Review Article

Strategies of Bacterial Isolation and Cultivation

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Abstract

For the study of bacterial virulence, antibiotic susceptibility and genome sequence a pure bacterial culture is remains required. A culture which contains only one species of microorganism is called a pure culture. The process of obtaining a pure culture by separating one species of microorganism from a mixture of other species is known as isolation of the organisms. Initially bacterial culture was based on incubation time, nutritional requirement, atmosphere and temperature. Further microbiologist rebirth the culture in clinical microbiology for intra cellular bacteria. New species of Rickettsia was allowed to grown through cell vial procedure. Fastidious bacteria like Tropheryma whipplei and coxiella burnetii grown in axenic media with amoebal coculture. By providing microaerophilc atmosphere in culture media mycobacterium cultivation time is decreases dramatically. Use of antioxidant provides anaerobic condition strictly to grow anaerobic bacteria.

Key words: Amoebal Co-culture, Axenic Media, Bacterial Culture, Virulence

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Introduction

A culture media is a medium used in microbiological laboratories to grow different types of microorganisms. Robert Koch proposed a pure culture used as basic media for all researches of bacterial diseases (Brock, 1999). A growth medium or culture medium is made up of different nutrients that are essential for microbial growth. Bacterial culture is very useful in the study of antibiotic resistance and effective treatment of diseases and establishing genomic sequences of different strains of bacteria (Boulos *et al.,* 2005; Fournier *et al.*, 2007). Pure bacterial culture provides the base for the proteomic studies to high light proteins that use in serological tests as antigen (Fournier *et al.,* 2013). Recently axenic media is developed for intracellular bacteria. Axenic media is a sterile media contain no living bacteria except the

desired bacteria (Omsland *et al.,* 2012). Rehydration-centrifugation (RC) methodology used for isolation of rare and zoosporic Actinobacteria (Delphine *et al.,* 2018).

Conventional Methods of Bacterial Isolation and Cultivation

Different types of microorganisms have their unique properties and require different nutrients for their growth. For the growth of bacteria 4 primary elements are required i.e. nutrients, atmosphere, temperature and incubation period (Marmonier, 2007).

Type of Media on the Basis of State

Solid Media

The addition gelatin or agar, has led to the design of solid culture media (Hitchens and Leikind, 1939). Solid medium contains 1.5-2.0% agar of or some other, mostly inert solidifying agent. Bacteria grow in solid media in physically informative way as colonies. Coagulated egg and coagulated serum can also be used to solidify bacterial culture which are used in Lowenstein- Jensen for Mycobacterium and corynebacterium culture respectively (Pfyffer, 2007; Funke and Bernard 2007).

Semisolid Media

Levine (1916) employed semi-solid agar (nutrient broth with 0.5 per cent agar) in culture tubes to determine motility in the Escherichia-Aerobacter group. Concentration of agar in this media is 0.5% or less. They are useful for cultivation of microaerophilc bacteria for motility determination and have soft custard like consistency. It is also useful in semisolid agar overlay method for the isolation and characterization of phage.

Liquid (Broth) Media

Liquid media do not have solidifying agents such as gelatin or agar but have specific amounts of nutrients. Broth medium serves various purposes such as propagation of large number of organisms, fermentation studies and various other tests, e.g. sugar fermentation tests, Methyl Red- Voges Proskauer (MR-VP) broth.

Preservation Culture Media

All the required basic nutrients for microbial growth are present in this culture media. This media is used to preserve microorganism for a long period of time. This media protects the microorganism against adverse environment so these microorganisms when needed can be used.

Enrichment Culture Media

It is liquid media and has essential nutrients for cultivation of specific microorganism. Enrichment media enhance the growth of a particular microorganism by providing it with the essential nutrients and rarely contain certain inhibitory substance to prevent the growth of normal competitors.

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Selective Medium

Principle

Selective media allow certain types of microorganisms to grow, and suppress the growth of other organisms. Individual colonies may be isolated in selective medium because it is agar based solid medium. Examples of selective media include-

a) **Thyayer Martin Agar:** used to recover *Neisseria gonorrhea* contains antibiotics. Thayer and Martin (1964) used Vancomycin, Colistin and Nystatin. Martin and Lester (1971) used an additional antibiotic Trimethoprim to make the medium selective.
b) **Mannitol Salt Agar:** used for identification of pathogenic staphylococci associated with acute infection (Murray *et al.,* 2003).
c) Pseudosel Agar (Cetrimide Agar): used to recover *P. aeruginosa* contains cetrimide (antiseptic agent) (Aylin *et al.,* 2012).
d) **MacConkey's Agar**: used for the isolation and differentiation of gram negative enteric bacilli (USPC, 2007), contains bile salt that inhibits most gram positive bacteria.
e) **Lowenstein Jensen Medium:** used to recover *M. tuberculosis* is made selective by incorporating malachite green. Lowenstein-Jensen Selective Medium reduces Contamination in *Mycobacterium tuberculosis* Culture (Kassaza *et al.,* 2014).
f) **Wilson and Blair's Agar** for recovering by the addition of dye brilliant green *Salmonella typhi* is rendered selective.
g) **Thiosulfate-Citrate-Bile Salts-Sucrose Agar (TCBS):** have high pH (8.5-8.6) used for isolating *V. cholerae* from fecal specimens and inhibit most of the microorganism.
h) **Potassium Tellurite Medium**: is a selective medium for isolation and cultivation of corynebacterium species (Scott, 1981; MacFaddin, 1985).

Differential Culture Media

Differential distinguish one microorganism type from another growing on the same medium (Washington, 1996). This media differentiate specific bacteria by other microorganism using identification marker. Certain media are designed in such a way that different bacteria can be recognized on the basis of their colony colour (Rouf *et al.,* 2017). The selective and differential culture media are opposites in nature that one inhibits the growth of other organisms while allowing the growth of some while the other does highlights one type but not kill the others. Blood agar is a common differential culture medium used to identify bacteria that causes haemolysis on blood. Examples of differential media include-

a) Mannitol salt agar (lactose fermenter and non-lactose fermenter)
b) Blood agar (various kinds of hemolysis i.e. α, β and γ hemolysis)
c) Mac Conkey agar (lactose fermenters, pink colonies whereas non- lactose fermenter produces pale or colorless colonies.
d) TCBS (*Vibrio cholerae* produces yellow colonies due to fermentation of sucrose)

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Advance Strategies for Bacterial Isolation and Cultivation

Resuscitation Culture Media

This is a special type of media which is used for growing microorganisms that are damaged and have lost the ability to produce due to certain harmful environmental factors Marthi (1994). This culture media provides nutrients that the organism had been deprived of to regain the organism. For example, if any microorganism that requires histamine for its growth its growth will be inhibited if cultivated in a medium lacking this essential component and it will start to grow again if the same microorganism is then placed in a medium consisting of histamine. An example of a commonly used resuscitation culture media is the tryptic soya agar.

Fermentation Media

It is a liquid selective media which is used to obtain a culture of a specific organism like yeast or a particular toxin. The fermentation media can also be differential but mostly it is selective in nature that is allowing the growth of one type while inhibiting the growth of others. Fermentation technology is widely used for the production of various economically important compounds which have applications in the energy production, pharmaceutical, chemical and food industry (Singh *et al.,* 2016). To monitor bioprocess and fermentation in biological and food applications Real-time analytical tools are becoming increasingly important. Traditional laboratory-based analyses need to reduce costs. Methods for bioprocess fermentation monitoring are spectroscopy-based (Roberts *et al.,* 2018).

Intracellular Bacterial Culture

Intracellular bacteria are responsible for the diseases with significant morbidity and mortality (Parola *et al.*, 2013). To grow intracellular bacteria, novel strategies were proposed at the end of the 20th century (Singh *et al.,* 2013). *Tropheryma whipplei*, an intracellular bacterium causing Whipple's disease, was grown in culture medium supplemented with amino acids. This medium was designed after sequencing of the *T. whipplei* genome, which lacks genes encoding the enzymes involved in the synthesis of some amino acids (Renesto, 2003). Recently, *C. burnetii*, the intracellular causative agent of Q fever, has been described as being cultivatable in an acellular chemically defined medium (Omsland *et al.,* 2009; Omsland *et al.*, 2011). *Chlamydia trachomatis* causative agent of lymphogranuloma venerum (LGV), *Chlamydophila pneumonae* and *C. psittaci* responsible for pneumonia were isolated in embryonated eggs or in animal inoculation. Cell culture system can be used to isolate intracellular bacteria (Gouriet *et al.,* 2005). Axenic medium is used for revival of intracellular pathogen culture (Singh *et al.,* 2013).

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Culture in Embryonated Eggs

Previously embryonated eggs were used to cultivate viruses. Few year later cox injected intracellular bacteria into yolk sac of embryonated egg and shows cultivation of bacteria (Cox, 1938). This culture system provides higher infective titre in comparison to mammalian tissue. The major disadvantage of the system is variations in its susceptibility and high susceptibility to contamination. Mostly antibiotic susceptibility study and isolation and propagation of *C. burnetii* and *Rickettsia* spp. occurs through this method. Moreover, this procedure requires antibiotic-free-diet embryonated eggs from 5 to 8 days of age, which are often difficult to obtain. This culture system was extensively used to isolate and propagate as well as to study antibiotic susceptibility (Raoult *et al.,* 1986; Raoult *et al.,* 1989). Recently cell culture system replaces this system due to more convenient.

Animal Inoculation

Despite improvements in culture media, some bacteria such as *M. leprae* and *T. pallidum* remain uncultivable except in animal models. *M. leprae* was first cultivated in mouse foot pad (Shepard, 1962). The yield can be increased by inoculation of bacteria in immunosuppressed mice (Scollard *et al.,* 2006). Nine-banded armadillo (*Dasypus novemcinctus*) is a natural host for *M. leprae* which exhibited manifestations of leprosy comparable to those in humans (Kirchheimer *et al.,* 1972) and 30% infected animals develop systemic infection mainly reticuloendothelial system affected. *T. pallidum* and Borrelia are difficult to cultivate in cell culture or in artificial medium, there cultivation is possible only after animal inoculation (Felsenfeld, 1965). For cultivating intracellular bacteria before the use of cell culture systems inoculation of animals was also used. Guinea pigs and mouse are the preferred animals for Mycobacterium tuberculosis (Orme and Ordway, 2016). This is expensive, time-consuming, and technically difficult technique. This technique can be applied only in specialized laboratories. Cross-contamination between infected and uninfected animals and low reproducibility are two major problem of animal inoculation. To isolate intracellular bacteria from specimens contaminated with other bacteria, however, animal models are still useful.

Coculture with Amoebae

Some intracellular pathogens such as mycobacteria, legionella and Chlamydia-like organisms are difficult to cultivate and because of poor growth even on selective media. These pathogens are often associated with amoebae, which serve as host-cell and allow the survival and growth of the bacteria Jacquier *et al.* (2013). Within both humans and animals free-living amoebae (FLA) are parasites causing a wide range of symptoms. They act as hosts of and vehicles for phylogenetically diverse microorganisms, called endocytobionts (Balczun and Scheid, 2017). There are two techniques that allow isolation and

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characterization of intracellular pathogens from clinical or environmental samples- the amoebal coculture and the amoebal enrichment.

Amoebal coculture allows recovery of intracellular bacteria by inoculating the investigated sample onto an amoebal lawn that can be infected and lysed by the intracellular bacteria present in the sample. Amoebal enrichment allows recovery of amoebae present in a clinical or environmental sample. This can lead to discovery of new amoebal species but also of new intracellular bacteria growing specifically in these amoebae. Both techniques act collectively to discover new intracellular bacteria that able to grow in amoebae.

Cell Culture

Intracellular pathogen grows and reproduces inside host cells. These pathogens can be divided into facultative intracellular parasites and obligate intracellular parasites Leon-Sicairos *et al.* (2015). Cell culture is now most common method for cultivation of intracellular bacteria. The susceptibility of bacteria varies according to their cell tropisms and optimal growth temperatures to different cells lines. For this purpose several eukaryotic cell lines can be used. Two types of cells may be used either cells cohering with each other, forming a cell monolayer or cells circulating in suspension. Several eukaryotic cell line of mammals and arthropods used. Genomic and proteomic study, antibiotic susceptibility can be done by this technique (Lagier *et al.,* 2015).

Axenic Culture Media for Intracellular Bacteria

Isolation in axenic culture is achieved by cultivating the species in an environment that was sterilized previously and was free from contaminating organisms. The use of new tools, such as genomic and transcriptomic analyses and the host cell-pathogen interaction have enabled the study of metabolic pathways, enhancing the success of axenic cultivation (Ogata and Claverie, 2005); Omsland *et al.*, 2013). Axenic culture media could have significant effects on the study of their pathogenicity, virulence, and antibiotic susceptibility and on the design of new diagnostic tools (Singh *et al.,* 2013). Coxiella burnetii has been described as being cultivable in an acellular chemically defined medium (Omsland *et al.,* 2009), a medium designed after a comprehensive analysis of the nutrient consumption of *C. burnetii*. CLUPS medium is a superior alternative culture medium, which is both economic and free of the interlot variability present in commercial TYIS-33 (Gonzalez-Salazar *et al.,* 2018).

Conclusion

Both conventional and modern approaches are used today for the cultivation of bacteria because no single approach can cultivate all type of bacteria. Older techniques as cultivation of basic media and animal inoculation are still useful to isolate and cultivate bacteria. Animal inoculation, are still used to propagate

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some bacteria and to isolate new bacterial species, such as "Candidatus Neoehrlichia mikurensis." Growth of large no of intracellular bacteria cell culture requires. Future prospects will be based largely on the development of axenic media, facilitating genetic manipulation, and on an understanding of microbial ecology and the pathogenicity of fastidious bacteria. Researchers should use a combination of both older and modern techniques to minimize cost and pure bacterial isolation of bacteria.

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