LJ) Media

- It's a selective medium used for the cultivation and isolation of ***Mycobacterium*** species.
- It was developed by **Lowenstein**
  - congo red and malachite green
  - inhibit unwanted bacteria.
- The present formulation - **Jensen's modification**
  - glycerol egg-based medium
  - malachite green

Composition of LJ Medium

Ingredients	**Amount**
Potato Flour (Potato Starch)	30.0 gm
L-Asparagine	3.6 gm
Monopotassium Phosphate	2.4 gm
Magnesium Citrate	0.6 gm
Malachite Green	0.4 gm
Magnesium Sulfate	0.24 gm
Glycerol	12 ml
Egg suspension	1000 ml
Distilled Water	600 ml

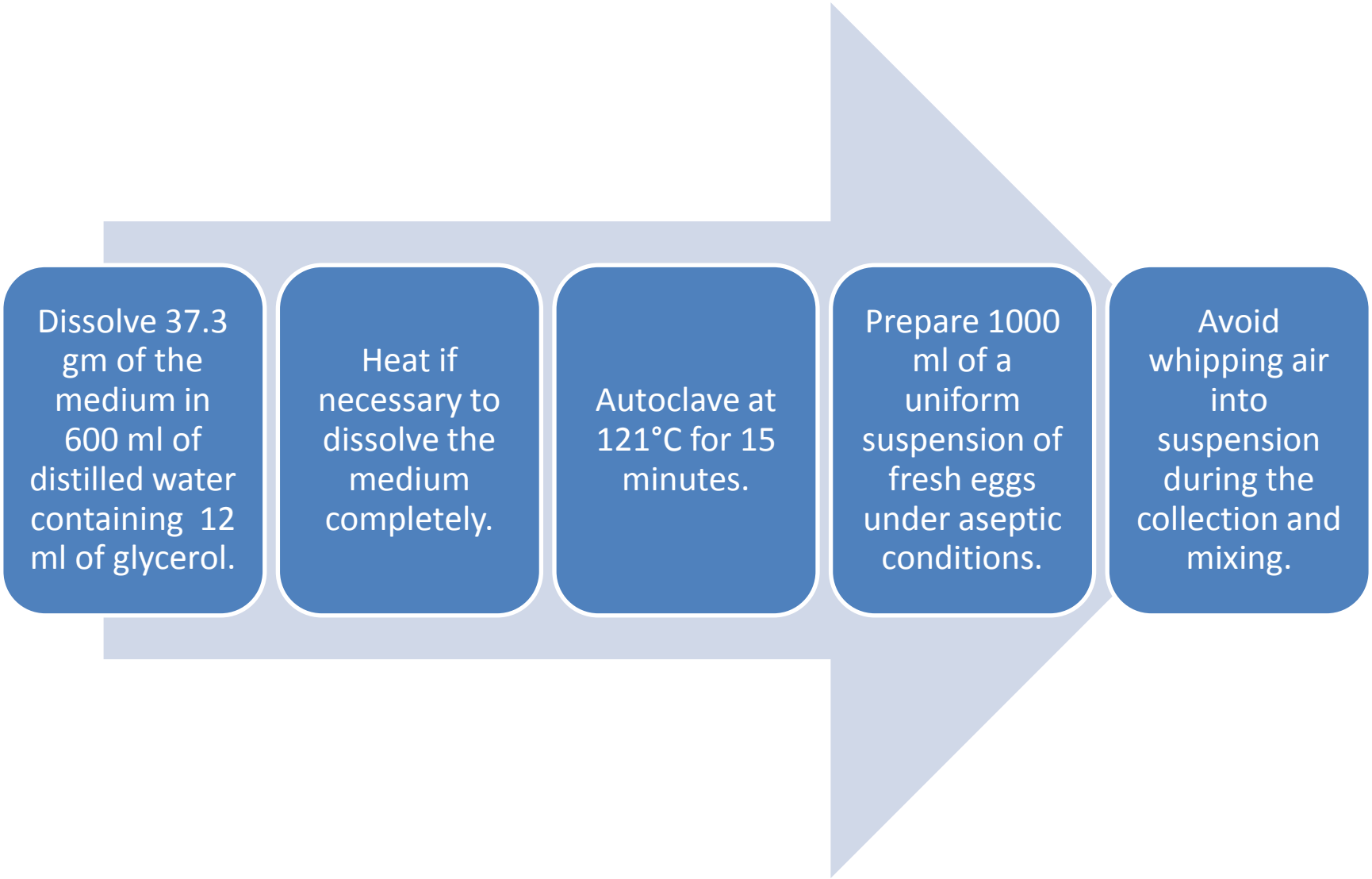
For cultivation of ***M. bovis*****, glycerol** is omitted and **sodium pyruvate** is added.

Principle of LJ Medium

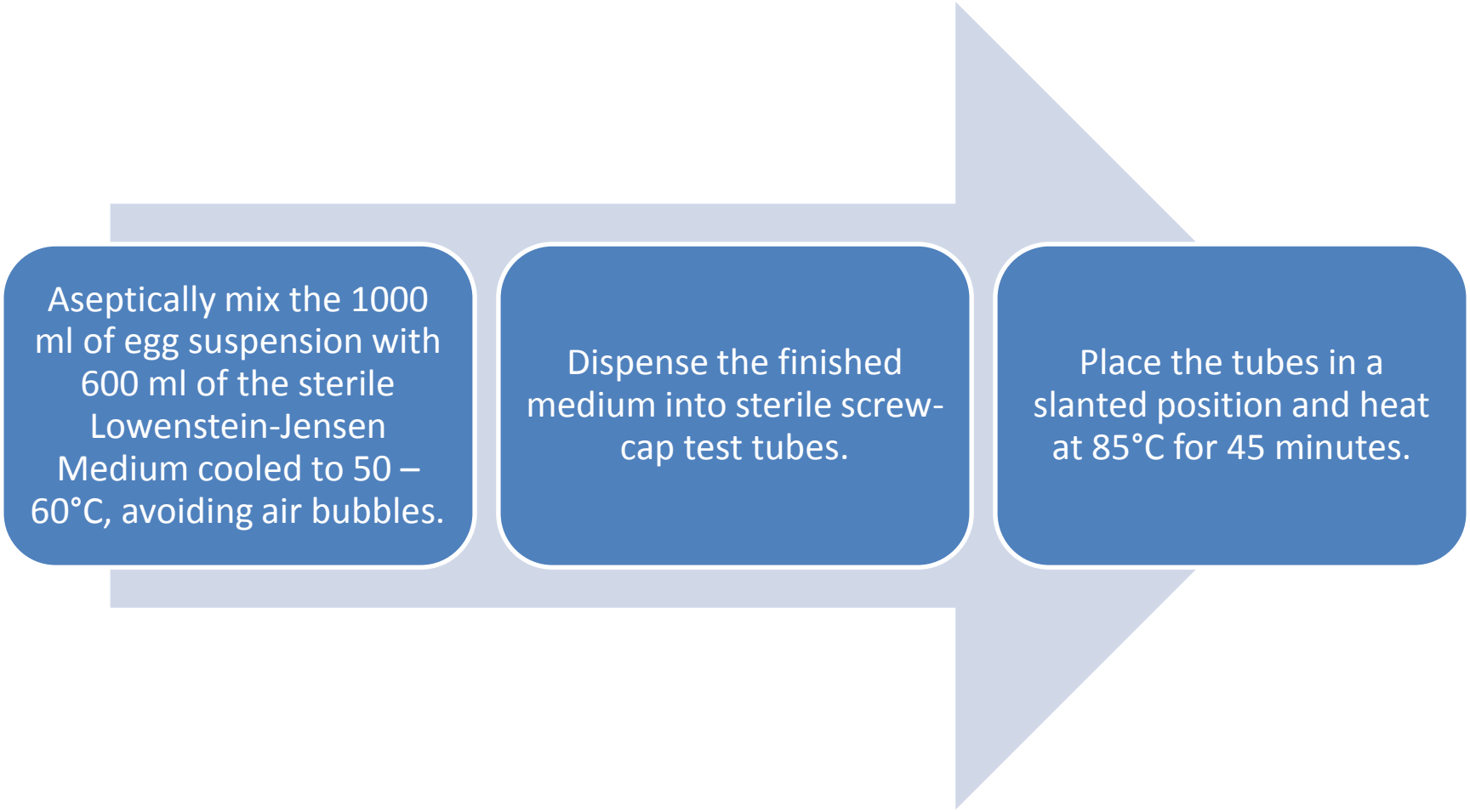
- **L-Asparagine** and **Potato Flour:**
  - nitrogen
  - vitamins
- **Monopotassium Phosphate** and **Magnesium Sulfate:**
  - enhance growth of organism
  - buffers

- **Malachite green:**
  - prevent the growth of the majority of contaminants
  - encouraging the growth of Mycobacteria.
- **Egg Suspension:**
  - fatty acids & protein
  - metabolism of Mycobacteria.
  - **egg albumin** coagulates when heated - solid surface for inoculation
- **Glycerol:** carbon source - tubercle bacillus

Preparation of LJ Medium:



Preparation of LJ Medium contd:

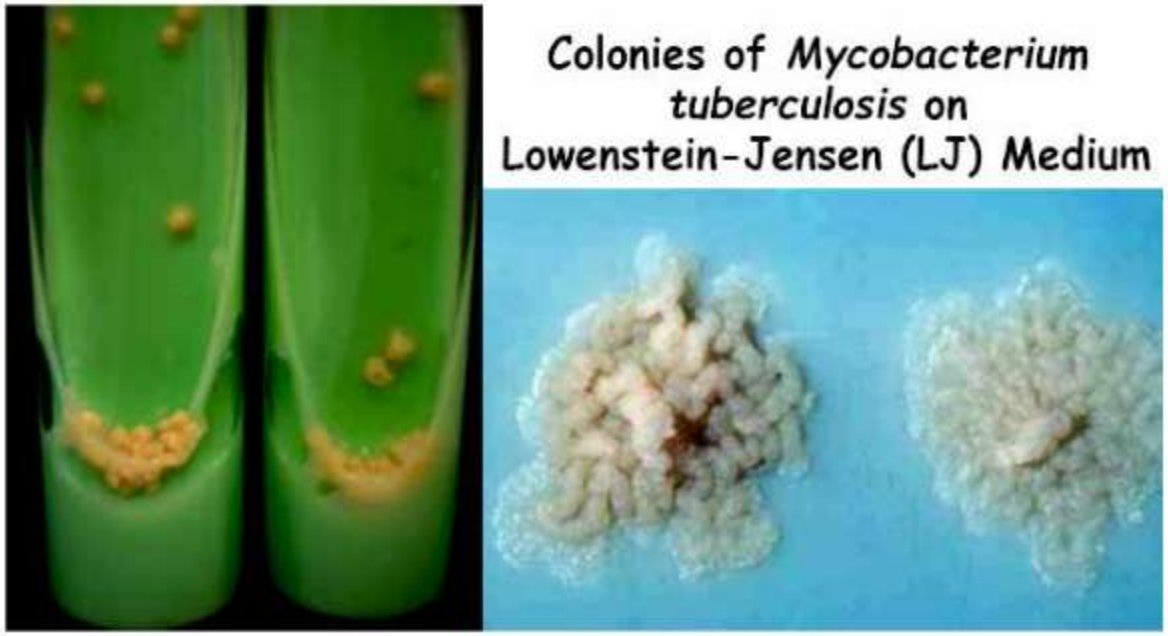


Uses of LJ Medium

- It is used for the diagnosis of Mycobacterial infections.
- It is used for testing antibiotic susceptibility of isolates.
- It is also used for differentiating different species of *Mycobacterium*
  - (by colony morphology, growth rate, biochemical characteristics and microscopy).

Colony Morphology on LJ Medium

- Cultures should be read within 5 to 7 days after inoculation and once a week thereafter for up to 8 weeks.
- Typical non pigmented, rough, dry colonies are seen on LJ medium.



Colonies of *Mycobacterium tuberculosis* on Lowenstein-Jensen (LJ) Medium

Limitations of LJ Medium

- It is recommended that biochemical and/or serological tests be performed on colonies from pure culture for complete identification.
- LJ Media require incubation in a 5-10% CO_2 atmosphere in order to recover Mycobacteria.
- Mycobacteria, for unknown reasons, are not recovered well from candle extinction jars.
- Negative culture results do not rule-out active infection by Mycobacteria.
- Due to nutritional variation, some strains may be encountered that grow poorly or fail to grow on this medium.
- Further tests are necessary for confirmation of *Mycobacterium* spp.

Mannitol Salt Agar (MSA)

- It is a selective and differential medium for the isolation and identification of ***Staphylococcus aureus*** from clinical and non-clinical specimens.
- Used for the detection and enumeration of coagulase-positive ***Staphylococci*** in milk, food and other specimens
- encourages the growth of a group of certain bacteria while inhibiting the growth of others.

Principle of Mannitol Salt Agar (MSA)

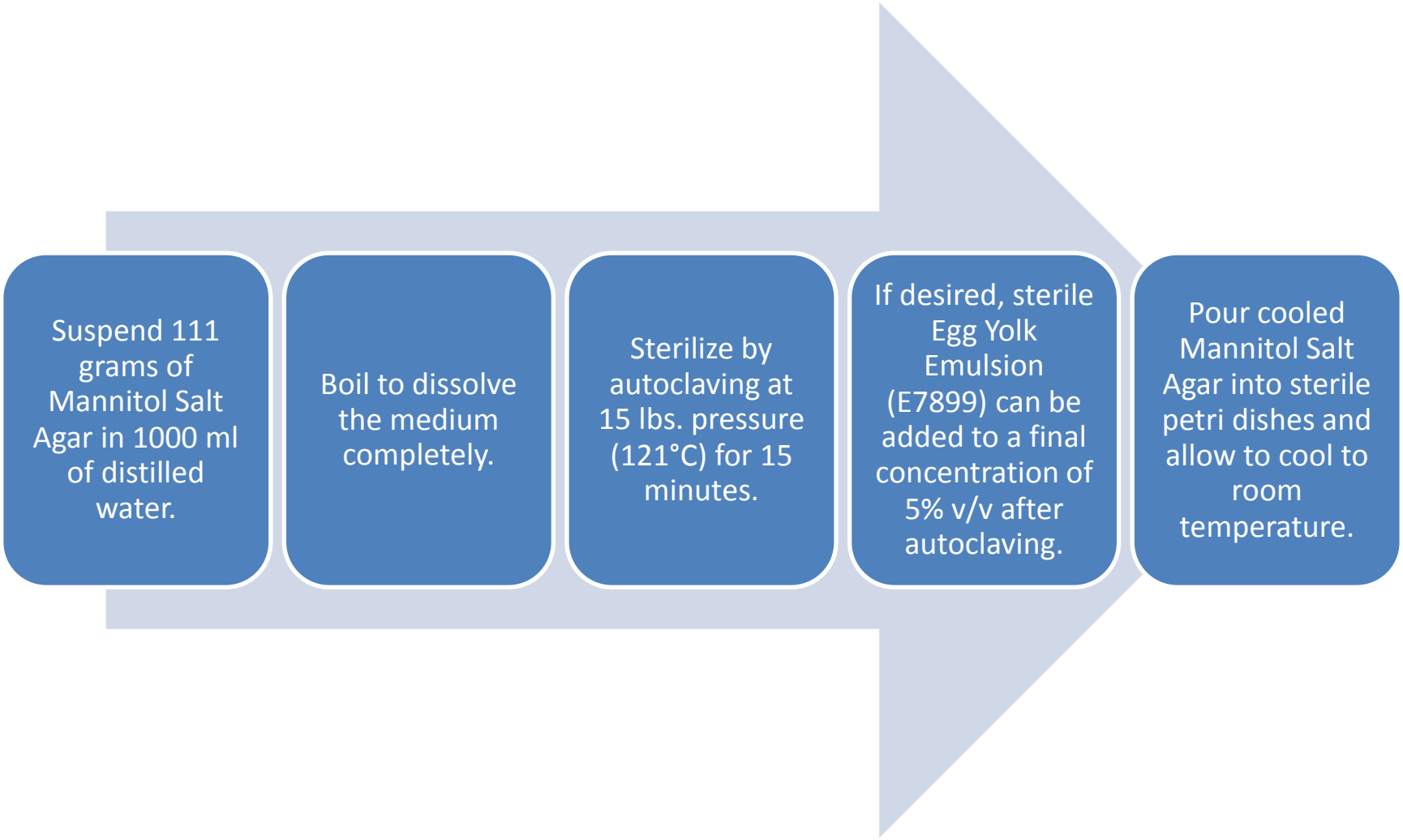
Contents	Function
beef extract, proteose peptone	provide essential growth factors and trace nutrients
sodium chloride 7.5%	partial or complete inhibition of other organisms
Mannitol	fermentable carbohydrate source Fermentation → acid production
	coagulase negative species of *Staphylococci* →small red colonies
phenol red	pH indicator: red at pH 8.4, yellow at pH 6.8
Agar	solidifying agent
Egg Yolk Emulsion 5% v/v	detection of lipase activity → staphylococci - Salt clears the egg yolk emulsion - lipase production → yellow opaque zone → colonies

Composition of Mannitol Salt Agar (MSA)

Ingredients	g/L
Proteose peptone	10.0
Sodium chloride	75.0
D- mannitol	10.0
Beef extract	1.0
Phenol red	0.025
Agar	15.0

Final pH 7.4 +/- 0.2 at 25ºC.

Preparation of MSA

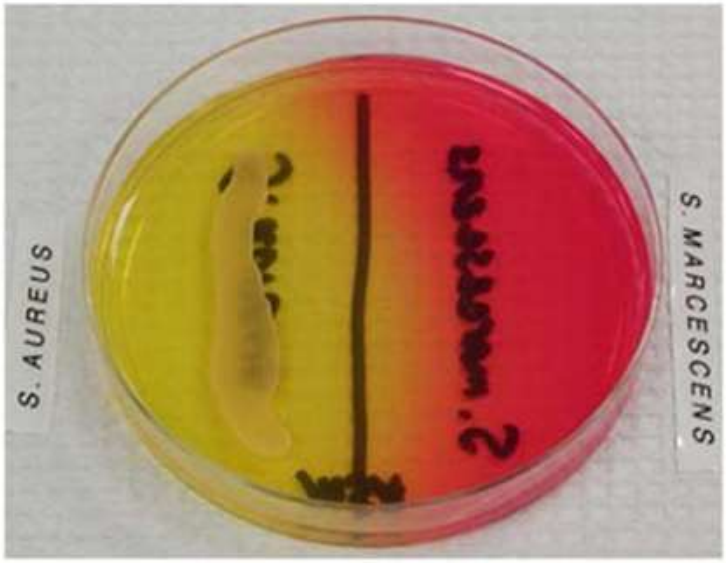


Results on MSA

Organisms	Results
Staphylococcus aureus	Yellow colonies surrounded by yellow zone
Staphylococcus epidermidis	Pink or Red colonies
Micrococci	Red colonies
Escherichia coli	No growth

Mannitol Salt Agar (MSA) for the isolation of *Staphylococcus aureus*

Yellow colonies of *Staphylococcus aureus*



Staphylococcus aureus and *Serratia marcescens* on MSA

Uses of MSA

- It is used for the selective isolation and differentiation of ***Staphylococcus aureus*** from clinical samples.
- It is also used for the enumeration of staphylococci in food and dairy products.
- This medium is also included in the Bacteriological Analytical Manual for cosmetics testing.
- It is also used in the bacteriological examination of swimming pool water, spas and drinking water using membrane filtration.

Limitations of MSA

- Other ***Staphylococcus* species** can grow on MSA and form yellow zone.
- Hence further biochemical tests are necessary for the **identification** of ***S. aureus***.
- High salt concentration inhibits other organisms except for **halophilic** marine organisms.

Limitations of MSA

- A few strains of *Staphylococcus aureus* may exhibit a **delayed fermentation** of mannitol.
- Negative plates should be re-incubated overnight before discarding.
- Presumptive *Staphylococcus aureus* must be confirmed with a **coagulase test**.

Culture medium used for Biochemical Tests

Biochemical Tests	Culture medium type
Triple Sugar Iron test	TSI Agar medium
Citrate Utilization Test	Simmon's Citrate Agar
Urease Test	Urea Agar

Triple Sugar Iron (TSI) test

- Most bacteria have the ability to ferment carbohydrates, particularly sugars.
- Sugar fermentation of Bacteria is used for identification and characterization purpose.

❑ Triple Sugar Iron (TSI) test:

- Determine the ability of microorganisms to ferment sugars such as glucose, lactose, and sucrose and to produce hydrogen sulfide.

Principle

- The triple sugar - iron (TSI) test uses Triple Sugar Iron Agar medium.

- Designed to differentiate among organisms based on:
  - Differences in carbohydrate fermentation patterns
  - Hydrogen sulfide production.

- Carbohydrate fermentation is indicated by:
  - Production of gas
  - Change in the colour of the pH indicator from red to yellow.

- **Carbohydrate utilization**:
- TSI Agar contains three fermentative sugars
  - Lactose
  - Sucrose

  } 1% conc.
  - Glucose – 0.1% conc.
- Phenol red – pH indicator in the medium
  - Fermentation of sugars –acidic products
  - Acidic pH: color changes from orange red to yellow
  - Oxidative decarboxylation of peptone – alkaline products
  - Alkaline pH: orange red to deep red

- Sodium thiosulfate and ferrous ammonium sulfate:
  - Detects the production of hydrogen sulfide and is
  - Indicated by the black color in the butt of the tube.
- Slant surface – varying degree of O_2 conc.
  - Aerobic – alkaline end products → slant area
  - Anaerobic fermentation – acid production → butt

- To detect fermentation of glucose only -
- the glucose conc. is one-tenth (0.1%) the conc. of lactose or sucrose (1%) .
- After depletion of the limited glucose, organisms will begin to utilize the lactose or sucrose.
- Acid production – yellow color – slant butt

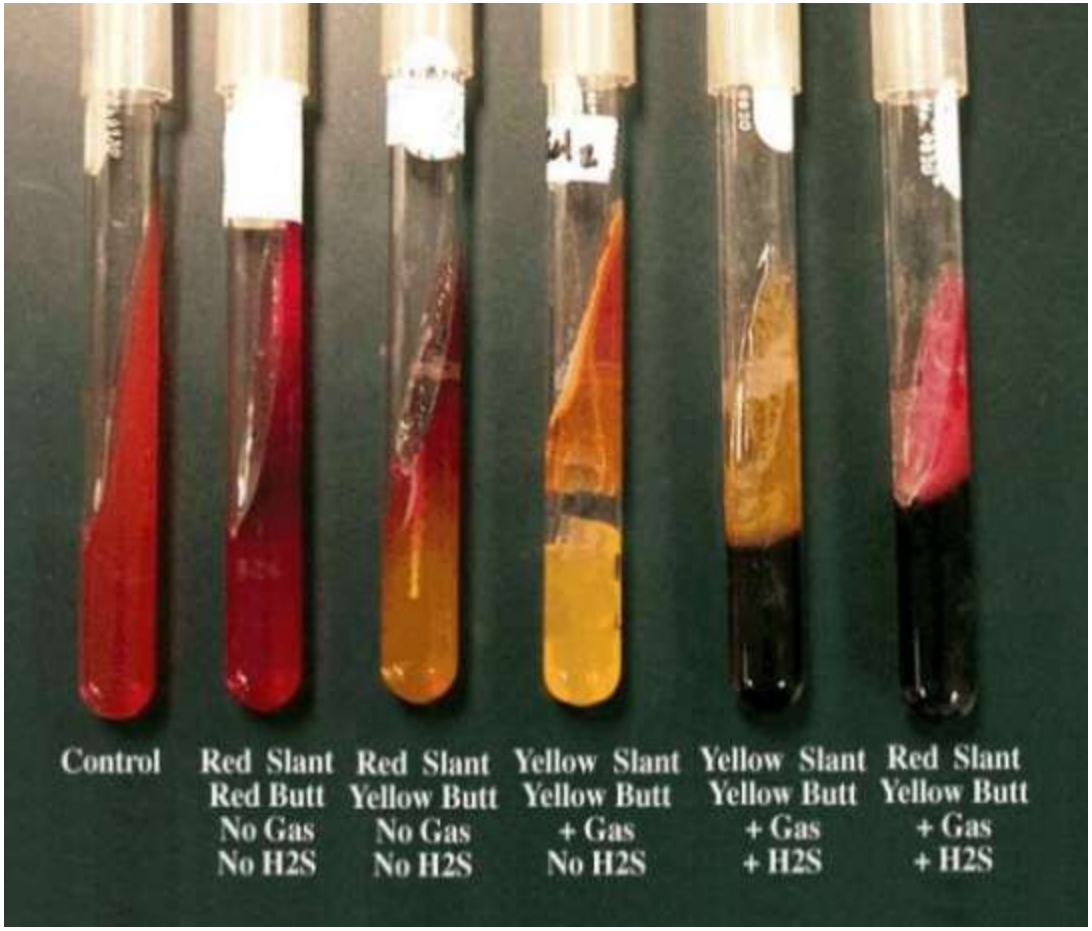
TSI agar medium

Composition	g/L
Enzymatic digest of casein	5 g
enzymatic digest of animal tissue	5 g
yeast enriched peptone	10 g
Dextrose	1 g
lactose	10 g
sucrose	10 g
ferric ammonium citrate	0.2 g
NaCl	5 g
sodium thiosulfate	0.3 g
phenol red	0.025 g
Agar	13.5 g
Distilled water	1000 mL
pH 7.3	

Method

- With a straight inoculation needle, touch the top of a well-isolated colony.
- Inoculate TSI by first stabbing through the center of the medium to the bottom of the tube
- and then streaking the surface of the agar slant.
- Leave the cap on loosely and incubate the tube at 35°-37°C in ambient air for 18 to 24 hours.
- Examine the reaction of medium.

Expected Results



Blackening of the medium: Occurs in the presence of H_2S

Gas production: Bubbles or cracks in the agar indicate the production of gas (formation of $CO_2$and H_2S)

Uses

- Differentiate members of the *Enterobacteriaceae* family from other gram-negative rods.
- Differentiation among *Enterobacteriaceae* on the basis of their sugar fermentation patterns.

Limitations

- It is important to stab the butt of the medium.
- Failure to stab the butt invalidates this test.
- TSI Agar must be read within the 18-24 hour stated incubation period.
- False-positive: read too early.
- False-negative: read later than 24 hours.
- TSI is not sensitive in detecting hydrogen sulfide compared to other iron containing mediums
  - Sulfide Indole Motility (SIM) Medium.

Citrate Utilization test

- Ability of bacteria to utilize citrate as a source of energy.
- This test is among a set of IMViC Tests (Indole, Methyl-Red, Vogues-Proskauer, and Citrate)
- Used to differentiate among the Gram –ve bacilli in the family *Enterobacteriaceae*.

Principle of Citrate Utilization Test

- **Citrate:** carbon source
- **Inorganic ammonium salts ($NH_4H_2PO_4$)** : nitrogen source
- Bacteria produce an enzyme - **citrate-permease** - converts **citrate** to **pyruvate**.
- **Pyruvate** can then enter the organism's metabolic cycle for the production of **energy**.
- Growth indicates utilization of citrate
- Intermediate metabolite in the **Krebs cycle**.

- Bacteria metabolize ⟶ **citrate**
- ammonium salts ⟶ ammonia ⟶ **alkaline**
- pH indicator: **bromthymol blue**
- Changes from **green to blue** at pH > 7.6.

Media used in Citrate Utilization Test

Simmon's Citrate Agar

Composition	**g/L**
Sodium Chloride	5.0 g
Sodium Citrate (dehydrate)	2.0 g
Ammonium Dihydrogen Phosphate	1.0 g
Dipotassium Phosphate	1.0 g
Magnesium Sulfate (heptahydrate)	0.2 g
Bromothymol Blue	0.08 g
Agar	15.0 g

Deionized water = 1,000 ml

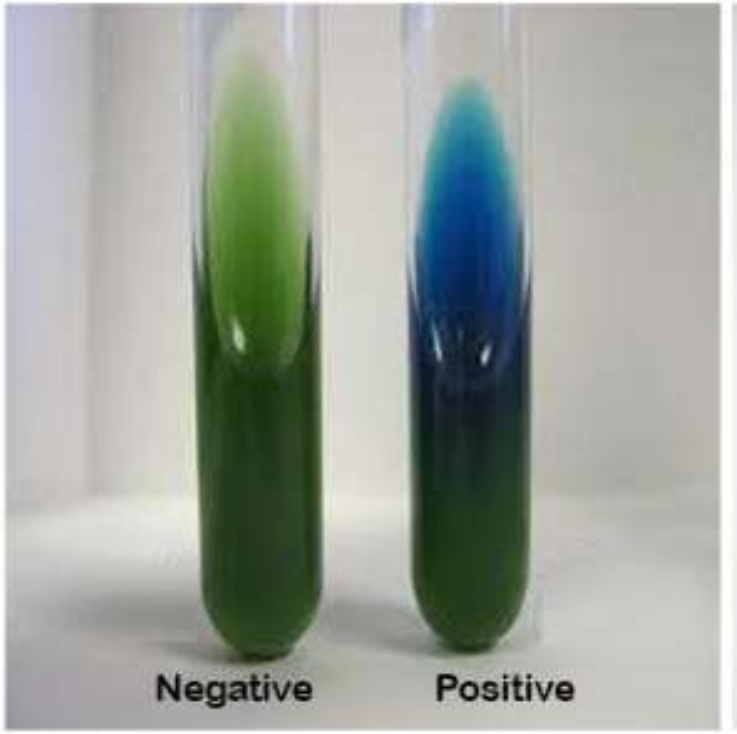
Final pH 6.9 ± 0.2 at 25°C.

Procedure of Citrate Utilization Test

- Streak the slant back and forth with a light inoculum picked from the center of a well-isolated colony.
- Incubate aerobically at 35-37°C for up to 4-7 days.
- Observe a color change from green to blue along the slant.

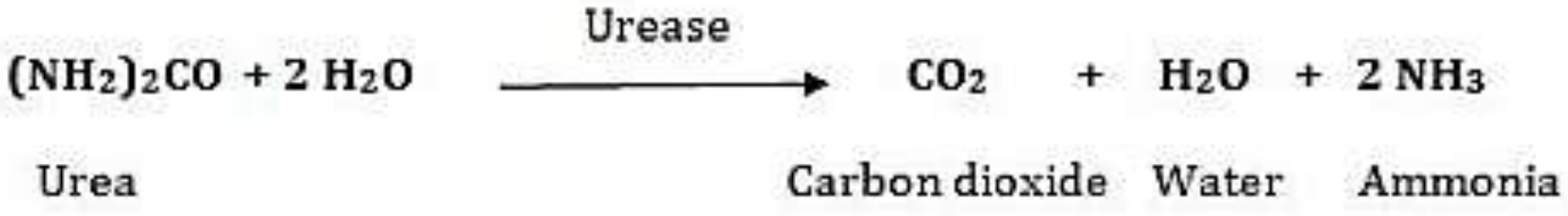
Result

- **Positive Reaction:** Growth with color change from green to intense blue along the slant.
  Examples: *Salmonella, Citrobacter, Klebsiella, Enterobacter, Serratia.*
- **Negative Reaction:** No growth and No color change; Slant remains green.
  Examples: *Escherichia, Shigella, Morganella, Yersinia*.



Urease test medium

- Urea Agar - developed by Christensen in 1946
- Differentiation of **enteric bacilli**.
- **Urease test:**
- Determines the ability of an organism to split urea by producing the enzyme **urease**.



Principle of Urease Test

- amino acids $\xrightarrow{\text{decarboxylation}}$ **urea**
- urea $\longrightarrow$ $NH_3 + CO_2$.
- **Ammonia** → medium pH – **alkaline**
- pH indicator : **phenol red**
- **light orange** $\longrightarrow$ **magenta (pink)**

  (pH 6.8) (pH 8.1)
- Rapid urease-positive organisms turn the entire medium **pink** within 24 hours.
- negative organisms: **no color change**
- **Yellow:** **acid production**.

Media used in Urease Test

Christensen's Urea Agar

Composition	**g/L**
Urea	20 g
Sodium Chloride	5 g
Monopotassium Phosphate	2 g
Peptone	1 g
Dextrose	1 g
Phenol Red	0.012 g
Agar	15 g
pH 6.7	

Preparation

- Dissolve the ingredients in 100 ml of distilled water and **filter sterilize** (0.45-mm pore size).
- Suspend the agar in 900 ml of distilled water, boil to dissolve completely.
- Autoclave at 121°C and 15 psi for 15 minutes.
- Cool the agar to 50-55°C.
- Aseptically add 100 ml of filter-sterilized **urea base** to the cooled agar solution and mix thoroughly.
- Distribute **4 to 5 ml** per sterile tube (13 x 100 mm) and slant the tubes during cooling until solidified.

Procedure of Urease Test

- Streak the surface of a urea agar slant with a portion of a well-isolated colony
- Leave the cap on loosely
- Incubate the tube at 35°-37°C in ambient air for 48h to 7 days.
- Examine for the development of a **pink color** for as long as 7 days.

Result Interpretation of Urease Test

Positive reaction:
Intense **magenta to bright pink** color
Examples:
Proteus spp, *Cryptococcus* spp,
Corynebacterium spp,
Helicobacter pylori,
Yersinia spp, *Brucella* spp

Negative Reaction:
No color change.
Examples: *Escherichia,*
Shigella, Salmonella, etc.

Thank you