



Bacterial culture media

There are various reasons why bacteria have to be grown (cultured) in the laboratory on artificial culture media. One of the most important reasons being its utility in diagnosing infectious diseases. Isolating a bacterium from sites in body normally known to be sterile is an indication of its role in the disease process. Indeed, isolating an organism from the clinical specimen is the first step in proving its role as an etiologic agent. Culturing bacteria is also the initial step in studying its morphology and its identification. Bacteria have to be cultured in order to obtain antigens from developing serological assays or vaccines. Certain genetic studies and manipulations of the cells also need that bacteria be cultured in vitro. Culturing bacteria also provide a reliable way estimating their numbers (viable count). Culturing on solid media is another convenient way of separating bacteria in mixtures.

Bacteria infecting humans (commensals or pathogens) are chemoorganoheterotrophs. When culturing bacteria, it is very important to provide similar environmental and nutritional conditions that exist in its natural habitat. Hence, an artificial culture medium must provide all the nutritional components that a bacterium gets in its natural habitat. Most often, a culture medium contains water, a source of carbon & energy, source of nitrogen, trace elements and some growth factors. Besides these, optimum pH, oxygen tension and osmolarity too have to be taken into consideration.

Some of the ingredients of culture media include water, agar, peptone, casein hydrolysate, meat extract, yeast extract and malt extract. While tap water is suitable for culture media, it must not be used if it contains high amount of minerals. In such situations, distilled or demineralised water should be used. Peptone is a byproduct of protein (plant or animal) digestion. Proteins are often obtained from heart muscle, casein, fibrin or soya flour and is digested using proteolytic enzymes such as pepsin, trypsin or papain. The final product contains peptones, proteoses and amino acids besides a variety of inorganic salts including phosphates, potassium and magnesium. Casein hydrolysate is obtained from hydrolysis of milk protein casein using HCl or trypsin. Meat extract is obtained by hot water extraction of lean beef and then concentrated by evaporation. Meat extract contains gelatin, albumoses, peptones, proteoses, amino acids, creatinine, purines, and accessory growth factors. Yeast extract is prepared from washed cells of bakers' yeast and contains wide range of amino acids, growth factors and inorganic salts. Malt extract is prepared by extracting soluble materials from sprouted barley in water at 55°C and concentrated by evaporation. It contains maltose, starch, dextrin, glucose and small amounts of protein and protein breakdown products and growth factors.

Initially, culture media were very simple; Louis Pasteur used simple broths made up of urine or meat extracts. Robert Koch realized the importance of solid media and used potato pieces to grow bacteria. It was on the suggestion of Fannie Eilshemius, wife of Walther Hesse (who was an assistant to Robert Koch) that agar was used to solidify culture media. Before the use of agar, attempts were made to use gelatin as solidifying agent. Gelatin had some inherent problems; it existed as liquid at normal incubating temperatures (35-37°C) and was digested by certain

bacteria. Bacterial culture media can be classified in at least three ways; Based on consistency, based on nutritional component and based on its functional use.

Classification based on consistency:

Culture media are liquid, semi-solid or solid. Liquid media are sometimes referred as “broths” (e.g nutrient broth).



Liquid media are available for use in test-tubes, bottles or flasks. In liquid medium, bacteria grow uniformly producing general turbidity. Certain aerobic bacteria and those containing fimbriae (*Vibrio* & *Bacillus*) are known to grow as a thin film called ‘surface pellicle’ on the surface of undisturbed broth. *Bacillus anthracis* is known to produce stalactite growth on ghee containing broth. Sometimes the initial turbidity may be followed by clearing due to autolysis, which is seen in pneumococci. Long chains of Streptococci when grown in liquid media tend to entangle and settle to the bottom forming granular deposits but with a clear medium. Culturing bacteria in liquid media has some drawbacks. Properties of bacteria are not visible in liquid media and presence of more than one type of bacteria can not be detected. Liquid media tend to be used when a large number of bacteria have to be grown. Culture media are suitable to grow bacteria when the numbers in the inoculum is suspected to be low. Inoculating in the

liquid medium also helps to dilute any inhibitors of bacterial growth. This is the practical approach in blood cultures. Culturing in liquid medium can be used to obtain viable count (dilution methods).

Any liquid medium can be rendered by the addition of certain solidifying agents. Agar agar (simply called agar) is the most commonly used solidifying agent. The word "agar" comes from the Malay word agar agar (meaning jelly). It is also known as kanten, China grass, or Japanese isinglass. Agar is chiefly used as an ingredient in desserts throughout Japan. It is an unbranched polysaccharide obtained from the cell membranes of some species of red algae such as the genera *Gelidium* and *Gracilaria*, or seaweed (*Sphaerococcus euchema*). Commercially it is derived primarily from *Gelidium amansii*. Agar is composed of two long-chain polysaccharides (70% agarose and 30% agarpectin). It melts at 95°C (sol) and solidifies at 42°C (gel), doesn't contribute any nutritive property, it is not hydrolysed by most bacteria and is usually free from growth promoting or growth retarding substances. However, it may be a source of calcium & organic ions. Most commonly, it is used at concentration of 1-3% to make a solid agar medium. New Zealand agar has more gelling capacity than the Japanese agar. Agar is available as fibres (shreds) or as powders.

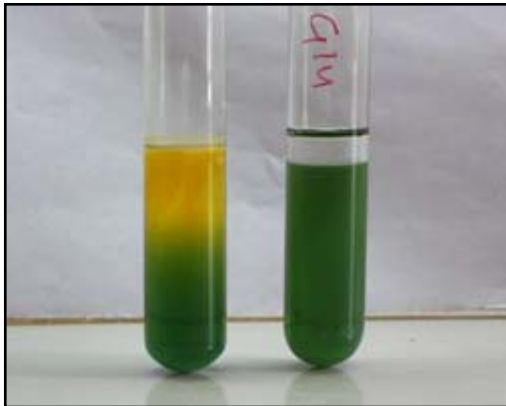
Agar fibres



Agar powder



For preparing agar in Petri plates, 3% agar (by weight) is added to the broth and autoclaved, when the medium is at $\sim 50^{\circ}\text{C}$, it is poured on to sterile Petri plates and allowed to set. For preparing agar containing media in test-tubes, the culture medium is mixed with 3% agar and heated with stirring to melt. This ensures that all the tubes get equal amounts of agar. These tubes can then be sterilized by autoclaving.



Reducing the amount of agar to 0.2-0.5% renders a medium semi-solid. Such media are fairly soft and are useful in demonstrating bacterial motility and separating motile from non-motile strains (U-tube and Cragie's tube). Certain transport media such as Stuart's and Amies media are semi-solid in consistency. Hugh & Leifson's oxidation fermentation test medium as well as mannitol motility medium are also semi-solid.

◀ Hugh & Leifson's OF medium



Sometimes, a culture system comprises of both liquid and solid medium in the same bottle. This is known as biphasic medium (Castaneda system for blood culture). The inoculum is added to the liquid medium and when subcultures are to be made, the bottle is simply tilted to allow the liquid to flow over the solid medium. This obviates the need for frequent opening of the culture bottle to subculture.

◀ Biphasic medium

Besides agar, egg yolk and serum too can be used to solidify culture media. While serum and egg yolk are normally liquid, they can be rendered solid by coagulation using heat. Serum containing medium such as Loeffler's serum slope and egg containing media such as Lowenstein Jensen medium and Dorset egg medium are solidified as well as disinfected by a process of inspissation.

Classification based on nutritional component:

Media can be classified as simple, complex and synthetic (or defined). While most of the nutritional components are constant across various media, some bacteria need extra nutrients. Those bacteria that are able to grow with minimal requirements are said to non-fastidious and those that require extra nutrients are said to be fastidious. Simple media such as peptone water, nutrient agar can support most non-fastidious bacteria. Complex media such as blood agar have ingredients whose exact components are difficult to estimate. Synthetic or defined media such as Davis & Mingioli medium are specially prepared media for research purposes where the composition of every component is well known.

Classification based on functional use or application:

These include basal media, enriched media, selective/enrichment media, indicator/differential media, transport media and holding media.

Basal media are basically simple media that supports most non-fastidious bacteria. Peptone water, nutrient broth and nutrient agar considered basal medium

Addition of extra nutrients in the form of blood, serum, egg yolk etc, to basal medium makes them **enriched media**. Enriched media are used to grow nutritionally exacting (fastidious) bacteria. Blood agar, chocolate agar, Loeffler's serum slope etc are few of the enriched media. Blood agar is prepared by adding 5-10% (by volume) to a basal medium such as nutrient agar or other blood agar bases. Since blood cannot be sterilized, it has to be collected aseptically from the animal. Animals have to be bled and the blood is collected in sterile containers with anticoagulant or glass beads. While sheep blood is preferred, blood from rabbit, horse and ox can also be collected. Human blood must be avoided since it may contain inhibitory substances including antibiotics. After the blood agar base is autoclaved, blood is added to the medium at temperature just above the solidifying point of agar. The mixture is then poured on to the plates and allowed to solidify. Blood agar is useful in demonstrating hemolytic properties of certain bacteria. Two major types of hemolysis are often seen on blood agar; beta and alpha hemolysis. Beta hemolysis is the complete lysis of RBC resulting in clearing around the colonies whereas alpha hemolysis is the partial lysis of RBC resulting in greenish discoloration around the colonies. Gamma hemolysis is a misnomer and it indicates non-hemolytic colonies. Chocolate agar is also known as heated blood agar or lysed blood agar. The procedure is similar to that of blood agar preparation except that the blood is added while the molten blood agar base is still hot. This lyses the blood cells and releases their contents into the medium. This process turns the medium brown, hence the name. This medium is especially useful in growing *Hemophilus* and *Neisseria*. Serum for medium can be obtained from animal blood but must be filtered through membrane or seitz filter before use.

Blood agar



Chocolate agar



Selective and enrichment media are designed to inhibit unwanted commensal or contaminating bacteria and help to recover pathogen from a mixture of bacteria. While selective media are agar based, enrichment media are liquid in consistency. Both these media serve the same purpose. Any agar media can be made selective by addition of certain inhibitory agents that don't affect the pathogen. Various approaches to make a medium selective include addition of antibiotics, dyes, chemicals, alteration of pH or a combination of these. Thayer Martin Agar used to recover *N.gonorrhoeae* contains Vancomycin, Colistin and Nystatin. Mannitol Salt Agar and Salt Milk Agar used to recover *S.aureus* contain 10% NaCl. Potassium tellurite medium used to recover *C.diphtheriae* contains 0.04% Potassium tellurite. McConkey's Agar used for Enterobacteriaceae members contains Bile salt that inhibits most gram positive bacteria. Pseudoseal Agar (Cetrimide Agar) used to recover *P.aeruginosa* contains cetrimide. Crystal Violet Blood Agar used to recover *S.pyogenes* contains 0.0002% crystal violet. Lowenstein Jensen Medium used to recover *M.tuberculosis* is made selective by incorporating Malachite green. Wilson & Blair's Agar for recovering *S.typhi* is rendered selective by the addition of dye Brilliant green. Selective media such as TCBS Agar and Monsur's Tellurite Taurocholate Gelatin Agar used for isolating *V. cholerae* from fecal specimens have elevated pH (8.5-5.6), which inhibits most other bacteria.

Wilson & Blair's agar



TCBS agar



Enrichment media are liquid media that also serves to inhibit commensals in the clinical specimen. Selenite F broth, tetrathionate broth and alkaline peptone water are used to recover pathogens from fecal specimens.

Certain media are designed in such a way that different bacteria can be recognized on the basis of their colony colour. Various approaches include incorporation of dyes, metabolic substrates etc, so that those bacteria that utilize them appear as differently coloured colonies. Such media are called **differential media or indicator media**. When a particular carbohydrate is incorporated into a medium and a mixture of bacteria inoculated on it, only that bacterium that can ferment it produces acid. This change in pH is detected by using a pH indicator incorporated in the medium and the bacterium that can ferment the sugar appears in a different colour. This approach is used in MacConkey's agar, CLED agar, TCBS agar, XLD agar etc. MacConkey's agar is the most commonly used media to culture and identify gram negative bacilli (especially enterobacteriaceae members). It contains bile salts (selective agent), lactose (sugar), peptone and neutral red (pH indicator), agar and water. Those bacteria that can ferment lactose produce pink coloured colonies where non-lactose fermenting colonies produce colourless colonies. Similarly, *Vibrio cholerae* produces yellow coloured colonies on sucrose containing TCBS medium. Reduction of potassium tellurite to metallic tellurium by *Corynebacterium diphtheriae* results in production of black coloured colonies on PT agar. Production of H₂S by *Salmonella typhi* results in production of black coloured colonies on Wilson & Blair's medium. *Enterococcus faecalis* produces black coloured colonies on bile esculin agar due to reduction of esculin to esculetin. Detection of hemolysis on blood agar can be considered as an indicator property of Blood agar.

MacConkey's agar with LF



MacConkey's agar with NLF



Clinical specimens must be transported to the laboratory immediately after collection to prevent overgrowth of contaminating organisms or commensals. This can be achieved by using **transport**

media. Such media prevent drying (desiccation) of specimen, maintain the pathogen to commensal ratio and inhibit overgrowth of unwanted bacteria. Some of these media (Stuart's & Amie's) are semi-solid in consistency. Addition of charcoal serves to neutralize inhibitory factors. Cary Blair medium and Venkatraman Ramakrishnan medium are used to transport feces from suspected cholera patients. Sach's buffered glycerol saline is used to transport feces from patients suspected to be suffering from bacillary dysentery. Pike's medium is used to transport streptococci from throat specimens.

Anaerobic bacteria need special media for growth because they need low oxygen content, reduced oxidation-reduction potential and extra nutrients.



Media for anaerobes may have to be supplemented with nutrients like hemin and vitamin K. Such media may also have to be reduced by physical or chemical means. Boiling the medium serves to expel any dissolved oxygen. Addition of 1% glucose, 0.1% thioglycollate, 0.1% ascorbic acid, 0.05% cysteine or red hot iron filings can render a medium reduced. Robertson cooked meat that is commonly used to grow *Clostridium* spp medium contain a 2.5 cm column of bullock heart meat and 15 ml of nutrient broth. Before use the medium must be boiled in water bath to expel any dissolved oxygen and then sealed with sterile liquid paraffin. Thioglycollate broth contains sodium thioglycollate, glucose, cystine, yeast extract and casein hydrolysate. Methylene blue or resazurin is an oxidation-reduction potential indicator that is incorporated in the medium. Under reduced condition, methylene blue is colourless.

Care must be taken to adjust the pH of the medium before autoclaving. Various pH indicators that are in use include phenol red, neutral red, bromothymol blue, bromocresol purple etc. Dehydrated media are commercially available and must be reconstituted as per manufacturers' recommendation. Most culture media are sterilized by autoclaving. Certain media that contain heat labile components like glucose, antibiotics, urea, serum, blood are not autoclaved. These components are filtered and may be added separately after the medium is autoclaved. Certain highly selective media such as Wilson and Blair's medium and TCBS agar need not be sterilized. It is imperative that a representation from each lot be tested for performance and contamination before use. Once prepared, media may be held at 4-5°C in the refrigerator for 1-2 weeks. Certain liquid media in screw capped bottles or tubes or cotton plugged can be held at room temperature for weeks.