EXPERIMENT 7 PREPARATION OF MEDIA

Structure

7.0 OBJECTIVES

After going through this experiment, you will be able to:

- explain the role of nutritional requirement in the cultivation of microorganism in laboratory;
- acquire knowledge about various types of microbiological media used for different purpose; and
- prepare the media for microbiological analysis.

7.1 INTRODUCTION

For any bacterium to be propagated for any purpose it is necessary to provide the appropriate biochemical and biophysical environment. The biochemical (nutritional) environment is made available as a *culture medium.* Media are the substances over which micro-organisms such as bacteria, viruses, fungi etc are cultured or grown to harvest in a larger amount. You all know that individual micro-organism is too small and therefore they are required in larger amount for their evaluation. Depending upon the special needs of particular bacteria (as well as particular investigators) a large variety and types of culture media have been developed with different purposes and uses. Culture media are employed in the isolation and maintenance of pure cultures of bacteria and are also used for identification of bacteria according to their biochemical and physiological properties.

7.2 EXPERIMENT

7.2.1 Principle

The ingredients in culture media range from pure chemical compounds to complex materials such as extracts or digests of plant and animal tissues. If all the ingredients of a culture medium are known, both qualitatively and quantitatively, the medium is called a **chemically defined medium**. These media are of great value in studying the nutritional requirements of microorganisms or in studying a great variety of their metabolic activities. In a **complex medium**, the exact chemical composition is not known, and such a medium is often prepared from very complex materials, e.g., body fluids, tissue extracts and infusions, and peptones.

Commonly Used Constituents in Microbiological Media:

i) **Carbohydrates:** Carbohydrates and alcohols are added as carbon and energy sources, which stimulate growth of microorganisms. Carbohydrates are also used to aid in microorganism identification and differentiation.

ii) **Agar:** Agar is the major solidifying agent used in bacteriological media. It is an impure polysaccharide gum obtained from certain marine algae. It is added as a powder at a more or less standard concentration (1.5% for plates and slanted media, 0.5% or less for "semisolid" media), usually after the other medium components have been added and dissolved in the water. Agar dissolves at approximately 100°C, and an agar-containing medium thus heated will not solidify until the temperature is brought down to about 43°C. Once solidified, the medium will not melt until brought back up to about 100°C.

iii) **Body Fluids:** Whole or defibrinated blood, plasma, serum or other body fluids are frequently added to culture media for the isolation and cultivation of many pathogens. Body fluids contribute many growth factors and/or substances which detoxify certain inhibitors.

iv) **Buffers:** These compounds are incorporated to maintain the optimum pH range of the organism. Substances like sodium and potassium phosphates and calcium carbonate prevent marked changes in pH which otherwise would result from microbial production of organic acids or bases.

v) **Extracts (Beef/Yeast):** Eucaryotic tissues (yeast, beef muscle, liver, brain, heart, etc.) are extracted by boiling and then concentrated to a paste or dried to a powder. These extracts are frequently used as a source of amino acids, vitamins and coenzymes, including many needed as growth factors by fastidious organisms. Trace elements and other minerals and usually some sugar are also present.

vi) **Peptones:** These complex mixtures of organic and inorganic compounds are obtained by digestion of protein-containing tissues of animals and plants such as meat scraps, beef muscle, gelatin, milk protein (casein) and soybean meal. Peptones primarily contain peptides and single amino acids. Being crude digests of complex materials, they contain a great variety of other organic and inorganic materials. Examples are Tryptone (a pancreatic digest of casein), Phytone (a papaic digest of soybean meal) and simply Peptone (a digest of beef muscle).

vii) **pH Indicators:** An acid-base indicator is often added to differential media to detect changes in hydrogen ion concentration during the growth of an organism such as in Carbohydrate Fermentation Broth, Kligler Iron Agar, Simmons Citrate Agar, MacConkey Agar and Glucose O/F Medium. Brom-cresol purple, brom-thymol blue and phenol red are commonly used; for each of these, an acidic pH turns the indicator a yellow colour.

viii) **Reducing Agents:** Certain chemicals may stimulate growth by reducing the oxidation-reduction potential in the environment. Cystine and thioglycollate are reducing agents often used for the cultivation of anaerobes.

ix) **Selective Agents:** Antimicrobial agents such as crystal violet, bile salts, brilliant green, potassium tellurite, sodium azide and antibiotics can be employed in selective media to suppress or inhibit the growth of certain groups of microorganisms while allowing growth of desired organisms. These agents are usually bacteriostatic.

Classification of Culture Media

A classification of media based on their respective uses as follows. Note that these categories can overlap.

a) A **minimal medium** is one, which supplies only the minimal nutritional requirements of a particular organism. As an example, a typical, prototrophic strain of *E. coli* is able to synthesize all of its cell components from a simple solution containing several "mineral salts" plus glucose as the source of carbon and energy – such as the medium given above. Minimal media vary in composition according to the minimal nutritional requirements of the particular species under study.

b) An **all-purpose medium** is rich in a wide variety of nutrients (including many growth factors) and will, therefore, support the growth of a wide range of bacteria. All-purpose media include Nutrient Agar, APT Agar, Plate Count Agar, Heart Infusion Agar, and Brain Heart Infusion Agar.

c) A **selective medium** supports the growth of desired organisms while inhibiting the growth of many or most of the unwanted ones – either by purposely adding one or more selective agents which "poison" certain types of organisms or by including or deleting certain nutrients such that the desired organisms and few others are able to grow. Examples on how these things may be accomplished are as follows:

   i) **MacConkey Agar:** This is an example of a medium where selective agents are added which directly suppress the growth of undesired organisms as much as possible. The particular selective agents chosen for this medium – bile salts and crystal violet – inhibit gram-positive bacteria, allowing the near-exclusive growth of gram-negative bacteria.

   ii) **Nitrogen-Free Broth:** Here the medium is made selective by the deletion of a required element; no nitrogen compounds are present. Therefore, the only organisms which can grow after inoculation into this medium are those, which can utilize gaseous nitrogen (N2) which diffuses in from the atmosphere. These are the nitrogen-fixing bacteria. While this medium does not utilize selective agents, it is still restrictive to an extensive number of various organisms.

   iii) **Succinate Broth:** In this example, a particular nutrient utilized by the desired organism – and few others – is included as the only carbon source. This medium is used for the enrichment of the purple non-sulfur photosynthetic bacteria; most other organisms tend not to metabolize succinate under the anaerobic conditions utilized. This is another example of a restrictive medium not utilizing selective agents.

d) **Differential Medium** is one which allows two or more different organisms to grow, but it contains dyes and/or other components upon which different organisms act in various ways to produce a variety of end products or effects, often detected by variations in colour. Examples:

   i) **MacConkey Agar:** This medium is used in plates. Organisms which ferment the lactose in the medium will lower the pH due to the production of acids. The pH indicator (neutral red) will turn red, and the colonies will consequently have a reddish appearance. Other colonies on the same plate which do not contain lactose-fermenting cells should appear whitish. (As this medium also appears in the above category, it is termed a selective-differential medium.)

   ii) **Carbohydrate Fermentation Broth:** This medium is used in tubes, usually with Durham tubes. Organisms which ferment the particular carbohydrate in the medium (e.g., glucose, lactose, sucrose) will cause the pH indicator to change colour. Also, if insoluble gas (H2) is produced during fermentation, a bubble will be seen in the inverted Durham tube.

   iii) **Other examples** of differential media include Motility Medium (exploiting a morphological characteristic – production of flagella), Nutrient Gelatin, Starch Agar, Kligler Iron Agar and Blood Agar.

Some of the common media used in microbiological examination of food products are as follows:

Nutrient Broth and Agar

Nutrient broth is a liquid medium commonly used for the cultivation of microorganisms which are non-demanding in their nutritional requirements e.g. water borne organisms, air, soil and dust flora. Addition of agar to the broth gives a solid medium (Nutrient agar) used for cultivation and determination of bacteria.

MacConkey's Broth and Agar

This is a selective medium used for detection, isolation and enumeration of coliforms and intestinal pathogens. The medium is used for performing the presumptive coliform test. Production of acid (indicated by the medium turning red or yellow) and gas in Durham tube is considered as indication of presence of coliforms in the given sample.

Eosine Methylene Blue (EMB) Agar

It is a differential plating medium, used for the isolation and differentiation of gram negative enteric bacilli. Typical coliform organism (*E. coli*) form bluish black colonies with green metallic sheen, while *Enterobacter aerogenes* forms mucid pink colonies. *Salmonella* and *Shigella* organisms form translucent, amber coloured or colourless colonies.

Endo Agar

Endo agar is used for the confirmation of the presumptive test for coliforms. Tubes of liquid media showing a positive presumptive reaction for coliforms are subcultured on to Endo agar. Lactose fermenting coliforms give rise to red colonies with a metallic sheen while non-coliforms form colourless colonies.

Tryptone Glucose Extract Agar

This medium is recommended as standard plate count agar for enumeration of bacteria in water and thermophilic bacteria in products.

7.2.2 Requirements

Generally following equipments, glass wares and chemicals are required for preparation of media.

i) Autoclave (Portable)

ii) Balance

iii) Heating mantle/ water bath

iv) pH meter

v) Laminar air flow

vi) Stirrer

vii) Pipettes

viii) Distilled water

ix) Media

x) Test tubes

xi) Beakers

xii) Cotton plugs

xiii) pH paper

xiv) Measuring cylinder

Nutrient Broth and Agar

i) Beef extract

ii) Peptone

iii) Agar

iv) Sodium chloride

v) Distilled water

vi) Sterilized test tubes, bottles and flasks

vii) pH indicator

viii) Lovibond pH comparator

ix) Autoclave

MacConkey's Broth and Agar

i) Sodium glycol or taurocholate or bile salt

ii) Peptone

iii) Lactose

iv) Sodium chloride

v) Andrade's indicator

vi) Bromocresol purple solution

vii) Test tubes

viii) pH indicator

ix) Flask

x) Lovibond comparator

xi) Autoclave

EMB Agar

i) Peptone

ii) Lactose

iii) Sucrose

iv) Dipotassiun Phosphate

v) Agar

vi) Eosin Y

vii) Methylene blue

viii) Test tubes or flasks

ix) Lovibond comparator

x) Autoclave

Endo Agar

i) Peptone

ii) Lactose

iii) Dipotassium phosphate

iv) Sodium sulphate

v) Basic fuchsin

vi) Distilled water

vii) Test tubes or flasks

viii) pH indicator

ix) Lovibond comparator

x) Autoclave

Tryptone Glucose Extract Agar

i) Tryptone

ii) Yeast extract

iii) Glucose

iv) Agar

v) Test tubes or flasks

vi) pH indicator

vii) Lovibond comparator

viii) Autoclave

7.2.3 Procedure

General steps for preparation of microbiological media usually involve the following:

1) Carefully weigh the proper amount of the dehydrated base medium or the correct proportion of constituent ingredients and dissolve in appropriate volume of distilled water and heat.

2) Determine the pH of the medium, and adjust if necessary with dilute acid or alkali.

3) If a solid medium is desired, add agar (1.5-2%) and boil the medium to dissolve the agar.

4) Distribute the medium into tubes or flasks. The amount of medium distributed per container should be limited so that no point within the volume of the medium is more than 2.5 cm from the top surface of the container.

5) Autoclave at 121°C for 15 minutes. Some media (or specific ingredients) that are heat labile are sterilized by filtration.

Nutrient Broth and Agar

The composition of medium is as follows:

Ingredient	Grams/ Litre
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Agar	15.0
Distilled water	1000 ml
Final pH	7.4 ± 0.2

- Weigh the ingredients and dissolve in 800 ml of water one by one (agar to be added only for nutrient agar).
- Boil the mixture to dissolve the medium completely.
- Cool to 50°C and adjust the pH with the help of Lovibond comparator using pH disc and appropriate pH indicator solution.
- Adjust the volume to 1,000 ml.
- Dispense into test tubes, bottles or flasks.
- Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

MacConkey's Broth and Agar

The composition of the medium is as follows:

Ingredient	Grams/ Litre
Peptone	20.0
Lactose	10.0
Sodium Taurochlorate	5.0
Bromcresol Purple or Andrade's Indicator	0.01
Distilled Water	1000 ml
Agar	15.0
Final pH	7.4 ± 0.2

- Weigh and dissolve the ingredients except lactose in 100 ml of water.
- Steam for 30 minutes.
- Adjust the pH to 7.4
- Add lactose.
- Filter through muslin cloth.

- Add Andrade's indicator to give clear amber colour or bromocresol purple to give a light purple colour.
- Dispense 8 ml into sterilized test tubes in which Durham tubes are placed inverted.
- Sterilize at 15 lbs pressure for 20 minutes.
- For preparing agar add agar to the broth medium, dispense into flasks or bottles and then sterilize.

EMB Agar

The composition of medium is as follows:

Ingredient	Grams/ Litre
Peptone	10.0
Lactose	5.0
Sucrose	5.0
Dipotassium	2.0
Phosphate	2.0
Agar	13.5
Eosin Y	0.4
Methylene Blue	0.065
Final pH	7.2± 0.2

- Suspend 36g in 1000 ml distilled water.
- Boil to dissolve the medium completely.
- Dispense and sterilize as mentioned earlier.
- Cool to oxidize the Methylene blue (i.e to restore its blue colour) and to suspend the flocculant precipitate which is an essential part of the medium.

Endo Agar

The composition of the medium is as follows:

Ingredient	Grams/ Litre
Peptone	10.0
Lactose	10.0
Dipotassium phosphate	3.5
Sodium sulphite	2.5
Basic fuchsin	0.5
Agar	15.0
Final pH	7.5 ± 0.2

- Suspend 41.5 g in 1000 ml distilled water.
- Heat to boiling to dissolve the medium completely.
- Distribute into the tubes or flasks and sterilize by autoclaving.
- Shake the flask to evenly disperse the characteristic flocculant precipitate that forms during heating.

Tryptone Glucose Extract Agar

The composition of medium is as follows:

Ingredient	Grams/ Litre
Tryptone	5.0
Yeast extract	3.0
Glucose	1.0
Agar	15.0
Final pH	7.0± 0.2

- Weigh and dissolve the ingredients in 1000 ml distilled water.
- Heat to dissolve the medium completely.
- Dispense into tubes or flasks and sterilize.

Composition of Different Media Generally Used for Microbial Study

1. ***Standard Plate Count Agar (SPCA)***

Tryptone	-	5.0g
Yeast extract	-	2.5g
D-glucose	-	5.0g
Agar	-	15.0g
Distilled water	-	1000ml
pH	-	7.0

2. ***Nutrient Agar for Bacterial count:*** It has already been discussed.
3. ***Potato Dextrose Agar for Fungal Count***

Potato, peeled and diced	-	200g
D-glucose	-	20g
Agar	-	15.0g

Boil 200g of peeled and diced potato for 1 hour in a litre of water. Filter and make up the volume to 1litre and add rest of the constituents.

4. *Violet Red Bile Agar (VRBA) for coliform count*

Yeast Extract	-	3.0g
Peptone	-	7.0g
Sodium Chloride	-	5.0g
Bile Salts	-	1.5g
Lactose	-	10.0g
Neutral red	-	0.03g
Crystal violet	-	0.002g
Agar	-	13.0g
Distilled water	-	1000ml
pH	-	7.4

7.2.4 Observations

The prepared media should be stored in a cool, dry place in the appropriate container. Observe the pH using the pH meter or colour indicator solutions. At times, tubes and flasks containing media may be incubated at 37°C for 48 hours before their use to detect and weed out any possible contamination.

7.2.5 Result

After performing this experiment you will observe that suitable media for growth of bacteria is nutrient agar (pH 6.8-7.0) or plate count agar (pH 7.0), whereas for yeasts and moulds it is potato dextrose agar (pH 5.6) and malt agar (pH 5.4).

7.3 PRECAUTIONS

- Only the fresh packs should be used for media preparation and packs should not be kept open for a longer as most of media powder are hygroscopic.
- Ensure before use that medium is not deteriorated physically and use before the expiry date printed on the label of the container.
- Only the clean glasswares should be used for preparation of media.
- Weighing should be accurate and pH must be adjusted before putting for autoclave.
- If possible, prepare media just before use. Repeated storage and autoclaving must be avoided.
- Avoid excessive heating or scorching of the medium. Agar media should not be held at high temperature (40° to 45°C) for longer time as agar tends to clump.
- The media must be cooled under laminar flow to a temperature range of 42-45°C before pouring over innoculum. Never cool under tap water or in septic area.
- If the medium is not used the same day it is prepared, store it properly in moisture proof containers to prevent drying of the medium.
- Prepare medium in such quantities that if stored, it will be used before loss of moisture through evaporation that becomes evident.

- To prevent contamination and excess evaporation of water from a medium in flask and tubes during storage, optionally fit aluminium foil or plastic with loose rubber bands before autoclaving in order to allow air to escape and to prevent the container from bursting.
- Avoid over loading autoclaves so that the rate of air exhaust and heating is not appreciably delayed.
- Flask or test tubes should be plugged with cotton or capped with paper.
- After sterilization gradually reduce the pressure within the autoclave (using no less than 15min) since liquids may be at a temperature above their boiling point at atmospheric pressure. If the pressure is lowered too rapidly, liquids may boil over and come out from the container.