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## An overview of microbial cell culture

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#### Abstract

A microbiological culture, or microbial culture, is a method of multiplying microbial organisms by letting them reproduce in predetermined culture media under controlled laboratory conditions. Microbial cultures are foundational and basic diagnostic methods used extensively as a research tool in molecular biology. Microbial cultures are used to determine the type of organism, its abundance in the sample being tested, or both. It is one of the primary diagnostic methods of microbiology and used as a tool to determine the cause of infectious disease by letting the agent multiply in a predetermined medium. Basic Constituents of Media for microbial cell culture include Inorganic salts, Carbohydrates, Amino Acids, Vitamins, Fatty acids and lipids, Proteins and peptides, Serum and Trace Elements. Further more culture media are classified on the basis of consistency viz., solid medium, solid media and liquid (broth) medium. Similarly synthetic and non synthetic medium constitute the classification based on composition while as on the basis of functional use, purpose and application microbial cell culture are classified as general purpose media, enrichment medium and selective and enrichment medium. This review article covers all the aspects of bacterial cell culture viz., preparation and storage of different culture media with suitable examples and conditions favouring the growth of the cultures viz., environment for microbial growth, aeration and mixing, sterilization etc. Besides components and working of fermentor employed for microbial cultures have been covered including the method of bacterial cultures like batch culture, feed batch culture, continuous culture and synchronous culture along with suitable figures and diagrams.

**Keywords:** culture, media, broth, agar, bioreactor, selective, microorganisms

#### Introduction

A microbiological culture, or microbial culture, is a method of multiplying microbial organisms by letting them reproduce in predetermined culture media under controlled laboratory conditions. Microbial cultures are foundational and basic diagnostic methods used extensively as a research tool in molecular biology. Microbial cultures are used to determine the type of organism, its abundance in the sample being tested, or both. It is one of the primary diagnostic methods of microbiology and used as a tool to determine the cause of infectious disease by letting the agent multiply in a predetermined medium. It is often essential to isolate a pure culture of microorganisms. A pure (or *axenic*) culture is a population of cells or multi cellular organisms growing in the absence of other species or types. A pure culture may originate from a single cell or single organism, in which case the cells are genetic clones of one another. For the purpose of gelling the microbial culture, the medium of agarose gel (agar) is used. Agar is a gelatinous substance derived from sea weed. A cheap substitute for agar is guar gum, which can be used for the isolation and maintenance. Culture media contains nutrients and physical growth parameters necessary for microbial growth. All microorganisms cannot grow in a single culture medium and in fact many can't grow in any known culture medium (Andrew, 2006)<sup>[1]</sup>.

#### Basic Constituents of Media

- Inorganic salts
- Carbohydrates
- Amino Acids
- Vitamins
- Fatty acids and lipids
- Proteins and peptides
- Serum
- Trace Elements

Each type of constituent performs a specific function as outlined below:

- **Inorganic Salts:** The inclusion of inorganic salts in media performs several functions. Primarily they help to retain the osmotic balance of the cells and help regulate membrane potential by provision of sodium, potassium and calcium ions. All of these are required in the cell matrix for cell attachment and as enzyme cofactors.
- **Carbohydrates:** The main source of energy is derived from carbohydrates generally in the form of sugars. The major sugars used are glucose and galactose, however, some media contain maltose or fructose. The concentration of sugar varies from basal media containing 1g/L to 4.5g/L in some more complex media. Media containing the higher concentration of sugars are able to support the growth of a wider range of cell types. Pyruvate is included in the formulation of some media, as an alternative energy source.
- **Amino Acids:** Amino acids are the building blocks of proteins. 'Essential' amino acids must be added to culture media as cells are not able to synthesize these themselves. The concentration of amino acids in the culture medium will determine the maximum cell density that can be achieved - once depleted the cells will no longer be able to proliferate.

In relation to cell culture, glutamine, an essential amino acid, is particularly significant. In liquid media or stock solutions glutamine degrades relatively rapidly. Optimal cell performance usually requires supplementation of the media with glutamine prior to use. Some media formulations include L-alanyl glutamine which is a more stable form of glutamine, and do not require supplementation. Adding supplements of non-essential amino acids to media both stimulates growth and prolongs the viability of the cells in culture.

- **Vitamins:** Serum is an important source of vitamins in cell culture. However, many media are also enriched with vitamins making them consistently more suitable for a wider range of cell lines. Vitamins are precursors for numerous co-factors. Many vitamins, especially B group vitamins, are necessary for cell growth and proliferation and for some lines the presence of B12 is essential. Some media also have increased levels of vitamins A and E. The vitamins commonly used in media include riboflavin, thiamine and biotin.
- **Proteins and Peptides:** These are particularly important in serum free media. The most common proteins and peptides include albumin, transferrin, fibronectin and fetuin and are used to replace those normally present through the addition of serum to the medium.
- **Fatty Acids and Lipids:** Like proteins and peptides these are important in serum free media since they are normally present in serum e.g. cholesterol and steroids essential for specialised cells.
- **Trace Elements:** These include trace elements such as zinc, copper, selenium and tricarboxylic acid intermediates. Selenium is a detoxifier and helps remove oxygen free radicals.
- Organisms that cannot grow in artificial culture medium are known as obligate parasites. *Mycobacterium leprae*,

*ricketsias*, *Chlamydias*, and *Treponema pallidum* are obligate parasites. Bacterial culture media can be distinguished on the basis of composition, consistency and purpose (Sharma *et al.*, 2013)<sup>[10]</sup>.

#### Classification of culture media used in Microbiology laboratory on the basis of consistency

1. **Solid medium:** Solid medium contains agar at a concentration of 1.5-2.0% or some other, mostly inert solidifying agent. Solid medium has physical structure and allows bacteria to grow in physically informative or useful ways (e.g. as colonies or in streaks). Solid medium is useful for isolating bacteria or for determining the colony characteristics of the isolate.
2. **Solid Media:** They are prepared with agar at concentrations of 0.5% or less. They have softcustard like consistency and are useful for the cultivation of microaerophilic bacteria or for determination of bacterial motility.
3. **Liquid (Broth) medium:** These media contain specific amounts of nutrients but don't have trace of gelling agents such as gelatin or agar. Broth medium serves various purposes such as propagation of large number of organisms, fermentation studies, and various other tests. e.g. sugar fermentation tests, MR-VR broth.

#### Classification of culture media based on the basis of composition

1. **Synthetic or chemically defined medium:** A chemically defined medium is one prepared from purified ingredients and therefore whose exact composition is known. Synthetic medium may be simple or complex depending up on the supplement incorporated in it.
2. **Non-synthetic or chemically undefined medium:** Non-synthetic medium contains at least one component that is neither purified nor completely characterized nor even completely consistent from batch to batch. Often these are partially digested proteins from various organism sources. Nutrient broth, for example, is derived from cultures of yeasts. A simple non-synthetic medium is capable of meeting the nutrient requirements of organisms requiring relatively few growth factors whereas complex non-synthetic medium support the growth of more fastidious microorganisms (Andrew, 2006)<sup>[11]</sup>.

#### Classification of Bacterial Culture Media based on the basis of purpose/ functional use/ application

Many special purpose media are needed to facilitate recognition, enumeration, and isolation of certain types of bacteria. To meet these needs, numerous media are available.

1. **General purpose media/ Basic media:** Basal media are basically simple media that supports most non-fastidious bacteria. Peptone water, nutrient broth and nutrient agar are considered as basal medium. These media are generally used for the primary isolation of microorganisms.
2. **Enriched medium (Added growth factors):** 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) blood to a blood agar base. Chocolate agar is also known as heated blood agar or lysed blood 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 of interest. Various approaches to make a medium selective include addition of antibiotics, dyes, chemicals, alteration of pH or a combination of these.

#### a. Selective medium

**Principle:** Differential growth suppression Selective medium is designed to suppress the growth of some microorganisms while allowing the growth of others. Selective medium is agar based (solid) medium so that individual colonies may be isolated.

#### Examples of selective media include

- Thayer Martin Agar used to recover *N.gonorrhoeae* contains antibiotics; vancomycin, colistin and nystatin.
- Mannitol Salt Agar and Salt Milk Agar used to recover *S.aureus* contains 10% NaCl.
- Potassium tellurite medium used to recover *C.diphtheriae* contains 0.04% potassium tellurite.
- Mac Conkey'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 (antiseptic agent).
- 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 and Blair's Agar for recovering *S. typhi* is rendered selective by the addition of dye brilliant green.
- Selective media such as TCBS Agar used for isolating *V.cholerae* from fecal specimens have elevated pH (8.5-8.6), which inhibits most other bacteria (Garrard *et al.*, 2013)<sup>[5]</sup>.

#### b. Enrichment culture medium

Enrichment medium is used to increase the relative concentration of certain microorganisms in the culture prior to plating on solid selective medium. Unlike selective media, enrichment culture is typically used as broth medium. Enrichment media are liquid media that also serves to inhibit commensals in the clinical specimen. Selenite F broth, tetrathionate broth and alkaline peptone water (APW) are used to recover pathogens from fecal specimens.

#### 4. Differential/ indicator medium: differential appearance

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. Differential media allow the growth of more than one microorganism of interest but with morphologically distinguishable colonies.

#### Examples of differential media include

- Mannitol salts agar (mannitol fermentation = yellow)
- Blood agar (various kinds of hemolysis i.e.  $\alpha$ ,  $\beta$  and  $\gamma$  hemolysis)
- Mac Conkey agar (lactose fermenters, pink colonies whereas non- lactose fermenter produces pale or colorless colonies)
- TCBS (*Vibrio cholerae* produces yellow colonies due to fermentation of sucrose)
- Anaerobic media: 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. Before use the medium must be boiled in water bath to expel any dissolved oxygen and then sealed with sterile liquid paraffin.

Robertson Cooked Meat (RCM) medium that is commonly used to grow *Clostridium* spp contains a 2.5 cm column of bullock heart meat and 15 ml of nutrient broth. 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 colorless (Heath *et al.*, 2007)<sup>[6]</sup>.

#### 6. Assay media

These media are used for the assay of vitamins, amino acids and antibiotics. E.g. antibiotic assay media are used for determining antibiotic potency by the microbiological assay technique (Kaur and Kaur, 2015)<sup>[8]</sup>.

#### Preparation and Storage of Culture Media

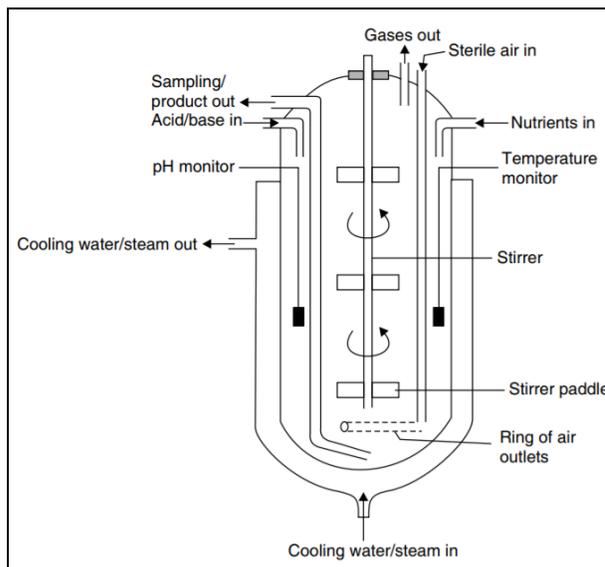
Care must be taken to adjust the pH of the medium before autoclaving. Various pH indicators that are in use include phenol red, neutral red, bromothymol blue, bromocresol purple etc. Dehydrated media are commercially available and must be reconstituted as per manufacturers' recommendation. Most culture 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, trypticase soya broth (TSB) 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 4-50C 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 (Knief *et al.*, 2011)<sup>[9]</sup>.

- Sterilization:** The media and culture vessel have to be sterilized to prevent the growth of unwanted microorganisms and thus contamination. If

laboratory scale experiments are carried out in 100 to 1000 ml flasks, or in lesser volumes such as 50 ml or 10 ml, the media along with the culture flasks or vials can be steam-sterilized with an autoclave. Depending on the quantity of the materials autoclaved, the sterilization can be carried out alternatively in a pressure cooker of convenient size. Steam-sterilization with an autoclave or pressure cooker is carried out at 120°C for 15 to 20 minutes under 15 psi pressure. When microbes are cultivated in a fermentor for large-scale operation, it is convenient to sterilize the fermentor as a whole with or without media. Media may be sterilized separately or in situ, in the fermentor itself. Steam is used for the sterilization of the media and fermentor, by passing the steam through the sterilization jacket or the coil around the fermentor. When the fermentor is sterilized without media in it, steam can be sparged into the vessel through all openings, allowing it to exit very slowly. Sparging is a process by which sterile air or steam is allowed to pass through the medium in the vessel with the help of a sparging device placed at the bottom of the fermentor. The steam pressure is held at 1.5 psi for 20 to 30 minutes while circulating or holding the steam within the vessel or in the jacket.

- Environment for microbial growth:** The nutrient composition of the medium, the ionic concentration of salts, pH, and temperature influence the growth of microorganisms in the culture and its metabolic state. Most of the bacteria grow at neutral pH, whereas yeast and fungi prefer acidic pH. Similarly, different organisms prefer different optimum temperatures for active growth and multiplication. The optimum temperature has to be maintained in the culture with the help of an incubator in the case of small-scale cultures and circulating water of the appropriate temperature through the jacket of the fermentor.
- Aeration and mixing:** Mixing of the broth is essential for the uniform distribution of the nutrients and the microbial population in the culture. Aeration is needed for the easy gas exchange between the medium and the environment. Aerated medium will be rich in oxygen. Aeration and mixing can be easily achieved by shaking the medium on a shaker in the case of small scale cultures (shake flasks cultures). In large-scale cultivation in bioreactors the transfer of oxygen to organisms is very difficult because it requires proper mixing. In fermentors, the proper mixing of cells, media components, and oxygen is achieved by stirring the medium with the help of a mechanical stirrer with baffles attached to it. Baffles help in maintaining turbulence. Microbial-free air passed through the media ensures proper aeration, and this forced aeration also helps in the mixing of media, cells, and oxygen.
- Fermentors:** These are bioreactors used for the cultivation of microbial cells on large scale under controlled conditions for industrial purposes. This closed metallic or glass vessel has the adequate arrangement for aeration, mixing of media by agitation, temperature control, pH control, anti-foaming, control of overflow, sterilization of media and vessel, cooling, and sampling (removal of sample, while the fermentor is on). Agitation of the media in the bioreactor may be through stirring or

aeration or both. This equipment is convenient for operation continuously for a number of days. The essential parts of a laboratory fermentor are given in Figure below:



**Fig 1:** A laboratory ferment showing the essential components

As indicated in the figure, the bioreactors are provided with controls for monitoring and adjusting the many physical and chemical parameters such as temperature, pH, nutrient composition, foaming, etc. Maximum cell growth and product formation can be achieved by controlling these parameters that assist cell growth and metabolism leading to high output of the product. A stirred tank bio-reactors the most commonly used bioreactor for microbial cultivation, in which the microbial medium is stirred with an impeller. A high density of metabolically active cells in the medium can result in sudden depletion of dissolved oxygen creating an anaerobic condition in the medium. This can result in serious consequences in the quality of the product or even in the type of products formed in the fermentation reaction. Similarly, the cell growth and product formation can alter pH of the medium, which can also create problems in the further growth and metabolism of the cell cultures. Rapid growth also results in the depletion of essential nutrients that directly link to the growth and metabolism that causes the production of the product. All these changes are monitored by the accessories of the fermentor or bioreactor and are accordingly indicated automatically. For example, whenever there is a change in pH from the optimum value, automatically a sufficient amount of acid or alkali is added to the media to keep the optimum pH constant. Similarly, if there is foaming in the media the sensor will detect the foam formation and accordingly, the antifoam agent is delivered into the medium to prevent the foaming. In addition to the industrial type of bioreactors or fermentors, there are fermentors of small volumes suitable for operating in the laboratories, known as laboratory fermentors. These laboratory fermentors are for 10 to 100 liters of volume and are used for optimizing culture conditions and nutritional parameters for better growth of cells and production of metabolites for conducting research studies in the laboratory.

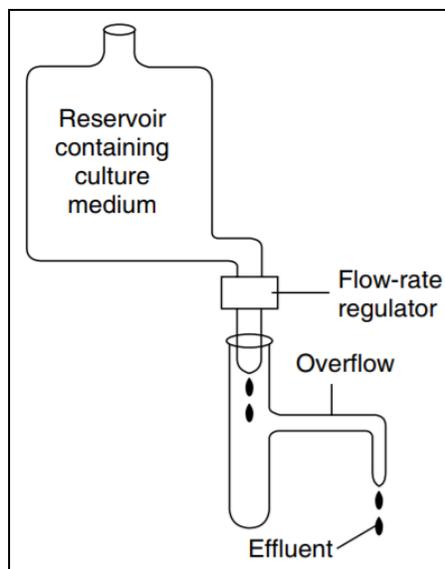
**Types of Microbial Cultures** The culturing of the microbial system can be achieved in different ways. The type of culture method sometimes depends on the type of the microbial system or on the type of the product that we expect. For

example, one can get two entirely different products from the same organism by changing the nutritional and other parameters or even culturing vessels.

- 1. Batch culture:** This is a small-scale laboratory experiment in which a microbial culture is growing in a small volume flask. It consists of a limited volume of broth culture in a flask inoculated with the bacterial or microbial inoculum and follows a normal growth phase. It is a closed-culture system because the medium contains a limited amount of nutrients and will be consumed by the growing microorganisms for their growth and multiplication with the excretion of certain metabolites as products. In batch cultures, the nutrients are not renewed and the exponential growth of cells is limited to a few generations. The growth phase of the culture consists of an initial lag phase, a log phase or the exponential growth phase, and a stationary phase. During the log phase the consumption of the nutrients will be the maximum resulting in the maximum biomass output with the excretion of the product. At the stationary phase the rate of growth decreases and becomes zero. This is because at the stationary phase the cells are exposed to a changed environment where there is only a small amount of nutrients and more cells along with the accumulation of metabolites, which may have a negative effect on the growth of the cells.
- 2. Fed-batch culture:** The batch culture can be made into a semi-continuous culture or fed-batch culture by feeding it with fresh media sequentially at the end of the log phase or in the beginning of the stationary phase without

removing cells. Because of this the volume of the culture will go on increasing as fresh media is added. This method is specially suited for cultures in which a high concentration of substrate is inhibitory to cell multiplication and biomass formation. In such situations the substrate can be fed at low concentrations to achieve cell growth. This method can easily produce a high cell density in the culture medium, which may not be possible in a batch fermentor or shake flask culture. This is especially important when the product formation is intracellular to achieve maximum product output per biomass.

- 3. Continuous culture:** Bacterial cultures can be maintained in a state of exponential growth over long periods of time using a system of continuous culture, designed to relieve the conditions that stop exponential growth in batch cultures. Continuous culture, in a device called a chemo-stat, can be used to maintain a bacterial population at a constant density, a situation that is, in many ways, more similar to bacterial growth in natural environments. This is a very convenient method to get continuous cell growth and product formation over a long period of time. In continuous culture, the nutrient medium including the raw material is supplied at a rate that is equal to the volume of media with cells and product displaced or removed from the culture. The volume removed and the volume added is the same. In effect there is no change in the net volume as well as the chemical environment of the culture. In a chemo-stat, the growth chamber is connected to a reservoir of sterile medium.



Schematic diagram of a chemostat, a device used for the continuous culture of microbes. The chemostat relieves the environmental conditions that restrict growth by continuously supplying nutrients to cells and removing waste substances and spent cells from the culture medium.

Once the growth is initiated, fresh medium is continuously supplied from the reservoir, the volume of fluid in the growth chamber is maintained at a constant level by some sort of overflow drain. Fresh medium is allowed to enter into the growth chamber at a rate that limits the growth of the bacteria. The bacterial cells grow (cells are formed) at the same rate at which bacterial cells (and spent medium) are removed by the overflow. The rate of addition of the fresh medium determines the rate of growth because the fresh medium always contains

a limiting amount of an essential nutrient. Thus, the chemostat relieves the insufficiency of nutrients, the accumulation of toxic substances, and the accumulation of excess cells in the culture, which are the parameters that initiate the stationary phase of the growth cycle. The bacterial culture can be grown and maintained at relatively constant conditions, depending on the flow rate of the nutrients. Density is constant in a turbidostat culture, which is also a continuous culture. Since the culture is fed with the fresh medium at specific rate, a

steady state of growth and metabolism is achieved. At a steady state, the cell multiplication and substrate consumption for growth and product formation occur at a fixed rate. The growth rate is maintained constantly. The formation of new biomass is balanced with the removal of cells from the outlet. Continuous culture is very suitable for the production of cell biomass and products, if it is excreted into the medium. It is widely used for the production of single-cell protein from liquid effluents as a byproduct of the waste treatment. The organic waste present in the effluent is converted into microbial biomass, which is known as single-cell proteins (Breslin and O'Driscoll, 2013)<sup>[2]</sup>.

#### 4. Synchronous cultures:

Synchronous cultures are composed of the population of cells that are the same stage of their life cycle. All the cells in the culture will divide at the same time, will grow for a generation time, and all will divide again at the same time. Thus, the entire population is kept uniform with respect to growth and division. It is not possible to analyze a single bacterial cell to obtain the information about growth behavior, Organization, differentiation and macromolecular synthesis. Synchronous culture provides the entire cell crop in the same stage of growth. Measurement made on such cultures are equivalent to the measurement made individual cells.

Synchronous cultures of bacteria can be obtained by a number of techniques. Two fundamentally different experiment approaches have employed. In the first approach, a Synchronous population of the cells can be sorted out according to age or size by physical separation of cells. In methods of the second type, a culture is induced by manipulating the physical environment or chemical composition of the medium to obtain asynchronously dividing the population. The techniques based on selection are preferable to those based on induction since induction is likely to introduce distortions in physiologic state of the cells (Garrard, 2013)<sup>[5]</sup>.

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