

## Review Article

# Inferences of Carbon Dioxide in Present-Day Cell Culture Systems: An Unacknowledged Problem and Perspectives

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## Abstract

The impact of cell culture technology has been immense in the field of research and pharmaceutical sciences. Suitable environmental conditions are paramount need in order to maintain the cell line in an *in-vitro* condition. Since the biological processes are highly susceptible to acid-base chemistry, variations among the factors will have a severe effect on cellular integrity that often leads to hypercapnia and senescence of the cells. pH regulation in the cell culture environment is a fundamental biological phenomenon of great significance for the growth and metabolism of cells. This review focuses on the implications of the two critical factors carbon dioxide (CO<sub>2</sub>), pH, and their correlated effects in the mammalian culture system. Bicarbonate buffer plays a vital role in maintaining homeostasis as dissolved CO<sub>2</sub> hydration occurs in bicarbonate (HCO<sub>3</sub><sup>-</sup>) and H<sup>+</sup> equilibriums. If pH is not controlled, inhibition of CO<sub>2</sub> causes acidification in the medium. In contrast, if pH is not regulated by integrating essential requirements, the equilibrium reaction shifts to the right towards the hydrogen ions that maintains the balance. Despite a few literatures exhibiting the role of carbon dioxide in cell culture, the present review distinguishes from them by showing the effects of CO<sub>2</sub> in the *in-vitro* environment in maintaining the pH balance and cellular integrity. Thus, ensuring the proper mechanism of the utilization of CO<sub>2</sub> and pH in the cell culture system will undoubtedly lead to the exploration of enormous concepts in the present crucial study.

**Keywords:** Hypercapnia; Senescence; Cellular metabolism; Bicarbonate-buffering system; Cell density; Intelligent fail-safe system

## Introduction

The condition of growth is major characteristics of living organisms and impersonating this technology *in-vitro* could be possible through tissue culture, which requires appropriate environmental conditions and culture medium in either liquid or solid form. Growth of tissue or cell separation of an organism is capable of evidently growing in *in-vitro* conditions from living tissues. Various researchers are trying to give presentable work in the field of animal cell cultures [1]. Primary cells proliferating from organs or tissue in the *in-vitro* environment resemble the parental cells, which reflect changes in the growing cells, organs, or tissues. Cell proliferation requires a suitable environment and type of media for culturing, and with the right constituents, the process becomes more accessible to culture, which is meant to achieve [2].

Suitable environmental conditions are significantly responsible for cell maintenance and growth. Cell culture technique has a considerable advantage to manipulate and reproduce under optimum environmental conditions [3]. In a simulated environment, the cell culture system involves mainly factors such as pH, ideal gas, temperature, and humidity, which allows cells to reproduce and grow. However, primary cells are typically preferred over continuous cell lines due to their physiological similarity in correspondence to the cells from living tissue. Furthermore, continuous cell lines undergo specific phenotypic and genotypic changes that lead to discrepancies during analysis. The cells obtained from living tissue in resemblance of primary cells, necessary for research can be studied

for their functions, development, defects, metabolic regulations, cell physiology, and conditions affecting the tissue of interest [4].

While working with human tissue, which is frequent pathogenic, biosafety, is essential, especially with the range of equipment required in carrying out the isolation of cells; purification and culture, as described for tissue culture laboratory. However, precautions are required to avoid infection of species by microorganisms in order to preserve aseptic conditions. Understanding basic cell-culture handling techniques, including suitable choice of culture media, and cryopreservation can be an essential factor in maintaining cultures. Thus, culture techniques can be significantly employed for screening drugs, disease diagnosis, and the development of vaccines [5]. Despite the plethora of research articles published in correlating the CO<sub>2</sub> and the cell culture system, there is no review so far present in the literature that describes the critical phenomenon of the effect of CO<sub>2</sub> in the *in-vitro* environment in maintaining the cellular integrity of the cells in our knowledge. The idea of this study was considered when we observed in the growing fibroblast cell lines under experimental conditions with the variation of CO<sub>2</sub> levels inside the incubator. As the level of CO<sub>2</sub> dropped below the optimum value of 5%, there were physiological changes observed in the morphology of the cell lines that finally lead to the loss of the adherence property. The color of the medium was also observed for the changes with variation in the CO<sub>2</sub> levels, which could be due to the variation in the pH, which is inter-correlated. This review emphasizes the correlative study of these two factors inside the cell culture system and their effects *in-vitro*.

**Table 1:** Basic components of media for cell culture.

S. No	Constituents	Functions	Source
1	Amino acids (L-glutamine)	Acts as the initial precursor for protein synthesis and proliferation of cells.	[12]
2	Inorganic salt (Ca <sup>2+</sup> , Na <sup>+</sup> , K <sup>+</sup> )	Retains the osmotic balance.	[13]
3	Proteins and peptides (Albumin and Fibronectin)	Protein in the blood helps to bind water, salts, free fatty acids, hormones, and vitamins, and transport them between tissues and cells.	[14]
4	Antibiotics (Penicillin, Streptomycin)	Used to control the growth of microbes and fungal infections.	[15]
5	Vitamins (A, D, E and K)	Acts as co-factor and helps in the growth of cells.	[16]
6	Growth factors	Promoting cell differentiation and division.	[17]
7	pH indicator (Phenol red)	For constant monitoring of media.	[18]
8	Buffer solution (HEPES)	Regulating pH, which is critical for optimum culture conditions.	[19]
9	Natural buffer (CO <sub>2</sub> )	For balancing CO <sub>2</sub> /HCO <sub>3</sub> <sup>-</sup> , content in the cell culture medium.	[19]

## Cell Culture Media

Cell culture media has significant advantages in the manipulation of the physiological environment (hormone and nutrient concentration) and physicochemical conditions (pH, temperature, O<sub>2</sub>, osmotic pressure, and CO<sub>2</sub> tension) for cell proliferation. For both growth and maintenance, proper cell culture environment is a necessity [1]. For the cells to grow *in-vitro* and maintain their life, it is essential to get it supplemented with all types of nutrient media. The balanced salt solution contains a proper mixture of nutrients to regulate optimum osmotic pressure and provides essential nutrition to the cells. The components of the cell culture medium include inorganic salts, serum, glucose, vitamins, and amino acids. The serum is used as growth factors, hormones, and attachment factors [6]. In the enzymatic reactions, metal ions act as co-factors and improve cell adhesion. Phenol red used as pH indicator, at the range pH 7.0-7.4 changes from orange (red) to yellow in acidic conditions and turn to purple in basic condition. The buffer solution used for pH balancing products, e.g., the Bicarbonate/HEPES buffer for normal growth conditions, retains precise parameters such as temperature (37°C), CO<sub>2</sub> supplement (5%) and relative humidity (95%) [7]. Transformed cell lines can see better growth at the slightly acidic condition at pH 7.0-7.4 and, whereas the basic state, substantiates a pH range of 7.4-7.7. Carbon dioxide regulates the pH in the medium, and balances bicarbonate (HCO<sub>3</sub><sup>-</sup>) and changes in the atmospheric CO<sub>2</sub>. A concentration of 5% of CO<sub>2</sub> is usually maintained in the air, and 10% of CO<sub>2</sub> is mostly used for experimental purposes [8].

Carbon functions as a source of energy, such as glutamine/ glucose. Organic compounds such as amino acids contain carboxyl (-COOH) and amine (-NH<sub>2</sub>) as a functional group along with the side chain [9]. Each amino acid contains R-group as specific [10]. The amino acid is the main component of the protein-synthesizing process. To promote the building blocks of proteins, it must contain vital elements of amino acid such as N<sub>2</sub>, C, and O<sub>2</sub>. For many enzymes, vitamins act as co-factors, and its function is essential in advancing cell growth and survival. Cell death or loss of productivity occurs due to absorbed vitamins. e.g., Vitamin-C, Vitamin-B2, Vitamin-B6, Vitamin-B7, etc. Basal media contains serum, as a source of growth and adhesion factors regulating the permeability of cell membranes and acts as a carrier of proteins, enzymes, lipids, micronutrients, and some trace elements [11] (Table 1).

## Factors Affecting Cell Culture

### Cell density

Cell density is considered to regulate the amount of cell differentiation in culture [20,21]. The transport of minor nutrients relies solely on the density of the cells and decreases as the density of cells increases and vice versa [22-24]. The relationship between cell growth and nutrient transport rate is, however, less clear. One of the critical events supporting superlative cell proliferation is backed by enhanced membrane permeability for vital nutrients [25,26]. However, rises in the absorption of low molecular-weight nutrients are correlated with growth initiation. At the same time, some of these transport improvements are not causally related to differences between quiescent and proliferation. [27,28].

### Media exhaustion

A media requires essential nutrients, hormones, and growth factors (serum), as well as osmotic pressure and pH maintenance for the cultural approach of medium affecting growth [1]. Once the organisms adhere to the cells, they grow faster and easily visible under the microscope. However, media, which includes serum, has some disadvantages such as specificity, standardization, high expense, stimulus or cellular activity, and growth inhibition. These downsides contribute to unintended results in cell culture, posing a considerable difficulty in performing cell culture experiments if the serum is not from the particular source, and can eventually lead to contamination [29]. Thus, contamination leads to changes in the color of the medium because of turbidity and changes in the pH, invariably causing cell death [30].

Serum factors protect cells from general strain induced by shear forces, air bubbles, and toxic components operates in minute quantity identical to metal ions and oxygen radicals [31,32]. Antibiotics suppress the contamination, which would change the cell morphology and genotype. In biochemical experiments, antibiotics are found lethal to cells. The cultures found contaminated in the laboratory are reported to be harmful owing to the removal of antibiotics. Hence, it is imperative to protect the cell from any type of contamination occurring, such as air, temperature, light, etc. as it is irreversible in the culture [33].

### Cell deterioration

Cell death may induce deterioration, which is the degradation of cultured cells; the frequent change in the required media could

**Table 2:** pH range shows different colour changes.

S. No.	Media colour	pH range
1	Phenol red to yellow	Below 6.8 (too acidic)
2	Red to pink	Above 7.0-7.7 (normal)
3	Bright fuchsia	Above 8.2 (too alkaline)

include toxic substances [30]. Mostly primary cell culture is subjected to decay; threats are attributed to the unobserved microbes. However, the cultured cells should be adequately maintained to avoid risks of infections [34].

Unfavorable impact on cells in culture can attain through a variety of biological and chemical contaminants, ranging from destruction of the culture, phenotypic changes to relatively minor changes in morphology, mutation, or growth rate. In cell cultures, there are divergent approaches to detect and lessen the risk of biological or microbial contaminants [35]. Improper handling, glassware, or other types of consumables or sourcing of cell culture reagents are the familiar sources of chemical contaminants. The matter of chemical contamination reduces through best practices in sourcing and handling of such materials and by keeping away the use of volatile solvents within incubators while maintaining cell cultures [36]. Environmental conditions include microorganisms, most of which are bacteria, the reason why laboratory experiments/instruments, such as garments or gloves, may contribute to cell contamination [30].

## pH

Normally, cell lines can be grown well at pH 7.4; among the cell lines, strains will differ, at very less variability. However, better growth is observed in transformed cell lines under acidic conditions such as pH 7.0-7.4. In basic state at pH 7.4-7.7, it prefers fibroblast cell lines typically [37].

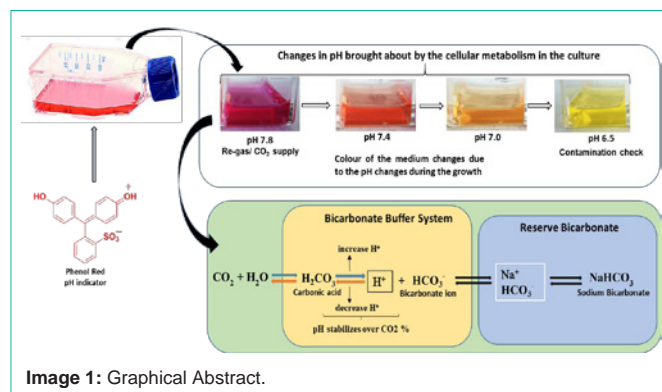
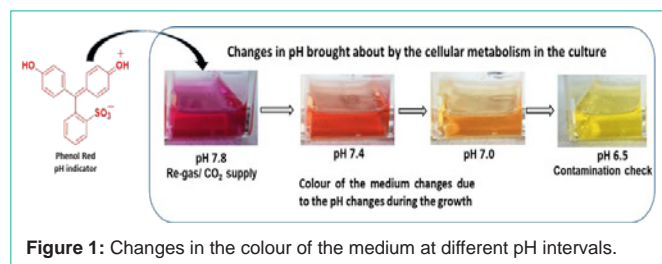
The medium changes color during cell growth when pH varies due to the metabolites produced by the cells. A phenol red indicator is commonly used as a balance to the media for cell culture, which reflects shifts in pH in the medium when the color changes to yellow hue in the very low pH (acidic) range [6] (Figure 1 and Image 1). When pH is too high (alkaline), its color shows purple, whereas, under the proper physiological condition, the pH color appears to be in orange-red hue [38] (Table 2).

## Temperature

Without an ideal temperature, it is challenging to grow cells in the culture medium, while supplementation with the required nutrients and ideal temperature will facilitate cytogenesis; thus, temperature plays a crucial role in cell development [39]. As temperature rises, cells often proliferate, and enzyme molecules can speed up metabolism by moving quickly. At a certain point, the value of all these activities, such as denature of enzymes and co-factors, cell confluence, detrimental effect (damage), and high rate values, leads to the cease of cellular growth [40]. In the isolation medium, cells require a host primarily to maintain cell culture as the optimum temperature is necessary [41]. Each species has its optimum temperature for the division that defines the character containing limits in microbes, as of that species maintained for growth. Cellular metabolism functions at the aspect of their optimal values of heat to which the ability of cells are increased

**Table 3:** The optimum temperature for various cell lines.

S. No	Type of cell line	Source	Temp. (°C)	Reference
1	<i>Spodoptera frugiperda</i> (sf-21)	Insect	27	[41]
2	<i>Trichopusia ni</i> (Tn-5)	Insect	27	[41]
3	Chinese Hamster Ovary (CHO)	Mammalian	36-37	[43]
4	Human embryonic kidney (HEK-293T)	Human	37	[44]
4	HF 205 and HF 210	Plant	25	[45]
5	<i>Spodoptera frugiperda</i> (sf-9)	Insect	27	[46]

**Image 1:** Graphical Abstract.**Figure 1:** Changes in the colour of the medium at different pH intervals.

in their size and divide rapidly [42] (Table 3).

## Buffering system $\text{CO}_2/\text{HCO}_3^-$

$\text{CO}_2/\text{HCO}_3^-$ , usually used as a buffering system in the media, is preferred as a physiological and biological approach in the research. The exogenous buffer may have undesirable consequences to prevent, for example, long-term  $\text{CO}_2$  gradient toxicity regimes a realistic transmembrane in the buffer such as glycolytic medium stimulus in  $\text{Ca}^{2+}$  binding receptors to produce  $\text{CO}_2$  in cells. [40,47-49]. Cellular homeostasis is responsible for membrane transport, whereas the  $\text{HCO}_3^-$  activates essential membrane ions additionally [50,51]. However, behavioral changes by the transduction mechanism modulate the extracellular pH as its sensitivity influences steady-state intracellular pH [43].

## Effect of $\text{CO}_2$ in Growth

In industries, cell culture reactors with carbon-dioxide maintain partial pressure of 150-200 mmHg, for inhibition of cell growth significantly, resulting, a proportional increase in osmolality by increased partial  $\text{CO}_2$  at constant pH, due to balance with bicarbonate ( $\text{HCO}_3^-$ ). The utilization of specific glucose and lactate production at 140mmHg of partial  $\text{CO}_2$  ( $\text{pCO}_2$ ) lowers by 40-50 %. At higher  $\text{pCO}_2$ , the constraint of glycolysis develops because of lower by-product levels and nutrient residue (higher) at lower cell density. It is

correlated with oxygen intake and cell-specific glucose increase. Thus, the culture system varies with the evident effects of  $p\text{CO}_2$  [52].

The production of  $\text{CO}_2$  occurs through catabolic reactions for the required synthesis of fatty acids, pyrimidines, and purines. Media generally consist of vitamins, amino acids, salts, and other substances, such as  $\text{NaHCO}_3$ , as an organic buffer substance. A balanced and stable pH value is required for the growth of cell cultures. Cell type varies from one cell type to another at the value of pH ideally during culturing. In an atmosphere of 5-10 %  $\text{CO}_2$ , cells are grown using buffered media with  $\text{NaHCO}_3$  (sodium bicarbonate) and with ideal maintained pH 7.2-7.4 [1].  $\text{CO}_2$ - $\text{HCO}_3^-$  buffer acts as a base medium in this condition, as pH is dependent on the dissolved  $\text{CO}_2$  and the delicately balanced medium, which is responsible for altering the pH medium as changes occur in the atmospheric  $\text{CO}_2$  [8]. Depending on the amount of concentration, the corresponding amount of  $\text{CO}_2$  is gassed into the selected medium, and the supply is monitored to continue throughout the cell culture process for the required pH stability. The pH change in the medium against buffers controls cell growth [53].

Hydration of dissolved  $\text{CO}_2$  occurs in equilibrium with  $\text{HCO}_3^-$  and  $\text{H}^+$ . If pH is not controlled, acidification will cause in the medium by inhibition of  $\text{CO}_2$ . If pH regulates by incorporating basic conditions, it shifts in the equilibrium reaction towards the right; in this case,  $p\text{CO}_2$  increases with osmolality [52] (Figure 2). The detrimental effect of cells shows high osmolality. However, the production of  $\text{CO}_2$  information is relatively studied in cultured cells [54,55].

## Role of $\text{CO}_2$ Incubator in pH Stability

$\text{CO}_2$  incubator is used for the culturing of cells for experimental purposes and maintains optimum pH under a sterile environment providing optimum temperature and moisture. In the medium, the carbonate buffer supports the constant pH release along with  $\text{CO}_2$  gas in the incubator. When the color changes in the media, changes can be seen in pH, indicating that the altering of  $\text{CO}_2$  levels has occurred [1]. Usually, 4-10 % of  $\text{CO}_2$  is used in the cell culture, and 5-7 % of  $\text{CO}_2$  is used in air. For the proper pH and osmolality to achieve, the medium has to maintain  $\text{HCO}_3^-$  concentration and  $\text{CO}_2$  tension [56]. For example, if two or more people use the same incubator, then there will be a slight disturbance while opening and closing the door. The chamber interior affects the parameters of  $\text{CO}_2$  concentration, temperature, and humidity; even if the door is kept open for about 30 seconds. The standard atmosphere (0.3%), which is attained with an immediate drop in  $\text{CO}_2$  concentration, shifts the pH value by itself within the growth medium. However, within a few minutes, the disturbances can remunerate rapidly for the climate balance. The reason for a highly adaptive and dynamic system with an IR sensor or adverse reactions in cell growth can appear when the system does not prevent it immediately. Measurement of the atmospheric air moisture is independent at an accurate time, and immediately  $\text{CO}_2$  contents are regulated [57].

Usually, after disturbance of air, it reduces relatively at the buildup humidity, in such conditions as often-opening doors or any other exposure to physical condition. However, the IR sensor with BINDER CB technology results in rapid recovery at times, and pH value ranging remains neutral. This phenomenon increases by

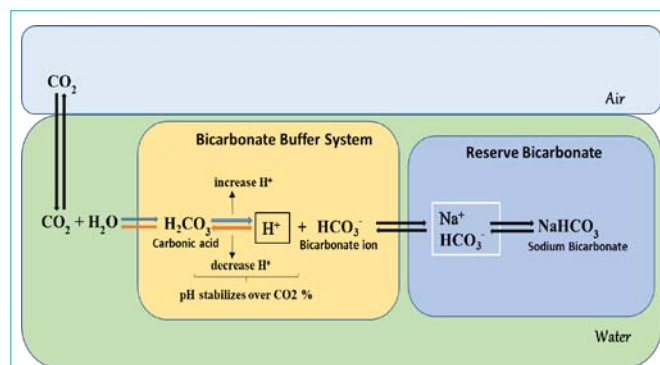


Figure 2: Mechanism of  $\text{CO}_2$  buffering system in cell culture.

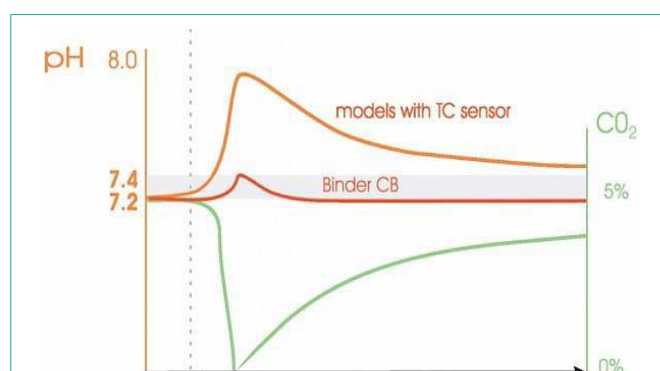


Figure 3: Rapid stabilization of the pH with an IR sensor vs. long recovery times in models with technologies for thermal conductivity sensors.

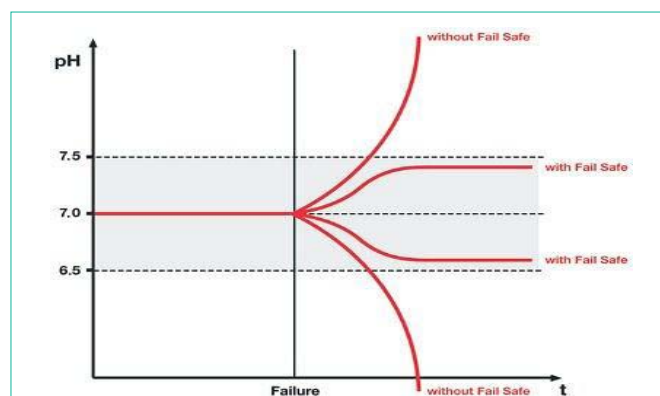
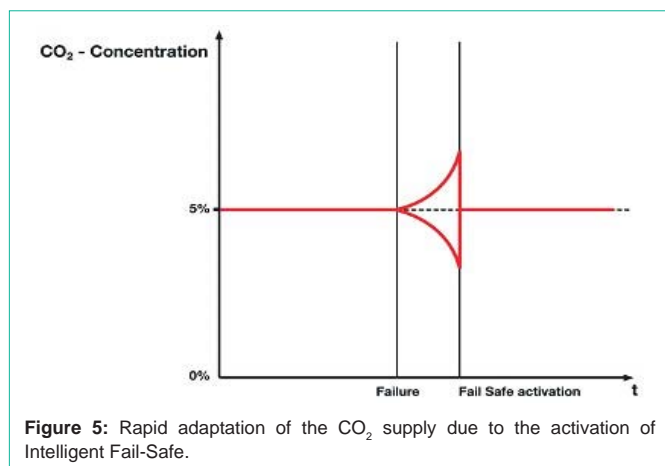


Figure 4: Possible consequences of a  $\text{CO}_2$  supply disturbance on the pH value of cell culture: the pH value may change to the alkaline range with a limited supply of gas (low  $\text{CO}_2$ ) or to the acid range with an excessive supply of gas (high  $\text{CO}_2$ ). Intelligent fail-safe ensures rapid harmonization.

opening the door. The kinetics explains the reaction of cell cultures when there is a disturbance in  $\text{CO}_2$  concentration. In an incubator, regulation of  $\text{CO}_2$  supply always is interpretative to cell safety. Above the tolerance limits, it is crucial to stop shifting the pH value into the alkali-acidic range during the long cultivation process at any time [57] (Figure 3).

Adverse effects in culture growth are attained after 4 hours post, which a downturn in the cell division as observed and can lead to cell death. Nevertheless, problems can occur when  $\text{CO}_2$  content changes are unnoticed in the incubator. In this regard, (Binder)



**Figure 5:** Rapid adaptation of the CO<sub>2</sub> supply due to the activation of Intelligent Fail-Safe.

Intelligent Fail-Safe System may be the right solution, which may prove irreversible when there is no one in the lab. In the standard incubator, gas consumption information is stored to obtain necessary CO<sub>2</sub> concentration and comparison of actual gas supply done with saved value consistently, during the operating the system [57] (Figure 4).

Intelligent Fail-safe controls the CO<sub>2</sub> supply where it is too much or less gas difference in injecting CO<sub>2</sub> and attains back to its original concentration value. Any changes in the gas supply, the system reacts immediately, signals before under concentration, or over-concentration of CO<sub>2</sub> is triggered (+ or - target value) and rapidly controls the pH value (Figure 5). The reliability of the pH value is ensured in all situations, including after a controller or sensor defect. The Fail-Safe device is referred to as “Intelligent” because it tracks the production of CO<sub>2</sub> individually and continuously throughout the process [57].

### Effect of CO<sub>2</sub> in Cellular Metabolism

Each cell has its mechanism and transport route in an organism, and the cells function effectively and efficiently when cellular respiration occurs appropriately in organisms [58]. The process of dilution of gas with the lipid bilayer occurs by a passive diffusion process. The gastric glandular cells in plants are impermeable to carbon dioxide; specific structures like aquaporin are found in the plasma membrane and the inner chloroplast membrane act as carbon dioxide (CO<sub>2</sub>) channels [59].

In eukaryotes, the role of carbon dioxide is very crucial in maintaining the plasma pH homeostasis via the buffering capability of carbonic acid. The elevated carbon dioxide levels lead to a condition called hypercapnia, which is the result of an imbalance in pH, leading to pathophysiological effects. Such effects appear at the organismal and cellular levels resulting in a varied response in many tissues, including lungs, kidneys, the central nervous system, the peripheral nervous systems, muscles, and blood cells [60].

The mechanism, such as glycolysis, protein synthesis, DNA synthesis, and exocytosis, a secretion, alter the proliferating cells' internal pH [61]. However, in an *in-vitro* experiment, which involves the growth of cells outside the body of the organism, high dissolved carbon dioxide [dCO<sub>2</sub>] concentration, can lead to a significant

problem causing growth inhibition, metabolic changes, and reduced productivity in mammalian cell culture [46]. The stabilization of the cytoplasmic pH in mammalian cells is controlled by the exchange of sodium and hydrogen [Na<sup>+</sup>/H<sup>+</sup>] and the presence of HCO<sub>3</sub><sup>-</sup> transport. Various enzyme systems, which are yet unknown, control the ionization states and activities, thus, affecting the pH. The development of the sophisticated techniques, which is involved in the measurement of the intracellular pH, such as microelectrodes [62,63], fluorescence [64-67], and nuclear magnetic resonance [68], made the understanding of the regulation of intracellular pH and its proliferation in cells very clear. However, many new dilemmas remain unknown.

A typically dCO<sub>2</sub> level in a high-density mammalian cell perfusion culture reaches as high as 200 mm Hg [69]. On the other hand, a dissolved carbon dioxide concentration of ca. 38mm Hg (5%) is adequate for mammalian cell growth [70]. Hence, the proliferative control of the pH is responsible for the characteristic phenotypic expression by the cells.

### Implications on Other Environmental Factors

In mammalian bioprocess, the respiratory product is CO<sub>2</sub>, which is, consequently, unavoidable. Cellular activity depends on 15-20 % of aeration, in which CO<sub>2</sub> fraction flow in the exhaust gas. Bioreactors filled the height up to 10-15 m and 0.5-2 bar with overpressure microbial fermentation, taking into consideration the 1-1.5 bar hydrostatic pressure. The pCO<sub>2</sub> value hits at 0.1-0.6 bar at the bottom of the bioreactors. Notably, such costs decrease if fresh air is correctly collected [41].

The experimental data revealed that the effects of biomass, growth of substrate yields, cell division, product development, and morphology reported changes in osmolality in the media with co-effects in combination, which were due to the elevated pCO<sub>2</sub> pressure point [71]. Individual phenotypic studies observed the rate of fundamental growth kinetics of strains showing a decline in the count of insect cells, as high dissolved CO<sub>2</sub> concentration were present, due to which growth was inhibited rather than cell death, as 90% of the cell were viable all times [41].

In large-scale bioreactors, cells frequently circulate when pCO<sub>2</sub> level changes, i.e., by pseudo-stationary scale is usually not stimulated. The cultivated mammalian cells are limited in contrast to the microbial application owing to the small scale of bioreactors and cell operation [72]. Many types of research have studied on elucidating of pCO<sub>2</sub> levels on microbes and mammalian cultures [42,73].

Monoclonal antibody development by hybridoma cell culture, performed at the industrial level, can achieve high cell density in large-scale reactors, a problem induced by CO<sub>2</sub> accumulation. The elevated level of pCO<sub>2</sub> is present with or without compensation of osmolality. Well, the synthesis of an antibody in plate cultures consists of glucose metabolism, and death in hybridoma cells, which is the consequence of a rapid increase in osmolality and partial pressure of carbon dioxide (pCO<sub>2</sub>), which often involve improvements in oxygen consumption. For further characterization, batch and continuous experiments are carried out in controlled bioreactors [52].

## Significance and Future Directions

Cell culture plays a crucial role in modern-day research, and thereby understanding the mechanism of the gross complexity within an organism can be simplified and easily understood using this technique. A significant role played by a researcher is observing in maintaining of an uncontaminated/less contaminated zone during the flow of the protocol. Following points may be of great significance in this regard:

- The mechanism of understanding the gross complexity within an organism can be simplified with the usage of an *in-vitro* setup of cell culture.
- The replication of sustainable environmental conditions plays a crucial role in modulating the changes desired for the growth, proliferation, or differentiation depending upon the need for an experiment.
- The usage of cell lines implicates and plays a crucial role in mimicking an *in-vivo* model and helps in understanding the modulations and changes effectively than a primary culture.
- The constitutive role of a CO<sub>2</sub> incubator is seen as a limiting factor in ensuring the maintenance of the pH and active development of the cell cultures.
- An approach to maintain a healthy and ethical culture is to practice a proper laboratory handling and media preparation protocol, ensuring a contaminant free media with an optimal seeding density to prevent exhaustion of nutrients for the cell.
- Along with the culture media, temperature, humidity, seed density of a particular cell type pH, and CO<sub>2</sub> along with the buffering system involved plays a vital role in the regulation of the variability among the cells. CO<sub>2</sub> incubator achieves the osmolality, pH, and maintaining moisture content.
- The cellular metabolism and production of competent metabolites carbon dioxide are essential. Alternation in it can result in pH imbalance leading to acidification and changing the osmolarity.
- The development of sophisticated techniques like NMR, microelectrodes, and fluorescence enables in the measurement of intracellular pH and requires a gross study to understand the unknown process yet to be discovered.

## Conclusion

The review portrays a descriptive organization of data upon the limitations of the use of carbon dioxide in an *in-vitro* condition. For the effective functioning of many tissues and cells, carbon dioxide is an essential factor; an attempt was made on the brief comparison of pCO<sub>2</sub>, dCO<sub>2</sub> and high levels of CO<sub>2</sub> upon the seeding density of the cells. Their role is least described in many reports and studied. The data collected regarding the protective effects of hypercapnia and efficiency of the carbon dioxide are less explored aspects, and hence a need to understand the process is required. An elaborate study focusing on the importance of adequate percentage (5%) pCO<sub>2</sub> to maintain cell culture and metabolic influx in an *in-vitro* system is lacking. In order to attain an understanding of cell physiology and develop cell culture processes, the emphasized areas need to be pondered upon and experimented, which would unravel various

aspects, which remains unexplored.

## Highlights

- Variations among the factors in the cell culture environment affect both the cellular and the physiological integrity of the cells.
- Reduction in cellular growth with the variation of CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> is a regular phenomenon in several biological processes, susceptible to acid-base chemistry.
- CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> levels limit anaplerotic reactions inside the biological systems.
- Imbalance in pH due to variation in CO<sub>2</sub> levels inside the cells leads to hypercapnia that integrates the pathophysiological effects.

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