



Mini-Review Article

Basic Techniques and Limitations in Establishing Cell Culture: a Mini Review

Priyabrat Swain^{1*}, Pramod Kumar Nanda², Sukanta Kumar Nayak¹, Sudhansu Sekhar Mishra¹

¹Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar-751 002, India;

²Indian Veterinary Research Institute, Eastern Regional Station, Belgachia, Kolkata-700 037, India

*Corresponding author: pswainy2k@yahoo.co.in

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ABSTRACT

Cell culture has become an indispensable tool in modern day science and is being innumerable applied in both fundamental and applied biological research. This technology involves isolation of cells from their natural (*in vivo*) location and propagation *in vitro* in favourable artificial conditions. Cell culture can either be short term (primary culture) or long term depending upon purposes. In several cases, cells are grown for short terms *i.e.*, for few days or weeks to obtain cells from target organs or tissues to pursue specific purposes. On the contrary, the long-term culture of cells is done to establish cell lines. Irrespective of the purpose and culture practices; optimal culture conditions in form of nutrients, media, supplements and growth factors is required to be maintained under controlled environment for *in vitro* growth of cells. Over the years, appropriate methods have been developed and standardized for culture of cells from all most all the tissues and organs of diversified species. However, the basic principles of cell culture techniques remain mostly unchanged since its introduction. This mini review illustrates the aspects that are critically required for cell culture and also focuses on the limitations, especially adaptation of cells to artificial environment for optimal growth and differentiation in order to form an established cell culture.

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INTRODUCTION

Cell culture is the complex process which involves isolation or removal of cells from their natural (*in vivo*) and their subsequent growth under controlled conditions in an artificial (*in vitro*) environment. Cultured cells, either as short term or as established cell lines, from a broad range of tissues and organs have many applications in fundamental and applied biological research (Keay, 1975; Hightower and Renfro, 1998; Marrec-Croq et al., 1999; Butler, 2005; Nema and Khare, 2012). These *in vitro* models form the biological basis of most alternative methods (Hartung, 2007) and are used to study cell differentiation, toxicology and pharmacology, cancer research thereby avoiding the use of laboratory animals. Further, cell culture is widely used as a diagnostic and research tool, particularly for diagnosis of viral diseases (Wolff, 1988) as isolation of pathogenic viruses from animals are totally dependent on the availability of a live host such as permissive cell cultures (Hetrick and Hedrick, 1993). Cell cultures are also used in vaccine manufacturing using recombinant DNA (rDNA) technology and other biological products such as production of immunobiologicals (monoclonal antibody, recombinant protein, lymphokines), cell and gene therapy, novel drugs selection and improvement etc. (Butler, 2005; Eibl et al., 2009; Nema and Khare, 2012). Because of such

broad range of applications, there is a growing need to develop and establish short term and continuous cultures from various commercially important animal species. The *in vitro* growth of cells requires the essential features of the conditions within the body that cells experience in living tissues for their optimal cell survival, growth and function (Cooke et al., 2008). To achieve this, optimisation of the *in vitro* culture conditions providing culture media, substrate, nutrients and growth supplements is required making a conducive environment for cells to function, grow and propagate in a realistic manner. This mini review emphasizes the role of various factors and also focuses on the limitations in establishing cell culture.

Historical Background

Tissue or cell culture as a technique was first devised at the beginning of the 20th century as a method to study the behaviour of animal cells *in vitro*. Prior to this, Roux (1885), an embryologist, was able to maintain embryonic chicken cells in warm saline for several days and established the principles of tissue culture. Carrel and Burrows (1911) demonstrated that the survival of cells isolated from blood and connective tissue can be made in serum and plasma. Harrison (1907) established the methodology of tissue culture and observed cell growth in clotted lymph fluid

from explants of frog embryo tissue by the “hanging drop” method. This was followed by the work of Carrel and his co-worker who succeeded in growing adult and embryonic tissues of warm blooded mammalian cells *in vitro* (Carrel and Burrows, 1910). Carrel (1912) expanded the possibilities of cell culture by keeping fragments of chicken embryo heart alive for over 3 months *in vitro*. To overcome the contamination problems, Carrel or T-flask, developed by Carrel and his co-worker in 1923, has become one of the important items of cell culture laboratories enabling the subculture of cells (Butler, 1991). The technique of trypsinization was exploited by Rous and Jones (1916) using trypsin (a proteolytic enzyme) to dissociate cells from tissue matrix. This facilitated the subculture of adherent cells to produce homogeneous cell strains and marked the start of animal cell culture in true sense (Moscona, 1952). Till to that period, culture of cells from primary explants of tissue dominated the field (Fisher, 1925). The developments that occurred in over ten years (1940–1950) made cell culture widely available and accepted as a tool for scientists. Notable among these are use of antibiotics to avoid contamination problems in cell culture and development of chemically defined culture media by Eagle (1955). These advancements in cell culture technology offered more scope to researchers to widely use cell, tissue and organ culture in their research as a result of which cell culture is routinely carried out in many laboratories throughout the world.

Cell Culture Models in Animals

Cell culture models are highly defined system, in which parameters and conditions can be regulated leading to more reproducible results in comparison to other studies. Over the last decades, these models are extensively used to study morphological, biochemical signaling processes, toxicity testing, study of reconstructed tissue equivalents with aim to reproduce the *in vivo* environment (Carere et al., 2002), transfection of cell lines with specific enzymes to study the biotransformation of compounds (Yoshitomi et al., 2001; Hashizume et al., 2009). Some of the types of cell culture models and or their applications are as follows.

Cell Culture Models for Disease Studies

Different *in vitro* cell culture models can provide an insight into the mechanisms of infection for various microbial pathogens (Duell et al., 2011). Of late, several culture models are available for studying neurodegenerative diseases in order to gain a better understanding of the disease and understand their underlying mechanisms to investigate new therapeutic strategies (Figlewicz et al., 2000). Further, these *in vitro* cell culture models might provide important insights about the pathogenesis of many disorders viz. cervical dysplasia–pluripotent stem cell models of human heart disease, human ES cell lines for Alzheimer’s disease drug discovery, cell culture model for Huntington’s disease and may also replace *in vivo* models to evaluate compounds or the screening of potential pharmacological agents against many disease conditions. Currently, a fast, efficient and reproducible cultured cell system from animal models for the screening and testing of compounds for the treatment and therapy of Abeta-associated diseases (AD) has been developed (Trinchese et al., 2004). Furthermore, cell culture models are reported to be versatile and extremely useful experimental models to investigate the biology of both the

normal and the pathological isoform of the prion protein of transmissible spongiform encephalopathies /prion diseases. However, only few cell culture models permissive to prion replication are available till date. Among them, mouse neuroblastoma cell lines (N2a) are most commonly used for screening as well as selecting possible therapeutic compounds so as to develop new strategies for the investigating specific diagnostic markers (Lawson et al., 2008).

Cell Culture Models Pathogen Interaction Studies

Cell culture models have several advantages over animal model for studying microbial pathogenesis and are now being considered as indispensable tools to study bacteria-induced cell death such as apoptosis or necrosis, lysis, adhesion, invasion, and cell-to-cell movement *etc.*, of different foodborne pathogens like *Listeria monocytogenes*, *E. coli*, *Clostridium perfringens*, *Vibrio sp.*, *Shigella sp.*, *etc.* Further, cytopathic effects and multiplication of avian flu virus (H5N1) and hepatitis A virus can also be studied using cell culture models (Salyers and Whitt, 2002; Bhunia and Wampler, 2005).

Cell Culture Models for Cellular Studies

Cell culture model is mostly used for oxidative stress related studies (Gille and Joenje, 1992), to test therapies against hyperglycemia-mediated oxidative stress and injury (Vincent et al., 2005), mitochondrial dysfunction of familial amyotrophic lateral sclerosis (Menzies et al., 2002) in cellular studies.

Cell Culture Models to Study Biological Barriers

Such types of models are successfully used in studying endothelial barrier permeability studies, ocular barriers studies, drug absorption and delivery (Garcia et al., 2011; Schneider et al., 2013).

Microfluidic Cell Culture Models for Tissue Engineering

Microfluidic techniques facilitate simultaneous manipulation and analysis of cultured cells, starting from a single cell to larger populations, intact tissues and used for drug research (Wu et al., 2010). With the recent advances, this technology has several advantages over conventional cell culture methods and holds great promise for the creation of advanced cell culture models (Meyvantsson and Beebe, 2008; Inamdar and Borenstein, 2011).

Cell Culture Models for Ageing Studies

Cell culture models are also used to understand the mechanisms of cellular senescence and their impact on human ageing process (de Magalhaes, 2004). In this aspect, the most widely used cell culture models for studying ageing is Hayflick’s.

Establishment of Cell Culture System

Cell culture refers to the removal of cells from an animal or plant and their propagation and cultivation *in vitro* in a suitable artificial environment. The process starts with primary culture of cells to achieve confluence or formation of monolayer in a culture flask supplemented with nutrients and growth factors. After achieving confluence, cells are subcultured or passaged routinely from primary to

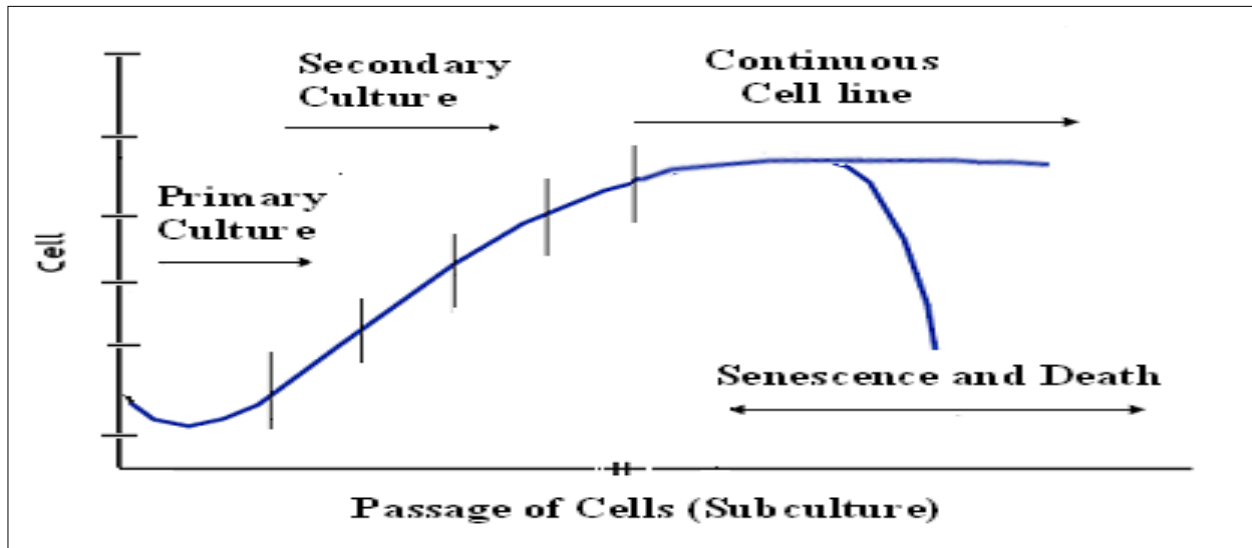


Figure 1: The growth kinetics of cells for generation of continuous cell lines from primary cell culture

secondary and secondary to tertiary till establishment of a continuous cell line (Figure 1). During this process of culture and subcultures, several factors regulate the success of cell culture practices. To establish primary cultures or continuous cell lines, culture conditions are required to be maintained in culture flask supplemented with all the essential nutrients, supplements, hormones and growth factors that are required for metabolic activities, growth and

proliferation of cells (Bettger and McKeehan, 1986; Butler and Jenkins, 1989; Davis, 2002). This, in turn, provides the microenvironment for *in vitro* growth of cells mimicking *in vivo* physiological conditions and maintaining the physical and chemical factors such as buffering systems, pH, oxygen, osmolality etc. (Figure 2).

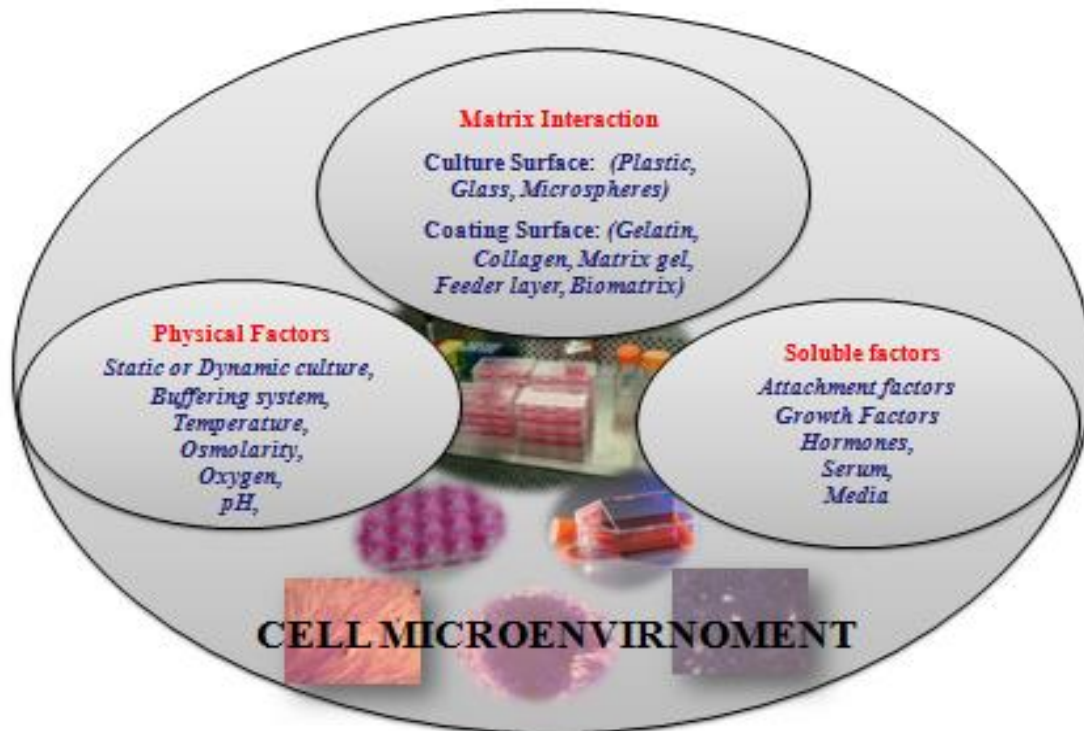


Figure 2: The basic requirements and factors governing cell culture

The initial stage to consider in cell culture is the isolation of the appropriate tissue. In general, cultures from embryonic tissues survive and proliferate in a better way as compared to those from the adult (Burgess et al., 1993), as they often have a lower growth fraction and a high proportion of non-replicating specialized cells due to the age and developmental stage of the animal. Furthermore, initiation and propagation of culture is difficult, and in most of the cases, the lifespan of the culture is often shorter. From this, it is clear that more dividing cells can be obtained from gonads, embryos than from skeletal muscle, and adult animals (Nicholson, 1989; Crane, 1999). But the choice of tissue and establishment of primary culture or cell line species depends upon the purpose of its application in cellular and molecular biology.

Cell Culture Methods

Cell cultures may be initiated by aseptic collection of normal, embryonic, malignant tissue, or from the organ of interest by surgical or enzymatic method and placing into a conducive culture environment for attachment, growth and proliferation. This is called a primary cell culture. This is the stage of culture after isolation but before the first subculture or passage of the cells (Freshney, 2010). To obtain primary cultures, explant and tissue dissociation (mechanical and enzymatic) methods are generally employed (Bols, 1991).

Explant Method

The culture of cells adopting explant method dominated the field of tissue culture for more than 50 years (Fisher, 1925; Parker, 1961). This is one of the simplest procedures, which involve finely chopping the tissue (explants of tissue) into fragments of no larger than 1–2 mm³. The tissue fragments (explants) are placed to a glass or treated plastic culture flask and once attached, further media is added to cover the explants completely (Wolf and Ahne, 1982). If successful, cells will migrate out from the explants and cover the growth surface eventually forming a culture. Attachment of the explants may also be promoted by treating the plastic culture flask with substrates or coating materials like collagen, fibronectin, gelatin, polylysine, other extra cellular matrix products (Mazia et al., 1975; Crouch et al., 1987) or treating with spent medium from another culture (Stampfer et al., 1980). One of the principal advantages of this method is that some aspects of the tissue's architecture can be preserved within the explant and the biochemical functions and hormonal responses of the tissue as closely as possible to those *in vivo* (Dils, 1994).

Enzymatic Dissociation

The most common method for cell isolation is the enzymatic digestion. In this method, suspension of cells is obtained by chopping the tissue or organs into small fragments followed by treatment with proteolytic enzymes, which may then be cultured as a monolayer on a solid substrate, or as a suspension in the culture medium (Unchern, 1999). The most obvious advantage of cell culture and of enzymatic dissociation in particular is that it makes individual living cells accessible and can be expanded to replicate cultures.

Proteolytic enzymes (also termed peptidases, proteases and proteinases) are capable of hydrolyzing peptide bonds in proteins (Mótyán et al., 2013). The purpose is that these enzymes digest connective tissue and the components of the

surrounding extracellular matrix facilitating the release of cells from a wide variety of tissues. Several enzymes viz. trypsin, collagenase, dispase, protease, pronase E, elastase, hyaluronidase are available in the market for the detachment of cultured cells, cell dissociation and cell component or membrane-associated protein isolation (Unchern, 1999). In this regard, crude enzymes are more effective but more toxic due to contamination with other proteases and prolonged exposure or treatment of cells with such enzymes can weaken their cell membranes and decrease their ability to attach to the substrate (McKeehan, 1977; Smets et al., 1979; Pleskach et al., 1994). On the other hand, increasing the purity of an enzyme gives better control and less toxicity but may result in less disaggregation activity (Freshney, 2010). Further, enzymatic dissociation using different proteases may generate differential yield, viability and efficiency of attachment of cells, as proteases differ in their specificities (Peakman et al., 1994). The efficacy of enzymes may also vary depending upon not only type of tissue, species and age but also due to the variations in time and temperature. Therefore, all these enzymes commercially available are not suitable for every tissue type. As there are variations, an optimized method must be developed for each species as well as for each tissue type for successful primary culture.

Mechanical Disaggregation

Cell isolation can also be achieved through mechanical dispersion using a pipette or syringe. By forcing the tissue through a sieve or syringe, individual or small clumps of cells can be obtained from the bulk of the tissue. Although, this technique produces cell suspension quicker than other methods, an adhesion factor is sometimes needed to assist their attachment to the substrate as the ability of cells to attach is reduced (Toullec, 1999). Further, this procedure causes a great deal of mechanical damage and produces less viable cells than any other method (Freshney, 2010). This method is recommended for soft tissues like brain and spleen.

Role of Nutrients and Other Factors

Media

To start with cell culture, media has an important role to play as cultured cells need either a completely natural or an artificial medium supplemented with some natural products for growth. In early days, natural media such as biological fluids (plasma, serum, lymph, amniotic fluid etc.), tissue extract (extract of chick embryo, liver, spleen, bone marrow and leucocytes) obtained from various biological sources were used to establish cell culture (Shenoy, 2007; Nema and Khare, 2012). The major disadvantage of using natural media is that it often results in poor reproducibility in cell culture due to lack in knowledge about its exact composition.

Artificial or synthetic culture media is constituted based on two parts: a basal nutrient medium and supplements. The balanced salt solutions (BSS) such as Dulbecco's PBS, Hank's BSS, Earle's BSS, phosphate buffered saline (PBS) form the basis of complex media. Besides, a complete culture media contains varying amounts and concentrations of nutrients, serum proteins, carbohydrates, and buffering agents including trace elements, amino acids, and vitamins either in a powder form or in an aqueous solution (Morgan et al., 1950). This

complex mixture only provides energy but also maintains physiological conditions influencing the growth of cells *in vitro* (Ham, 1984; Freshney, 2010).

The media most commonly used to establish cell culture are Medium 199 (M-199), Dulbecco's Modified Eagle's Medium (DMEM), Eagle's Minimum Essential Medium (E-MEM), Hank's MEM (H-MEM), Iscove's Modified Dulbecco's Medium (IMDM), Ham's nutrient mixtures (F-10 and F-12), Leibovitz (L-15), Roswell Park Memorial Institute (RPMI)-1640 and Glasgow MEM, (Lester, 2007; Arora, 2013). But apart from being a nutrient source, the media in use should also be sterile, isotonic, provide buffering capacity and maintain physiological conditions of culture. To achieve the correct pH and osmolarity, each type of media has a recommended bicarbonate concentration and CO₂ tensions. On the other hand, the HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffered medium is suitable, when culturing cells in atmospheric air to maintain cell viability. Therefore, selecting appropriate media for a particular cell type is required during cell culture, as their characteristics and compositions vary. For example, M-199 developed by Morgan et al., (1950), is used for culturing chick embryo fibroblasts. DMEM, a modified version of Eagle's medium (Eagle, 1955) and created by Dulbecco and Freeman (1959) is used to achieve high-density growth of cultured cells. In contrast, F-12 formulated by Ham (1965) is required for clonal growth of low-density cells whereas Roswell Park Memorial Institute (RPMI 1640) medium is recommended for lymphoblastoid cell lines. On the other hand, L-15, a medium developed by Leibovitz (1963), contains galactose and sodium pyruvate instead of glucose and the cells can be grown without CO₂. As the source of culture medium can have a major impact on the health and viability of cultured cells (Allen et al., 1985; Dodson and Mathison, 1988), each laboratory must determine the basal nutrient medium that suits best to the cell type depending upon the objective of culture (growth, survival, differentiation, production of desired proteins).

Other Additives in Media

Serum

Serum, a mixture of hundreds of proteins, is a traditional supplement for mammalian cell cultures (Paranjape, 2004). Serum not only acts a source of several essential factors, attachment factors, but also improves the spreading of cells on the plastic culture substrate (Holmes, 1967; Yamada and Olden, 1978) and provides nutrients and hormones stimulating their growth (McKeehan et al., 1976). Serum modifies physico-chemical properties (viscosity and osmolality) of the culture medium and helps in protecting labile essential nutrients in it (Ham, 1981). Besides, serum also maintains pH buffer (Barnes and Sato, 1980; Cheng et al., 1993) and inhibits activity of proteolytic enzyme such as trypsin.

Among different commercially available sera, fetal bovine serum (FBS) and newborn calf serum (NBCS) are most common and widely used in media to carry out cell culture work of various species (Wolf and Quimby, 1969; Amemiya et al., 1984; Ostrander et al., 1995). But due to high demand, limited availability and high cost, culture of cells using sera of other species such as goat, horse and chicken have also been done by several workers (Chaudhuri and

Chakravarty, 1983; Castillo et al., 1991; McFarland, 1992; Nanda et al., 2009). Although serum offers many advantages, its constituents vary from lot to lot and may contain inadequate levels of cell-specific growth factors and an over abundance of others which may be cytotoxic resulting in poor growth of cells (Cheng et al., 1993; Freshney, 2010). Further, there is a risk of contamination with infectious agents (viruses, prions, fungi and mycoplasma or degradative enzymes. To overcome the problems of cytotoxicity, contamination, non-availability and ethical issues, a wide range of serum-free, chemically defined, animal-derived component free (ADCF); protein free media are available (Hamilton and Ham, 1977; Barnes and Sato, 1980; Stoll et al., 1996; Zimmerman et al., 2000; Brunner et al., 2010) for use in cell culture. But these media, apart from being costly, require supplementation with special ingredients to function as cell nutrients (Whitford, 2005).

Amino Acids and Glutamine

The importance of amino acids in culture media for survival, proliferation and growth of animal cells of cells *in vitro* has long been recognized as cells cannot synthesize these by themselves (Ehrensvar et al., 1949; Eagle, 1959). Out of many amino acids available, glutamine is most commonly used as additive in cell culture and it serves as a secondary energy and carbon source for metabolism (Lane and Bennett, 1987; Newsholme et al., 2003). But L-glutamine is only stable for about 3 weeks at 4 °C in cell culture medium and converts to a form that cannot be used by cells. Further, its stability depends upon time, temperature, and pH of the solution. Therefore, most commercially available media are formulated with free L-glutamine, which is added to media just before use at varying concentrations (up to 4mM) depending upon with cell culture type and duration (Pasioka and Morgan, 1959; Arora, 2013).

Antibiotic and Antimycotic Solution

One of the major problems encountered during cell culture is contamination of culture from microbes and/or fungi, either during dissection and tissue preparation or subsequent culture (Vierck et al., 2000). As the fungi and bacteria grow fast, they can be evident through visualization due to a sudden increase in turbidity and change in colour of media, affect the growth kinetics, viability and cell numbers, deplete nutrients level, add non-cellular material resulting in cell lysis and death (Cobo et al., 2005; Dev et al., 2011).

Further, if culture is contaminated by mycoplasma, the organisms have the capability to alter the morphology, function, metabolism, and growth and attachment cells to the culture vessel (McGarrity, 1976). Apart from these, presence of chemical contaminants (impure media, serum or organic compounds) in the cell culture media uncleansed storage vessel, pipettes, glassware due to malfunction in the sterilization procedures may cause unfavorable effects to the cells (Freshney, 2010). As far as selection and isolation of cells from tissue or organ is concerned, the contamination risk is low only removed aseptically. But in general, external tissues and organs pose a greater risk of contamination whereas internal organs are considered sterile with the exception of the digestive tract (Lester, 2007).

To prevent bacteria and/or fungi contaminations during cell culture, apart from following aseptic techniques

and good laboratory practices, supplementation of the culture media with antibiotics and/or antimycotics is often done. A wide range of suitable preparations are available from relatively specific antibiotics, e.g., penicillin/streptomycin solutions, to broader spectrum antibacterial/antimycotic agents such as kanamycin or amphotericin B (Martinez-Liarte et al., 1995). However, the antibiotics chosen should not be toxic to the cells in culture and their application and concentration may be decided depending on the type of contamination experienced in the individual laboratory (Lelong-Rebel et al., 2009). But higher doses of antibiotics could alter the phenotype or genotype of the cells and their prolonged use often cause negative impact on cultured cells affecting the cellular DNA as well as protein synthesis process which in turn interfere with cell metabolic processes (Kuhlmann, 1995; Toullec, 1999)

Physico-Chemical Properties of Culture Media

The culture media should have optimum range of pH, oxygen, osmolality, temperature and adequate buffering system to support good growth and proliferation of cultured animal cells.

pH

Animal cells placed in a nutritionally complete tissue culture medium grow well when the medium is buffered at a pH in the range 7.2 to 7.4 (Williamson and Cox, 1967). However, a slight variation in pH level is essential depending on the type and origin of cells. For instance, some normal fibroblast cells require pH of 7.4 – 7.7, whereas transformed cells do better at pH 7.0 – 7.4 (Eagle, 1973). Although, Eisinger et al (1979) reported that at pH 5.5, epidermal cells could be maintained, but this level has not been universally adopted. On the other hand, most cultured cells stop growing when pH falls from 7.0 to 6.5 and start their losing viability between pH 6.0–6.5. In such cases, the medium should be changed (Freshney, 2010). To overcome this problem, it is important to measure the exact pH of the culture media. Further, as most culture media are buffered using bicarbonate, the pH level of the medium can be checked by a pH indicator like phenol red. Accordingly, the media may be changed / replenished, if its color turns yellow (acid) or purple (alkali) during cell culture. However, it is advisable not to use media with phenol red for studies using serum-free media formulations, as it is reported to interfere with the sodium potassium homeostasis. To neutralize its effect, serum or bovine pituitary hormone may be included in the culture medium (Karmiol, 2000).

Oxygen

Oxygen plays an important role in the physiology of cells by regulating their function, differentiation, and survival. Although cultured cells depend on the dissolved oxygen obtained from the surrounding medium to maintain metabolic activities, it is sparingly soluble in cell culture media. At higher concentration, dissolved oxygen inhibits the growth and metabolism of a number of cell types and may be toxic to cells due to the generation of free radicals. Additions of selenium and free radicals scavengers (glutathione) are reported to reduce oxygen toxicity (McKeehan et al., 1976). Further, proliferation of cells lowers the dissolved oxygen concentration in culture media, which in turn drops the pH due to release of lactic acid as a

by-product of cell metabolism (Hanson et al., 2007). From this it is clear that too much medium inhibits oxygen diffusion resulting in slow cell growth where as too little may lead to cell death. As both dissolved oxygen and pH are good indicators of cell growth, their optimum concentration or level should be maintained throughout the culture period as the rate of cellular growth and metabolism are strongly dependent on these two factors (Miller et al., 1988).

Osmolality

Osmolarity is an important process variable during *in vitro* cultivation of mammalian cells. Cell structure and function can be influenced by the osmolality of the cell culture medium. Therefore, maintaining an appropriate osmolality in the culture medium is required for proper growth and function of cells (Kruse and Paterson, 1973). It should be similar to the osmolality of the natural environment of the animal cells, in the range of 260–320mosmol/kg. Once an osmolality is selected for a medium, it must be maintained at that level (+/-10mosmol/kg), as the change in osmolality of culture medium causes detrimental effects on cell, either delay the cell growth or accelerate its cell death (Freshney, 2010). In case of any change in osmolality of culture medium due to addition of acids, bases to the medium; it should be measured using osmometer and adjusted accordingly.

Temperature

Like pH, optimum temperature (37 °C) is required for the proper growth of the animal cells. However, the incubation temperature depends on the species and type of cells being cultivated, as cells from certain cultures like skin may require lower temperatures (Unchern, 1999). At lower temperatures, although the growth is arrested, cells can survive at as low as 4 °C temperature. Lowering the culture temperature of cells reduces glucose and glutamine consumption, proteolytic degradation rates and improves their tolerance against shear stress (Chen et al., 2004). At higher incubation temperature (>37 °C), cultured cells lose their viability (Khaparde and Roychoudhury, 2012). The enzymes of the cell at such temperature denature or become inactive whereas at low temperature the CO₂ concentration increases decreasing pH level (Freshney, 2010), thereby influencing growth of cells. A consistency in the temperature of cultured animal cells is to be maintained to get desired results.

Buffering Systems

The purpose of a buffer in cell culture is to maintain pH within a very narrow range and resist changes in pH in when small amounts of acid or base are added. The buffers to be used in cell cultures must possess distinctive characteristics like high water solubility and minimal organic solvents solubility. Further, they should not permeate into cell membranes, exhibit any toxic effects towards cells and have pKa values at or near physiological pH (Mohan, 2003). The most commonly used buffer in tissue culture media is the bicarbonate and phosphate systems but in some cases, tris buffers are also in use (Eagle, 1971). Amongst these, bicarbonate buffer offers advantages because of its low toxicity, low cost and nutritional benefits to the culture and maintains pH near 7.4 (Freshney, 2010). Apart from maintaining pH, bicarbonate buffers also helps in glucose metabolism and proper functioning of ion and

acid–base transporters (Bonarius et al., 1995; Kanaan et al., 2007). But buffers when used with *in vitro* biological systems have their advantages and disadvantages. For example, cell culture using bicarbonate buffer (pKa of 6.3 at 37 °C) needs to be maintained at an appropriate temperature and gas mixture (typically, 37 °C, 5–10% CO₂) in a cell incubator. On the other hand, phosphates in phosphate buffer (pKa, 6.9 at 37 °C) form insoluble complexes and inhibit the activity of some enzymes whereas tris buffer (pKa of 7.9 at 37 °C is reported to be cytotoxic at higher concentrations (Williamson and Pox, 1967). As each medium has a recommended CO₂ tension and buffer concentration to maintain correct pH and osmolality, buffers should be tested for side effects, if any, on cells types before use (Coutu and Schroeder, 2013). To overcome the problems, a new range of zwitterionic buffers like N–tris (hydroxymethyl) methyl–2–aminoethanesulphonic acid (TES), N–2–hydroxyethylpiperazine–N’–ethanesulphonic acid (HEPES) has been developed which exhibit low interference with biological processes. These buffers, covering a pKa range from 6 to 8 are very soluble, have low binding capacities for divalent cations and are stable (Good et al., 1966). Amongst these, HEPES has superior buffering capacity (pKa = 7.3 at 37 °C) and does not require a controlled gaseous atmosphere (Shipman, 1969). But it is toxic to some cell types at higher concentrations and relatively expensive (Zigler et al., 1985). Further, media containing HEPES can be photoactivated, if exposed to normal fluorescent lighting, producing hydrogen peroxide and free radicals that are toxic to cells (Wang, 1976; MacMichael, 1986).

Growth Substrates

Attachment of cells to culture vessels is a critical step for *in vitro* culture of primary cells (Freshney, 2010). This is because most normal cells and explants are anchorage–dependent and therefore, attachment to a substrate is required for their replication and subsequent growth (Folkman and Moscona, 1978; Danen and Yamada, 2001). Although glass, because of its optical properties and surface charge, was the substrate or matrix of choice during early days, it has now been replaced by synthetic plastic, usually polystyrene. But due to its hydrophobic nature, these materials do not provide a suitable surface for cell attachment (Curtis et al., 1983). To overcome the problem, now days, these materials are treated either through wet chemical processing, plasma treatment (glow discharge, corona, and flame) or radiation treatment (ultraviolet radiation and laser treatment) to produce a charged wettable surface (Guruvenket et al., 2003).

Considerable progress has also been made for identification and characterization of specific matrix proteins (glycoproteins) like collagen, gelatin, fibronectin, laminin and serum spreading factors (vitronectin) that promote and/or influence *in vitro* cell attachment to the surface or substratum of the culture vessel to induce adhesion and rapid spreading of cells (Damsky et al., 1984; Crouch et al., 1987). These materials improve the adhesive properties of the culture flask bottom to which negatively charged cells attach (Freshney, 2010). This helps to produce an extra cellular matrix, facilitates attachment, proliferation and further spreading of the cells (Kleinman et al., 1987). Apart from these, the substrate can also be conditioned by

treating with serum, plasma or spent medium obtained from the same or another culture (Stampfer et al., 1980). Conditioning the surface of the culture dish with biocompatible materials like Silicon nitride also facilitate growth of cells (Medina Benavente et al., 2014). However, the culture substrate or other biomaterials used as substrate should be compatible and not exert any adversely effect on the cells through leaching of toxin (Laluppa et al., 1997).

In recent times, there is a growing interest for culture of cells in three dimensional (3–D) system within a polymeric matrix (also termed scaffold) as these materials mimic the *in vivo* conditions and increase cell–cell interactions compared to normal two dimensional cell culture (Abbott, 2003; Harrington et al., 2013). Various synthetic biodegradable polymers including poly–lactic acid (PLA), polyglycolide (PG), and their co–polymers are most widely used in the fabrication of polymeric scaffolds (Mikos et al., 1993; Mikos et al., 1994a, Mikos et al., 1994b; Carletti et al., 2011). Amongst these, polymeric scaffold materials (PLA) blended with natural polymers like alginate, gelatin and dextran have been explored with success for adhesion, attachment and proliferation of fibroblast cells in primary culture by Nanda et al (2014).

Growth Factors

Growth factors are special polypeptides with stimulating effects on growth and division of certain types of cells. They act as signals and facilitate cell proliferation and differentiation mechanism (Tingjun et al., 2003). Cells from multicellular organisms are dependent upon exogenous signals. But growth factors are present in negligible amount in the nutrient medium (nanogram and pictogram level per millilitre of serum). So, they have to be added in the media for survival, growth, and proliferation to promote *in vitro* proliferation and differentiation of specialized cells. Over the years, considerable attention has been devoted to the role of peptide growth factors in the regulation of poultry and mammalian cell growth (Cyrino and Mulvaney, 1999). Notable major peptide growth factors (GF) are fibroblast GF, epidermal GF, platelet–derived GF, vascular Endothelial GF, Insulin GF (IGF–1, IGF–2) and transforming GF (Florini and Magri, 1989; Ewton and Florini, 1990; Florini et al., 1991). These growth factors are mostly used in serum–free media and act either individually or synergistically with each other, or with other factors like proteins (bovine serum albumin), polyamines, hormones (insulin and hydrocortisone) and nutrients (cholin, linolic acid etc.) to promote *in vitro* proliferation and differentiation of cells (Freshney, 2010).

Limitations

Although media, nutrients, physico–chemical, growth & attachment factors play an important role in establishment of cell culture and subsequent subcultures; these may or may not give rise to an established cell line. It is because of the fact that normal cells in primary culture do not proliferate indefinitely but instead are mortal and can only divide a limited number of times and die out after a fixed number of population doubling. This is known as the Hayflick limit. Often referred to as crisis, the cells enter the state of senescence whereby cells move from actively dividing to a non–dividing state (Bols and Lee, 1994; Crane, 1999). This stage is determined by a number of intrinsic

factors regulating cell cycle, such as pRb and p53 (Munger and Howley, 2002), and is accompanied by shortening of the telomeres as almost all cells and tissues, with the exception of post-mitotic cells show progressive shortening of telomeres with increased age (Wright and Shay, 2002; Ben-Porath and Weinberg, 2004). As a result, the protective ends of the chromosomes, the telomeres, gradually shorten with each cell cycle and when a critical telomere length is reached, the cell is unable to divide and the cell enters an irreversible state of quiescence (Barker et al., 2000). This is thought to involve the tumor suppressor gene p53, which arrests cell cycle progression (Kuilman et al., 2010). Cells that overcome the senescent state and have the potential to divide indefinitely in culture are referred to as immortalised (Crane, 1999). Therefore, for a cell line to become established or continuous, it must undergo transformation or immortalization. This can be induced through chemical mutagens, infection with a transforming virus or through transfection (Takarada et al., 1989; Bodnar et al., 1998; Guo et al., 2003; Butler and Nowak, 2004). Immortalization of cells or development of continuous cell line from normal cells requires telomere maintenance apart from optimal culture conditions as pointed out by several researchers (Mathon et al., 2001; Herbert et al., 2002; Wright and Shay, 2002). From this, it is clear that media, attachment factors, growth factors and other supplements simply optimize the conditions for cell proliferation and provide more opportunities to develop a continuous cell culture, but do not ensure development of a cell line from normal tissue.

Cell Line Database

- Cell-culture Database : <http://cell-lines.toku-e.com>
- Databases On Medicine And Molecular Biology: <http://www.meddb.info>
- Cell Line Data Base (CLDB): (HyperCLDB) : <http://bioinformatics.istge.it/cldb/cldb.php>
- Bio-Information Web A complete information of Web-resources of Biology : <http://bioinfoweb.com/CLDB-list-Y.htm>
- American Type Culture Collection (ATCC): <http://www.atcc.org>
- Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ): <http://www.dsmz.de>
- European Collection of Cell Cultures (ECACC): <http://www.hpacultures.org.uk/collections/ecacc.jsp>
- Japanese Collection of Research Bioresources (JCRB): <http://cellbank.nibio.go.jp>
- RIKEN Bioresource Center Cell Bank: <http://www.brc.riken.go.jp/lab/cell/english/g>
- AltTox. Org Non-animal Methods for Toxicity Testing: <http://www.alttox.org/ttrc/resources/databases.html>

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