HAMILTON


## DISSOLVED $CO_2$ SERIES:

# Should $CO_2$ Be a Critical Process Parameter?

White Paper


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# Abstract

Dissolved carbon dioxide ($CO_2$) is a Critical Process Parameter (CPP) in biopharma production processes according to PAT guidelines. By influencing other parameters such as extracellular and intracellular pH, it has an effect on different metabolic pathways which are involved in cell growth or in product formation and quality. Carbon dioxide in elevated concentrations can, for example, inhibit mammalian cell culture growth and reduce the titer of their products (e.g. monoclonal antibodies or viruses), as well as undermine their therapeutic effectiveness (e.g. mAbs with undesired glycosylation patterns). Too high concentrations of $CO_2$ show detrimental effects on microbial fermentations too. Carbon dioxide must be controlled in real-time within the proper range of saturation for each specific bioprocess application. As a CPP it has to be controlled in real-time because it influences Key Performance Indicators (e.g. viable cell density, product titer) and Critical Quality Attributes (e.g. glycosylation patterns). This white paper details the complex role of $CO_2$ in the bioreactor and the control strategies that can be used to achieve the desired level in upstream processes.

The paper is divided into three chapters. The first lays the foundation by describing the many roles of $CO_2$ in a bioprocess and how it develops throughout the process. The second chapter analyzes why dissolved $CO_2$ is considered a CPP for different cultures and fermentations. Finally, the third chapter provides an overview of how to control dissolved $CO_2$ in real time and how doing so can improve bioprocess performance.

**Keywords:**

Dissolved Carbon Dioxide, $CO_2$, $pCO_2$, Bioprocesses, pH Control, Mammalian Cell Cultures, Microbial Fermentations, CHO, Vero Cells, Microbials, Viable Cell Density, Product Titer, Lactate Shift, Osmolality, PAT, Critical Process Parameter, Real-Time Control, CPP, KPI, CQA, mAb, Vaccines, and Glycosylation.

# 1. $CO_2$ Fundamentals: The Cell Culture Example

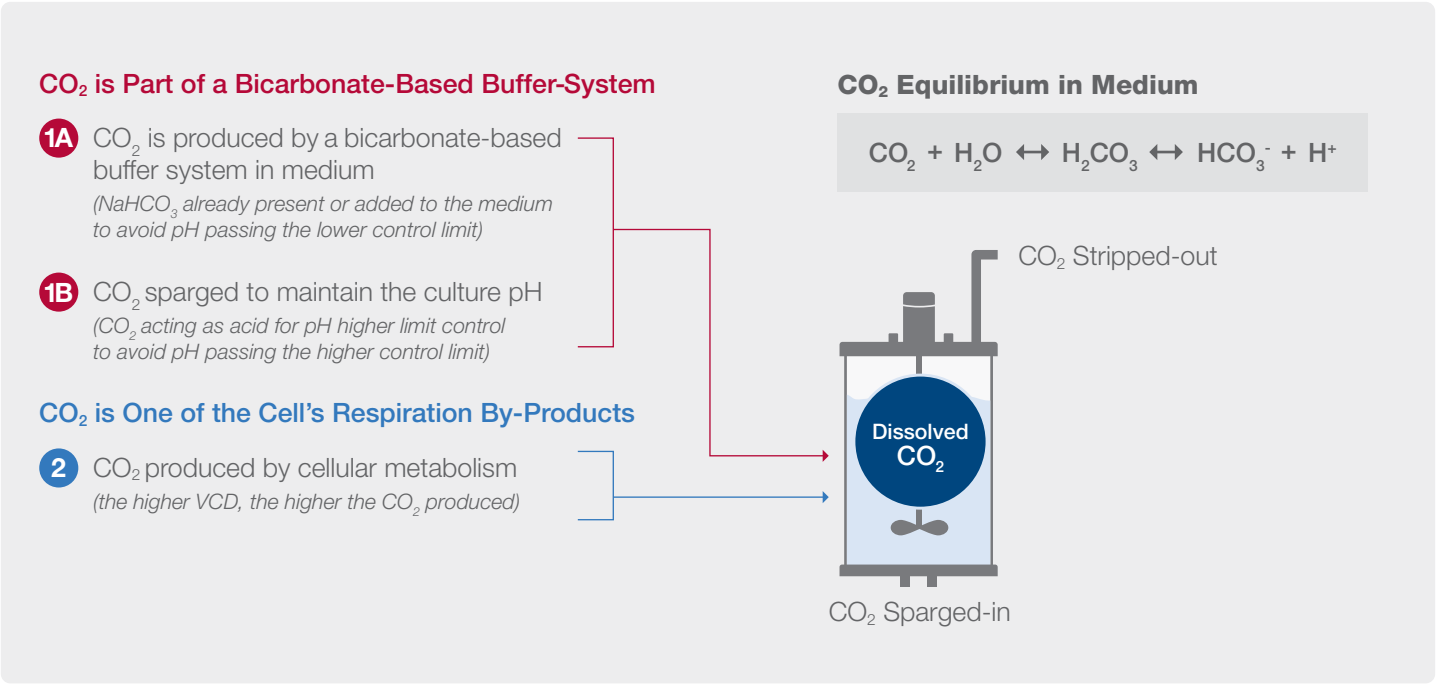



*Figure 1: Dissolved $CO_2$ in cell culture bioreactor. It is both produced by cellular metabolism and it is part of the buffer system.*

Carbon Dioxide ($CO_2$) is an important part of bioproduction. It typically increases in concentration in the bioreactor throughout a biopharmaceutical process. This is valid for almost all aerobic bioprocesses, both animal cell cultures and microbial fermentations. The organisms involved in such processes produce $CO_2$ as part of the respiration process. The $CO_2$ then dissolves in the bioreactor medium. In cell culture, dissolved $CO_2$ ($DCO_2$) also commonly plays an important role in the culture's buffering system as shown in Figure 1.

Due to the multiple roles it plays in the bioreactor, $CO_2$ is more complex to understand and control in a bioprocess than parameters such as $O_2$. It is important to understand where $CO_2$ is involved to understand why, by the PAT guidelines[1], it is defined as a Critical Process Parameter (CPP). It is necessary to break down such complexity and first dig deeper into how $CO_2$ gets into the bioreactor. This white paper will include discussion of this complex balance in both cell culture and fermentation, with the first chapter focusing primarily on cell culture.

# 1.1 $CO_2$ to Control pH in Buffer Systems

## 1.1.1 Principle of the Bicarbonate-Based Buffer System

Cell cultures require an environment with a very narrow pH variation deadband, for optimal results. Maximum viable cell density and productivity are only achieved when the intracellular medium, cytosol, is stable within the physiological pH range. This intracellular pH tends to align with the extracellular pH, better called the medium pH. Therefore, bioprocess engineers try to optimize the intracellular pH by controlling the medium pH. The necessary pH set-point value for bioreactor media usually lies between 6.4–7.4 while the deadband is often ± 0.1 pH (or sometimes as low as ± 0.05). The optimal pH can vary based on cell line, desired product, and other factors. To maintain this tight range, bioprocesses include a buffer system. Laboratory studies commonly work with industrially-produced biological buffers such as HEPES, while production environments typically use $CO_2$/bicarbonate ($HCO_{3-}$)-based physiological buffers[2] The $CO_2$/$HCO_{3-}$-based buffer system is more commonly used for its similarities to the system organisms naturally use to keep the optimal physiological pH. For example, this system is present in mammalian blood to prevent detrimental pH changes resulting from gas, nutrient, and metabolite fluctuations.

The bicarbonate buffering works through Le Chatelier's principle as shown in Figure 2.

**Le Chatelier's Principle:**

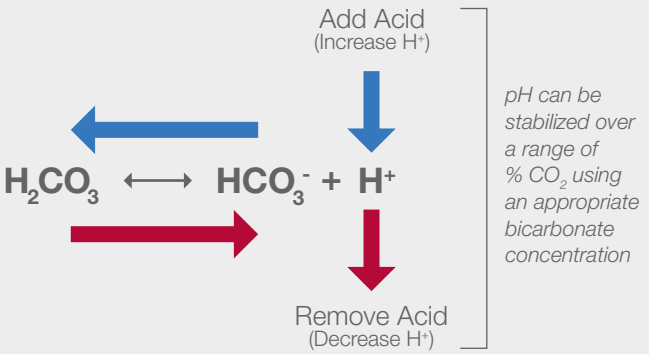



*Figure 2: The principle of bicarbonate buffering. Exemplified from https://www.phe-culturecollections.org.uk.*[3]

Increased acidity in the medium is an indication of an increase in hydrogen ions ($H^+$). Free bicarbonate ions react with free $H^+$ ions to form carbonic acid ($H_2CO_3$), shifting the reaction to the right of Stoichiometric Equation 1 and stabilizing the pH.

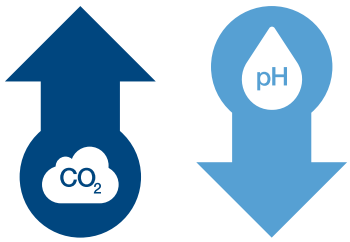
**Stoichiometric Equation 1:**

$$HCO_3^- + H^+ \leftrightarrow CO_2 + H_2O$$

Medium with a Physiological pH Between 6.8–7.4

Therefore:

- An increase in bicarbonate ions will drive the medium to an increase in pH.
- An increase in the partial pressure of $CO_2$ ($pCO_2$) and resultant increase in $DCO_2$ will lower the pH.




6.8 < Cell Culture Physiological pH < 7.4

Equation 1 and Figure 2 also show that the physiological pH can still be maintained at different $DCO_2$ levels, providing that the amount of $NaHCO_3$ in the medium formulation matches the foreseen needs of the specific bioprocesses. For example, to reach pH ≈ 7 in a medium commonly used for cell culture, such as RPMI, a concentration of 1.9–2.2 g/L $NaHCO_3$ is needed when the nominal $pCO_2$ expected in the bioreactor is 5%. Another medium such as the DMEM will allow nominal $pCO_2$ up to 10% when starting with $NaHCO_3$ up to 3.6 g/L.[4] As a consequence, it is critical to note that tightly controlling pH is not sufficient for ensuring consistent $CO_2$.

In aqueous solution at pH 7, $DCO_2$ occurs mainly in two inorganic forms: free aqueous carbon dioxide ($CO_2$ (aq)) and bicarbonate ion ($HCO_{3-}$). The solubility of $CO_2$ in aqueous solutions is dependent on $pCO_2$ in the headspace as described by Henry's law.

**Henry's Law Equation:**

$$H_{CO_2} = \frac{C_{CO_2L}}{P_{CO_2}} \left[ \frac{mmol}{L\ bar} \right]$$

Henry's law helps to illustrate why $DCO_2$ is generally referred to in terms of partial pressure of $CO_2$ ($pCO_2$), as opposed to molecular concentration.

> **Common units of measure of partial pressure include mbar, KPa, mmHg, or % saturation. The unit of measure in $CO_2$ literature generally varies by field: biology-focused literature, for example, tends to use "mmHg" and "% Saturation." To facilitate the comprehension of this white paper, a conversion table has been included in the glossary under the term "Dissolved $CO_2$".**

Based on Henry's law, $pCO_2$ influences the exchange of $CO_2$ between the intracellular cytoplasm (where metabolic reactions happen) and the extracellular medium. A large gradient between the $pCO_2$ in the medium and the intracellular cytoplasm will cause a flow of $CO_2$ into the cells, changing the intracellular pH. Disequilibrium of $CO_2$ in the cytosol brings the intracellular pH out of physiological range. This perturbs the metabolic patterns resulting in lower productivity or even apoptosis.

The partial pressure of $CO_2$ in the medium is critical for the cell's well-being, so it is important to also take into account that it is influenced by many bioprocess parameters in parallel to its use as a buffer system component. Examples include medium salinity, temperature (Figure 3), and hydrostatic pressure. For example, large production bioreactors have high variability in hydrostatic pressure between the top and bottom of the reactor. All of these factors need to be considered for proper understanding of $CO_2$ profiles in the bioreactor.

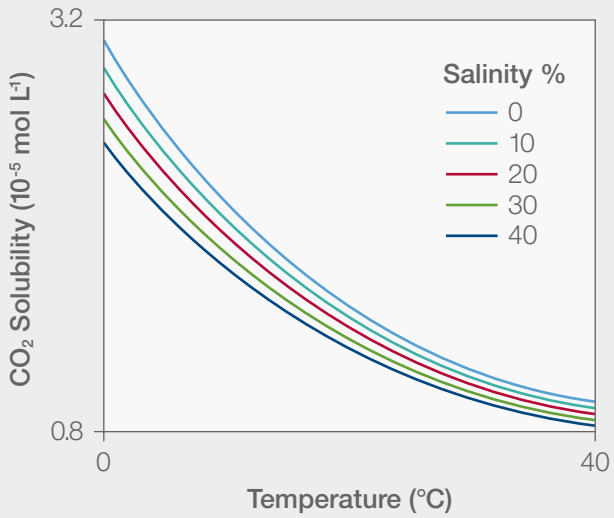



*Figure 3: Solubility of carbon dioxide in water at different temperatures. Exemplified from book "Carbon Dioxide Sensing."*[5]

## 1.1.2 pH Regulation by $CO_2$ Sparging

As discussed in the previous section, stable intracellular pH in the desired range will favour culture growth and product expression. Maintaining this stable pH set-point within the complexity of the bioreactor requires a finely tuned control strategy throughout the entire process. Without a control strategy, the medium tends to become acidic. Buffers are included in all media formulations to maintain the desired pH. In this section, we describe how the buffer system works in industrial bioprocesses.

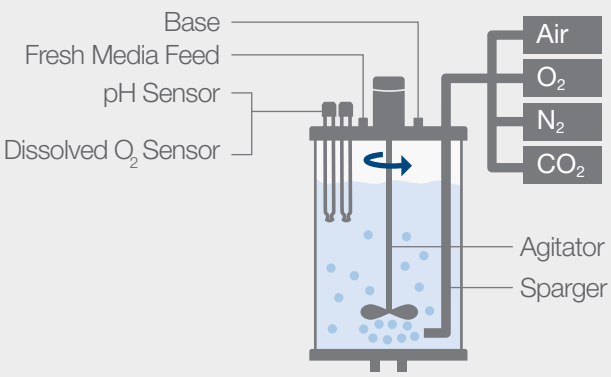
To reach the desired pH value, an initial concentration of $NaHCO_3$ is added to the bioreactor at the beginning of the bioprocess. Throughout the process, the buffer maintains optimal culture pH. In some cases, the buffer system is regulated with addition of Brønsted acids (e.g. HCl) to lower the pH or Brønsted bases (e.g. NaOH) to raise the pH. This is a widely used strategy to keep pH constant within a bioreactor, but it comes with potential deficiencies. For example, continuously adjusting by such additions will increase the osmolality of the media throughout a process. Osmolality may affect each process differently, but it is commonly believed to negatively impact culture productivity (i.e. final protein titer).

The majority of cell cultures used to produce monoclonal antibodies (mAbs) or vaccines now use a $CO_2$-based buffering system in place of a typical Brønsted acid to overcome these deficiencies. $CO_2$ can be added to the bioreactor in three main ways:

- As an overlay to the headspace
- Sparged in from the bottom or
- Formed from the addition of $NaHCO_3$.

$DCO_2$ is used to control the buffer capacity and ultimately the pH of the medium, so it needs to be added in appropriate amounts.

With the addition of $CO_2$ Equation 1 can be rewritten as Equation 2 shown in Figure 4 below:




**Stoichiometric Equation 2:**

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$$

Control Medium within ± 0.1 pH Deadband

*Figure 4: Bioreactor with sparged gases.*

# 1.2 By-Product of Cell Culture Metabolism

In addition to the intentional processes discussed above, $CO_2$ also accumulates in the bioreactor as a by-product of cell culture metabolism. The biostoichiometry of these reactions in mammalian cultures can be summarized by 2 major processes. The first process is respiration, in which glucose is oxidized to produce the fundamental molecule for intracellular energy exchange, ATP (Equation 3, Figure 5). The second process generates cell growth and therapeutic proteins, such as mAbs (Equation 4, Figure 6).

Both metabolic processes involve the reactions of glucose with oxygen and other molecules to produce carbon dioxide as a by-product. A small portion of the $CO_2$ produced by the cells in these processes is reused in synthesis processes such as the formation of fatty acids or the cell membrane, but the majority is released from the cell to the surrounding medium. This leads to a gradual increase in dissolved $CO_2$ throughout the process if left uncontrolled.

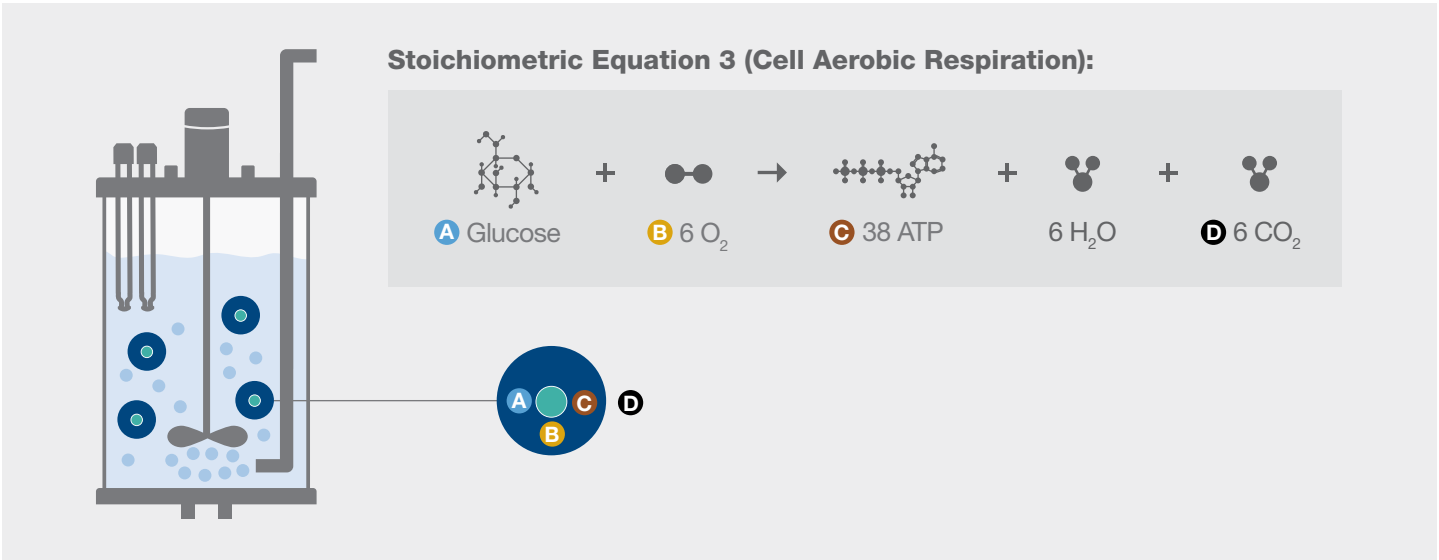



*Figure 5: Cell aerobic respiration simplified.*

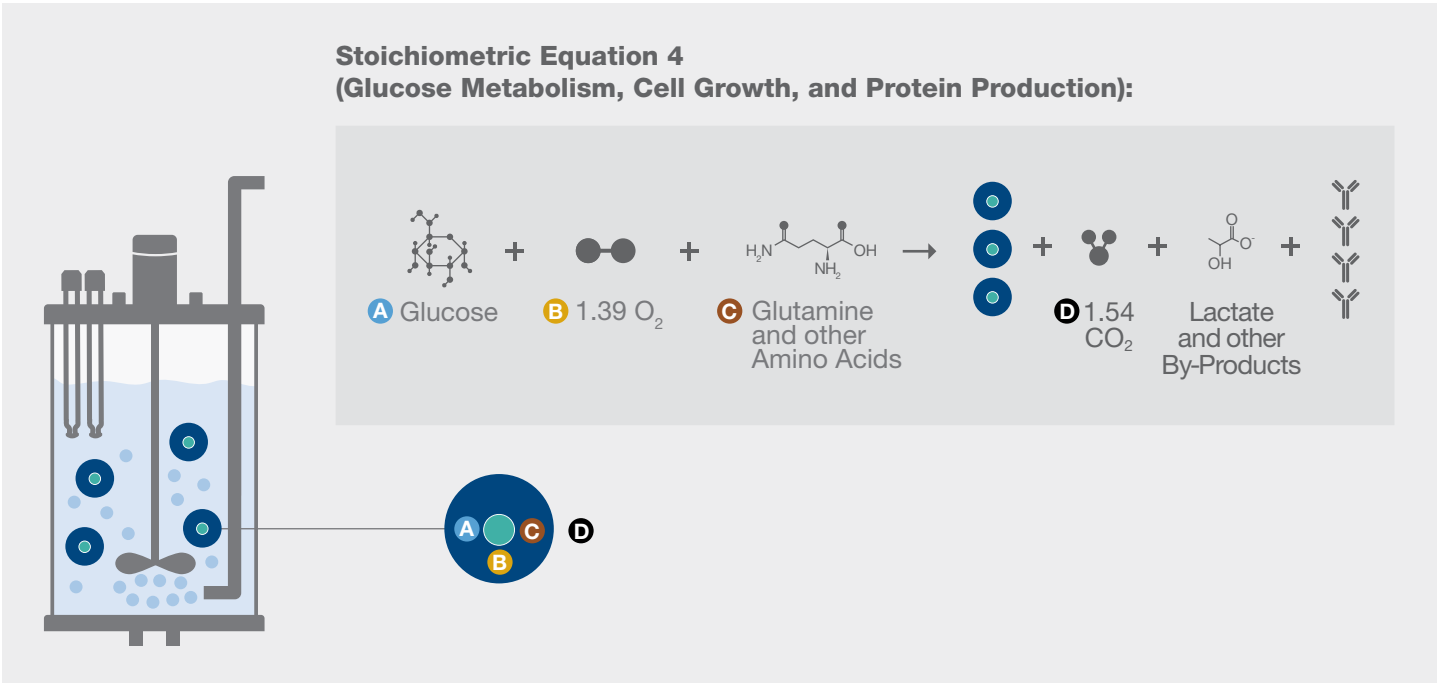



*Figure 6: Glucose/Lactate metabolism, cell growth, and protein production simplified.*

These reactions help to demonstrate the relationship between oxygen consumption, viable cell density, product titer, and carbon dioxide production, as well as lactate production. The determination of dissolved oxygen and $CO_2$ produced/consumed is therefore relevant to assess the correct deployment of a bioprocess. This is necessary especially for the accurate determination of parameters such as Oxygen Uptake Rate (OUR), Carbon Evolution Rate (CER), and Respiratory Quotient (RQ).[6]

# 1.3 $pCO_2$ and pH Evolution During a Bioprocess

$CO_2$ has a multifaceted relationship with the pH of the media, as discussed. It is a typical product of cellular respiration and is a major component of the buffer systems (both contributing to increased acid formation in solution). There are also other contributors to increasing media acidity, such as lactate, which interact with $CO_2$ and the buffer system that need to be considered. This section will discuss the interplay of these factors and the expected behavior of each parameter throughout a typical process.

## 1.3.1 Lactate and $pCO_2$ Interaction Influences the Culture pH

In addition to $CO_2$ and other products, the metabolism of glucose produces lactate, as simplified in Figure 6. Lactate is strongly produced during the growth phase and accumulates in the bioreactor. It acts as a Brønsted acid to produce $H^+$ ions, thus reducing the medium pH. This effect is counterbalanced by the buffer system, as long as there is sufficient buffer capacity. If left unneutralized, the lactate accumulation will become toxic for the culture.

As long as the bioprocess runs as expected, cell cultures, when entering the stationary phase, start to consume their own produced lactate as a supplement to or replacement of glucose as a carbon source. This change is called the lactate shift and is generally seen as desirable. This shift prevents lactate accumulation and simultaneously reduces the required glucose feed. This key event is also connected to increased culture longevity and final product titers.[7]

If the lactate shift is going to occur in a process, it typically begins when the culture is entering the stationary phase. Studies have shown that elevated $pCO_2$ (compared to

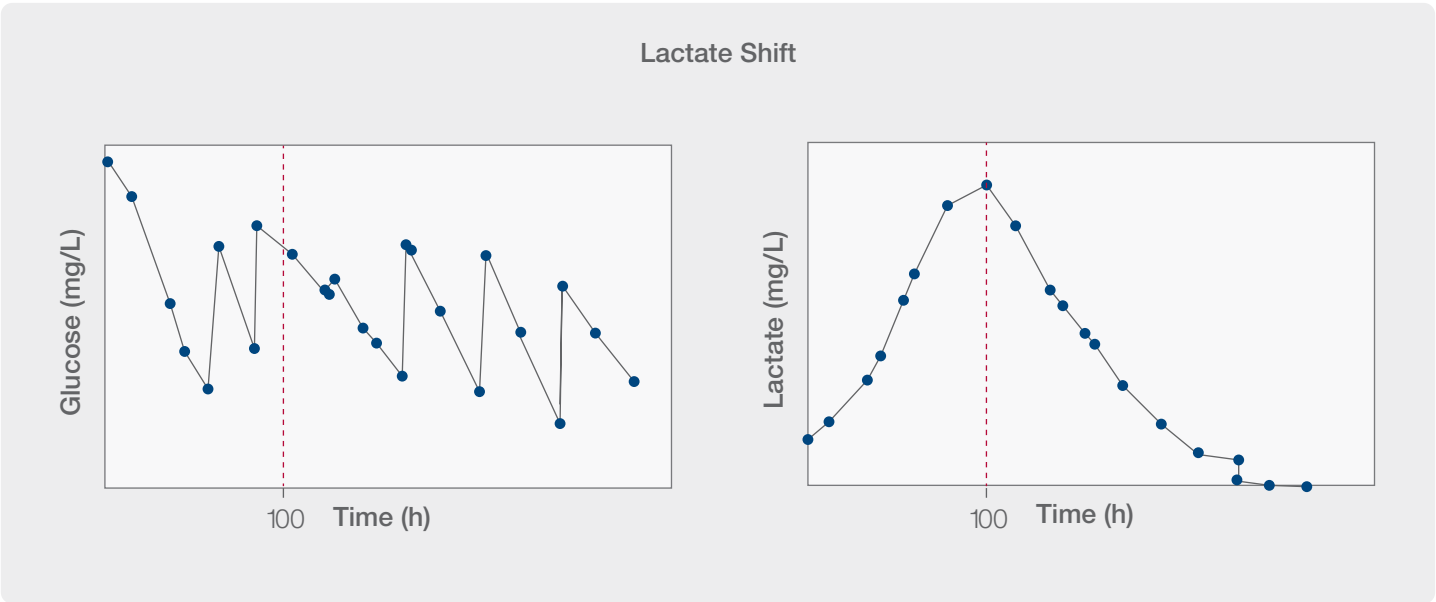
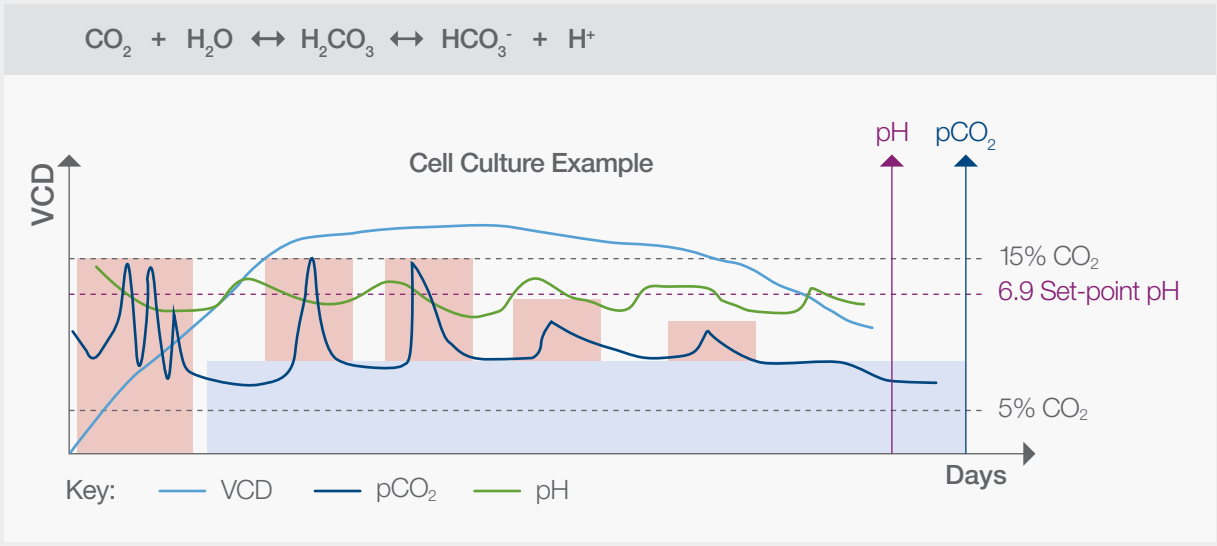



*Figure 7: A healthy cell culture is expected to shift consumption from glucose to lactate after a certain time; 100 hours in this example.*

control cultures with lower $pCO_2$ values) in batch and fed-batch cultures leads to the delay or the complete absence of this shift. In such a situation the accumulation of lactate has to be continuously compensated with addition of base in order to keep the desired pH level.[27] The continuous addition of base will increase the medium osmolality, which, as previously mentioned, may have toxic or counterproductive effects. In other words, identifying and controlling a proper $pCO_2$ level for the specific bioprocess will ensure that the lactate shift happens (or happens earlier), resulting in enhanced productivity.

### 1.3.2 How Do pH, $pCO_2$, and VCD Develop During Cell Culture?

The critical roles of pH and $CO_2$ in a bioprocess are complicated and intertwined,[8] primarily because $CO_2$ is used to regulate pH. This may lead to the false assumption that, by controlling pH, the $CO_2$ is also under control. Figure 8 illustrates that this is not the case. In this representative example,[9,10] pH is controlled at 6.9 with a deadaband of ± 0.1. The $CO_2$ profile, however, has significant variations that may be relevant to the process. Figure 9 depicts the relationship of $CO_2$, base addition, and lactate with pH of the media.





**Sparge $CO_2$**

At the beginning of the culture, the medium is slightly basic: $CO_2$ is sparged to lower the pH of the optimum set-point.


**Strip $CO_2$**

VCD is at its maximum during the stationary phase. The high VCD produces too much $CO_2$ and decreases pH below the set-point. This has to be neutralized by base addition ($NaHCO_3$) or by $CO_2$ stripping. $CO_2$ only needs to be sparged when pH eventually increases (peaks in graphic).

*Figure 8: Simplified development of pH, $pCO_2$, and VCD in cell culture fed-batch when pH is controlled and $pCO_2$ is just monitored.*

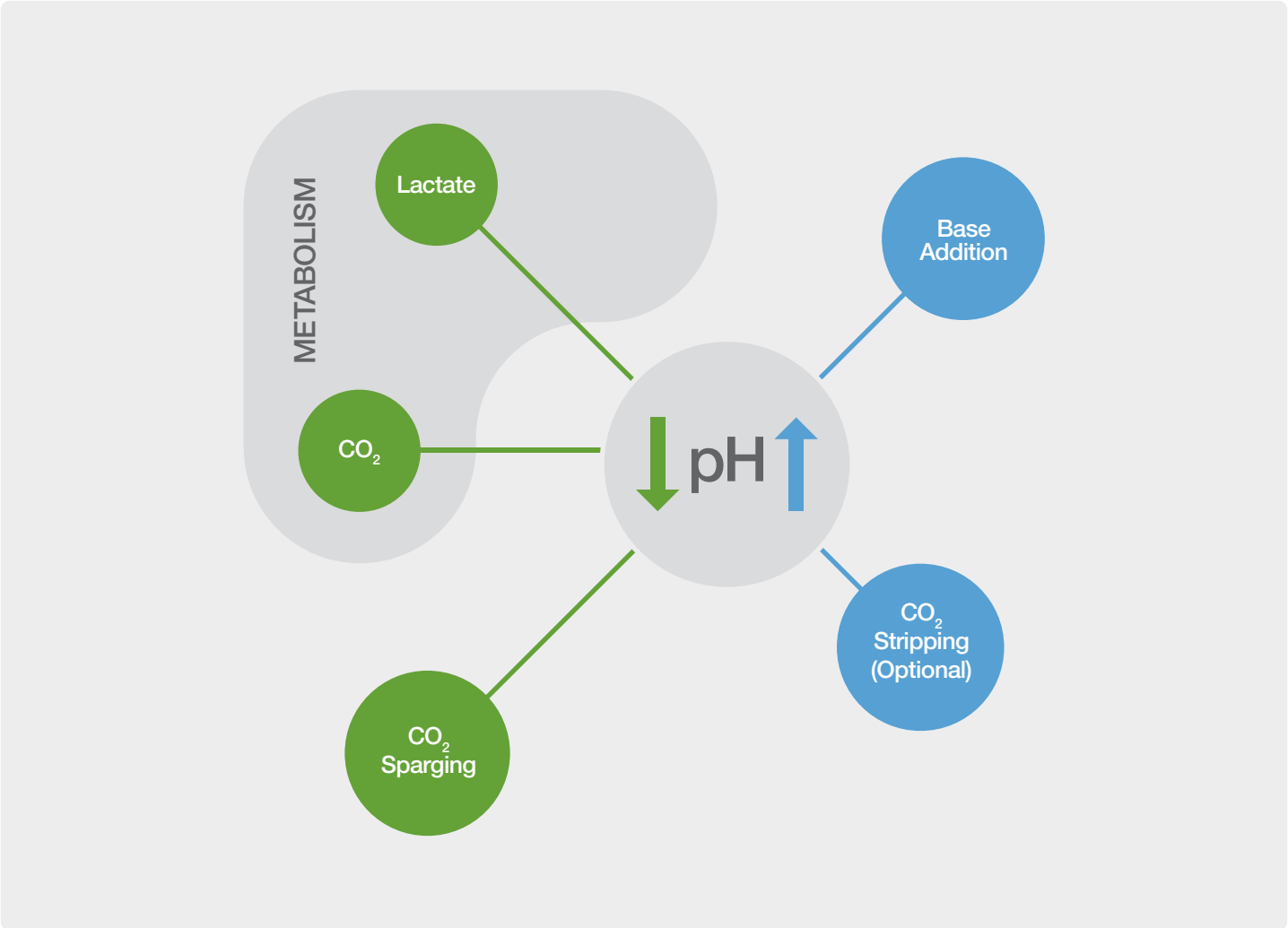



*Figure 9: Key effects on the pH of a cell culture.*

The information in this chapter provides the details necessary for understanding why $DCO_2$ is a Critical Process Parameter, which will be outlined in Chapter 2, followed by the beneficial effects of real-time $CO_2$ control, covered in Chapter 3.

# 2. $CO_2$ as a Critical Process Parameter

The $CO_2$ profile during the bioprocess is an indicator of cell culture well-being, as described previously, but more than that it is a Critical Process Parameter (CPP). In accordance with the Process Analytical Technologies (PAT) Initiative[1] this means that $pCO_2$ (along with parameters such as pH and temperature) is an actionable parameter to be controlled at the bioreactor.[11] CPPs have a direct impact on Key Performance Indicators (KPIs), such as viable cell density, as well as on Critical Quality Attributes (CQAs) of the final product, such as glycosylation patterns.[33] As a CPP, $CO_2$ should have a defined set-point and a defined profile for the ideal batch, often referred also as the "golden batch." This section will explore what that looks like for different applications and why it matters.

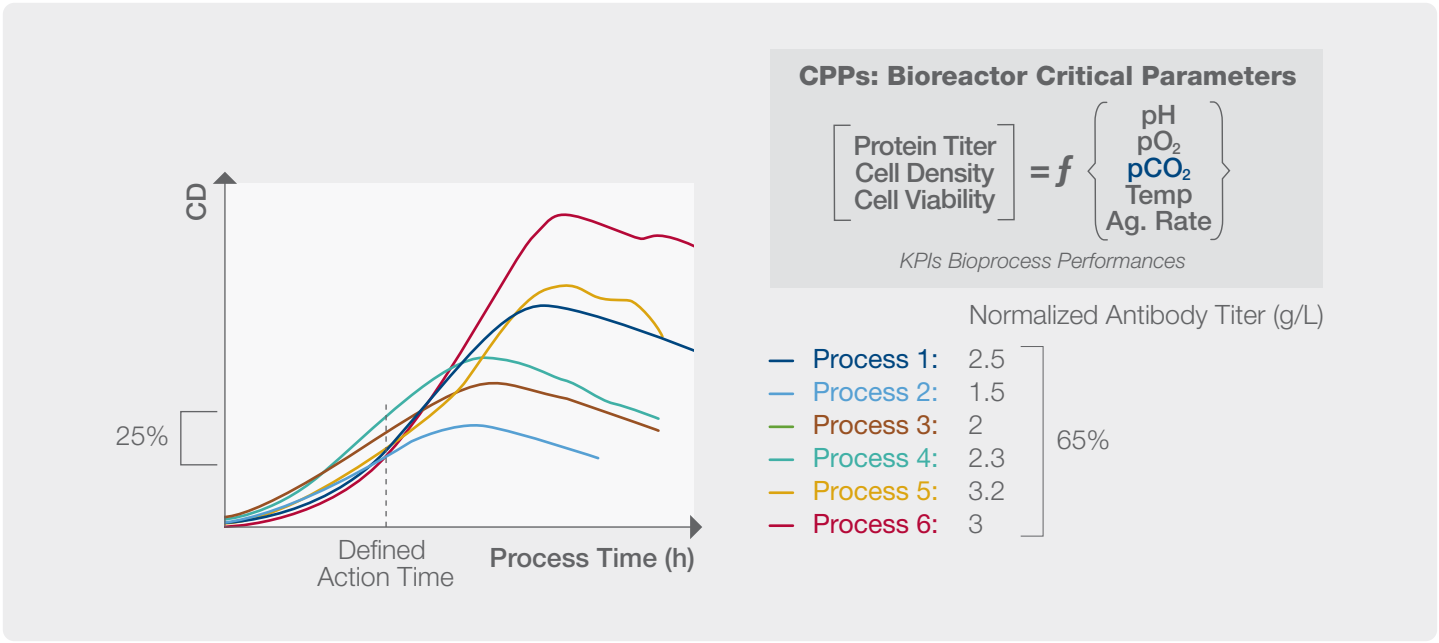



*Figure 10: PAT Critical Process Parameters affecting bioprocess' Key Performance Indicators.*

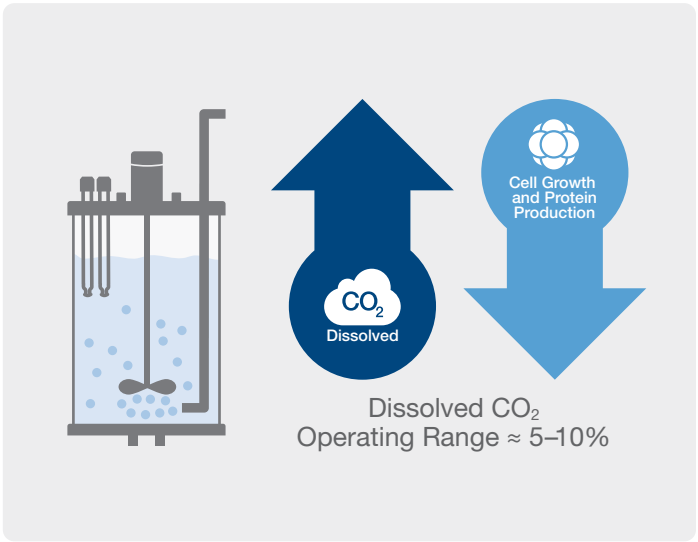



*Figure 11: Simplification of $pCO_2$ detrimental/toxic levels on cell culture. Exemplified from WuXI Biologics, Understanding the Role of Dissolved $O_2$ and $CO_2$ on Cell Culture in Bioreactors.*[12]

# 2.1 Impact on Cultures in General: Not Too High, Not Too Low

As with all bioreactor CPPs (e.g. DO, pH), too much or too little $CO_2$ can be detrimental to a bioprocess. The mechanism of interaction between carbon dioxide and cells or microorganisms impacts the optimal range, which varies between culture types. The impact and optimal ranges will be discussed in the following sections.

When there is not enough dissolved carbon dioxide in a process there are two major consequences. Firstly, the pH buffering system will not retain its buffer capacity. Secondly, the anaplerotic reactions (those that form metabolic intermediates) that rely on $CO_2$ as a reactant will be limited. Both consequences negatively impact the cell viability and ultimately the bioprocess productivity. Literature suggests that this lower limit typically exists at $CO_2$ levels below 5% saturation.[11] It can happen, for example, when mass transfer is not properly controlled and too much $CO_2$ is stripped or neutralized. However, due to the several possible sources of $CO_2$ in the bioreactor, this is an uncommon occurrence.

Far more frequent is the presence of too much $CO_2$ in the bioreactor. When its concentration is too high, it has a toxic effect on cell cultures and fermentations that is detrimental to cell viability and final product titer.[13] The buffer system keeps the medium pH at the physiological level by compensating for the excess carbon dioxide, but only to capacity. Above that point, the cell metabolism will slow and eventually cease to function, leading the cells to apoptosis. Increasing the buffer capacity through addition of a base (e.g. $NaHCO_3$) to compensate for this also increases the overall osmolality of the media. As mentioned, increased osmolality also produces a detrimental effect on cell viability and product titer, and thus should be implemented with caution. Each bioprocess has a specific tolerance, but 20% saturation is a common toxic level in mammalian cultures.

The degree of sensitivity and the tolerable levels of $CO_2$ vary widely between culture type (e.g. mammalian vs. bacterial) and application, but common to all processes is the existence of an optimal $CO_2$ level, above or below which can be detrimental.[14]

# 2.2 Specific Impact According to Culture/ Fermentation Type

Cell cultures (such as CHO or Vero) tend to be more sensitive to high $pCO_2$ than bacterial fermentations are,[15] but all bioprocesses have a level of $DCO_2$ which proves toxic in the worst cases or, at minimum, negative for productivity. It is important to note that not only are there huge differences between cell culture and fermentation, but that even different strains of the same cell type have different optimal levels of $CO_2$. Table 1 summarizes general limits for different organism types as determined by a survey of current literature. The following section summarizes literature findings of $CO_2$ impact on culture/fermentation viability and, where possible, productivity (e.g. product titer/quality).

## 2.2.1 Cell Culture (Mammalian)

Mammalian cell cultures are typically used for production of therapeutic proteins or vaccines. For example, Chinese Hamster Ovary (CHO) cells are often used in mAb production while Vero cells (from African Green Monkey Kidney Epithelium) produce many vaccines. Some cultures have shown a 60% decrease in growth rate when carbon dioxide is at 30% saturation (≈ 300 mbar), well above the optimal level.[15] In regards to CHO cells specifically, experiments have shown a decrease of 20% cell viability in perfusion cultures. Other studies show similar effects in hybridoma cell cultures.[15] Recent studies of $CO_2$ impact on growth of culture for T-cell therapy have also been published.[16] In these cases, $CO_2$ levels as low as 20% show negative impact both on viability and metabolism.

In general, different $CO_2$ levels have an impact on specific productivity.[17] Values of $pCO_2$ above optimal levels have been shown to cause up to 70% decrease in protein titer. In regard to product quality, or therapeutic effect, elevated $HCO_{3^-}$ levels (required to buffer the increasing $CO_2$) have a detrimental effect on important CQAs, such as a 40% decrease in glycan values.[27]

## 2.2.2 Fermentations

### Bacteria

Like mammalian cells, bacteria also suffer at elevated dissolved carbon dioxide levels. Bacteria are more resistant to higher $CO_2$ concentrations though, so the detrimental effects are mainly limited to growth rate. For example, the growth rate of *Bacillus subtilis* is inhibited up to 40% at 17% $pCO_2$ *E.Coli* has been shown to have a significantly lower conversion rate of glucose to biomass when the bioreactor aeration contains $CO_2$ in excess of 20%.[15] When the $DCO_2$ is increased to 30%, maximum growth rate is reduced by 30%, and the fermentation has twice the acetate formation. A dependency between $pCO_2$ and growth has also been observed in cultures of *C. glutamicum* when used to produce L-Lysine: In pressurized bioreactors, when ranging from about 20% to 80% $CO_2$, the growth dropped about 40%.

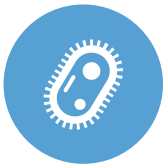
With bacterial fermentations, there is another important aspect connected with $CO_2$ as a CPP. When bacterial fermentations (such as *E. Coli*) use simple molecules like glucose as the carbon source, the simple molecules are consumed very quickly in order to minimize production of the detrimental by-product acetate. The consumption is so rapid that direct glucose monitoring is not sufficient for establishing automated feeding. An indirect KPI such as the Carbon Evolution Rate (CER) needs to be used instead.[18] The direct determination of $DCO_2$, which is necessary for accurate estimation of CER, is therefore a prerequisite to establish automation in bacteria bioprocesses.

### Yeasts

Compared to mammalian and bacterial processes, yeast cultures are less sensitive to high $pCO_2$. However, at 50% $CO_2$ a severe growth decrease of up to 40% can be seen. Cultures of *S. Cerevisiae* have been found to divide much more slowly with $pCO_2$ at 50% (500 mbar). Such a high level of $CO_2$ is also connected to high amounts of ethanol, a metabolic by-product which also impedes growth. Even so, the growth reduction goes up to 25% only when the $pCO_2$ goes over 60% (600 mbar). It should be noted that detrimental effects on growth have been observed primarily in aerobic cultures.[19] Anaerobic cultures exhibit very little effect from high $pCO_2$.

For yeast processes, $DCO_2$ is important for correct calculation of the Respiratory Quotient (RQ). The RQ reflects the distribution between respiration and other reactions that produce $O_2$ and consume $CO_2$. RQ can be used to describe the culture metabolic state. RQ near 1 indicates oxidative (aerobic) growth on glucose, RQ larger than 1 describes overflow metabolism (also aerobic), and an RQ less than 1 indicates ethanol consumption.[20]

**Table 1. $pCO_2$ Average Toxic Levels According to Culture/Fermentations**

| Culture Type | Too High $pCO_2$ | $pCO_2$ Average Toxic Levels* |
|---|---|---|
| *Mammalian*  | Up to -60% Growth Rate<br>Up to -70% Protein Titer<br>Up to -50% Product Quality (Glycosilation) | >20% (>200 mbar)<br>(> 30% in Continuous Culture) |
| *Bacteria*  | Up to -40% Growth Rate | > 30% (150/300 mbar) |
| *Yeast*  | Up to -25% Growth Rate | > 50% (500 mbar) Can Survive and Adapt Even at Higher Dissolved $CO_2$ |
| *Fungi*  | Up to -36% Antibiotic Content | > 15% (150 mbar) |

**Consolidated from the scientific literature referenced in the text and detailed in the references at the end of the white paper.*

**Fungi**

In fungal processes, growth inhibition can be observed as low as 8% $CO_2$ saturation (80 mbar), though that number may be higher such as in *Penicillium chrysogenum*. The mechanisms of this inhibition are not yet fully identified, but they may be related to interference with metabolic precursor synthesis. This in turn affects production. Too much $pCO_2$ has a negative impact on precursors of Penicillin, therefore hinders the production of this antibiotic. The same has been found with cultures of *Acremonium chrysogenum* used to produce Cephalosporin C. In this case, starting a culture above 15% $DCO_2$ reduced antibiotic production up to 36%.[15]

The detrimental effects of nonoptimal $CO_2$ described above illustrate why $DCO_2$ is critical and that there is a need to accurately monitor and control it. In fact, $DCO_2$ control is more than a tool to protect against the detrimental toxic effects explained; it is also a tool for bioprocess optimization. This will be detailed in the next chapter.

# 3. Monitor and Control $CO_2$ in Real-time

It should now be clear what the roles of $CO_2$ in bioprocesses are and why it is critical to research the optimal $CO_2$ development profile for each process. In order to maximize the benefits of this profile and avoid detrimental effects of suboptimal conditions, $CO_2$ needs to be monitored and controlled in real-time, ideally in-situ/in-line.[21] Continuous $CO_2$ monitoring can help to immediately indicate issues in the bioprocess (e.g. metabolism alterations, contaminations), while automated control ensures that $CO_2$ is at the optimal level throughout the entire process. This chapter will discuss control strategy considerations and detail the tangible benefits of real-time monitoring and control of $DCO_2$, including higher yield and more efficient scale-up.

## 3.1 Control Strategy Considerations

In order to explain the benefits gained from controlling $CO_2$ in real-time, it is important to state one point: *there is no universal strategy which can be applied to all bioprocesses*. In other words, there is not one $pCO_2$ value or one control strategy which is optimal for every cell culture or fermentation. To this point, consider the differences between $CO_2$ set-point in the case of two different feeding strategies (i.e. fed-batch and perfusion).

- **Fed-Batch (or Batch) Processes:**
  Currently the most common bioprocess type for cell culture and fermentations. Uncontrolled $CO_2$ profiles vary widely with scale in these processes. Production scale bioreactors often have relatively high volumes, regularly in the thousands of liters. Larger volumes result in reduced surface area-to-volume ratios and contribute to slower mass transfer, resulting in higher $CO_2$ accumulation at larger volumes. These bioprocesses experience detrimental effects as low as $pCO_2$ ≈15%. The media generally requires a lower buffer capacity than a perfusion reactor.
- **Perfusion Processes:**
  Gaining popularity in bioproduction using cell cultures. Typically, perfusion reactors employ media with high buffer capacity. The high cell density and long accumulation time for $CO_2$ require productivity in presence of up to 30% $pCO_2$ in solution.

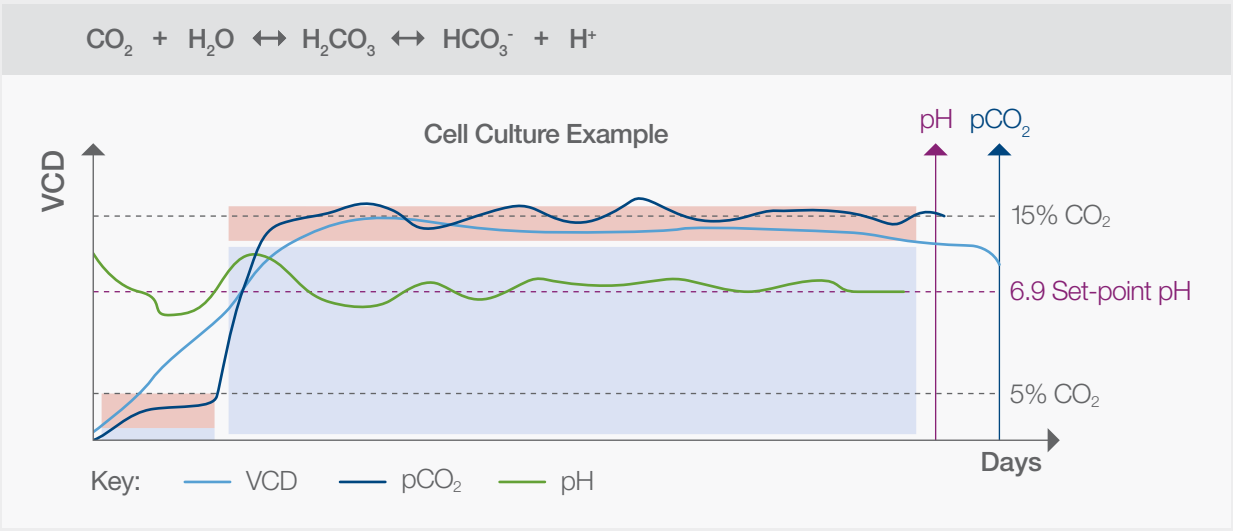
The optimal set-point for each application has to be identified. After that, the strategy chosen to control it plays an important role. When a bioprocess is at risk of accumulating a detrimental or even toxic $CO_2$ level, strategies need to be put in place to neutralize it or strip it from the media. This is a challenge because process mass-transfer parameters such as the $K_LA$ are determined and used to optimize oxygen transfer first and then $CO_2$ mass transfer.

$CO_2$ is 20 times more soluble in aqueous media than $O_2$ and therefore requires a different approach. Otherwise, the optimization of oxygen transfer will conflict with $CO_2$ regulation. As an example, optimal addition for oxygen mass-transfer utilizes a sparger producing small air bubbles to enhance the total exchange surface. Efficient sparging/stripping of $CO_2$, however, would be best achieved with larger air bubbles. Since double spargers are not common to all bioreactors (one for air and one for $N_2/CO_2$), especially at production scale, there is a risk of accumulating $CO_2$ to detrimental levels without a proper $CO_2$ control strategy in place.

In summary, it is important to consider all impactful bioprocess parameters when determining the optimal $CO_2$ control strategy. Relevant factors include:

- Cell Strain
  (e.g. $pCO_2$ tolerance)
- Medium Formulation
  (e.g. buffer composition, nutrients)
- Bioprocess/Bioreactor Type
  (e.g. batch, fed-batch, perfusion)
- Sparger Type
  (e.g. single or double, large or small bubbles)
- Bioreactor Scale/Volume

Each combination of these factors requires a specific identification of the optimal $pCO_2$ and the strategy to control it. However, common to all bioprocesses is that real-time control of $CO_2$ is required for maximum productivity. Figure 12 illustrates the benefits gained from $CO_2$ control. When compared with the uncontrolled $CO_2$ of Figure 8, the controlled process shows significantly prolonged production phase at maximal VCD. The graphic also shows that tighter control of $CO_2$ enables tighter control of pH.[9, 28] This will be further explained in the following sections, 3.2 and 3.3, with examples.





### Sparge $CO_2$

At the beginning of culture, the medium is slightly basic: $CO_2$ is sparged to lower the pH to the optimum set-point. In the stationary phase $CO_2$ is controlled in real-time to a set-point as well as pH. In this case, there is no huge variation of $CO_2$ and pH control results fine-tuned and better in control too.


### Strip $CO_2$

In the stationary phase the VCD is maximal and stripping is required only for fine-tuning. Yet, due to stable conditions of both pH and $pCO_2$, the culture keeps maximal VCD for a longer period in the same batch.

*Figure 12: Exemplification of the development of pH, $pCO_2$, and VCD in cell culture fed-batch when pH and $pCO_2$ are both controlled.*

# 3.2 Production Productivity: Longer Viable Phase and Maximized Product Titer

Optimizing $CO_2$ set-point and real-time control strategy for each bioprocess maximizes its productivity and thus should be done in the R&D and PD stages. Tight $CO_2$ control has been shown to substantially benefit bioprocess productivity. For example, one study investigated two equivalent fed-batch cultures, one with $pCO_2$ controlled at 10% and one in which $CO_2$ was left to accumulate to 20%.[9] The $CO_2$ control resulted in a longer productive phase and higher protein titer (Figure 13), even with equivalent pH control (6.85 ± 0.05).

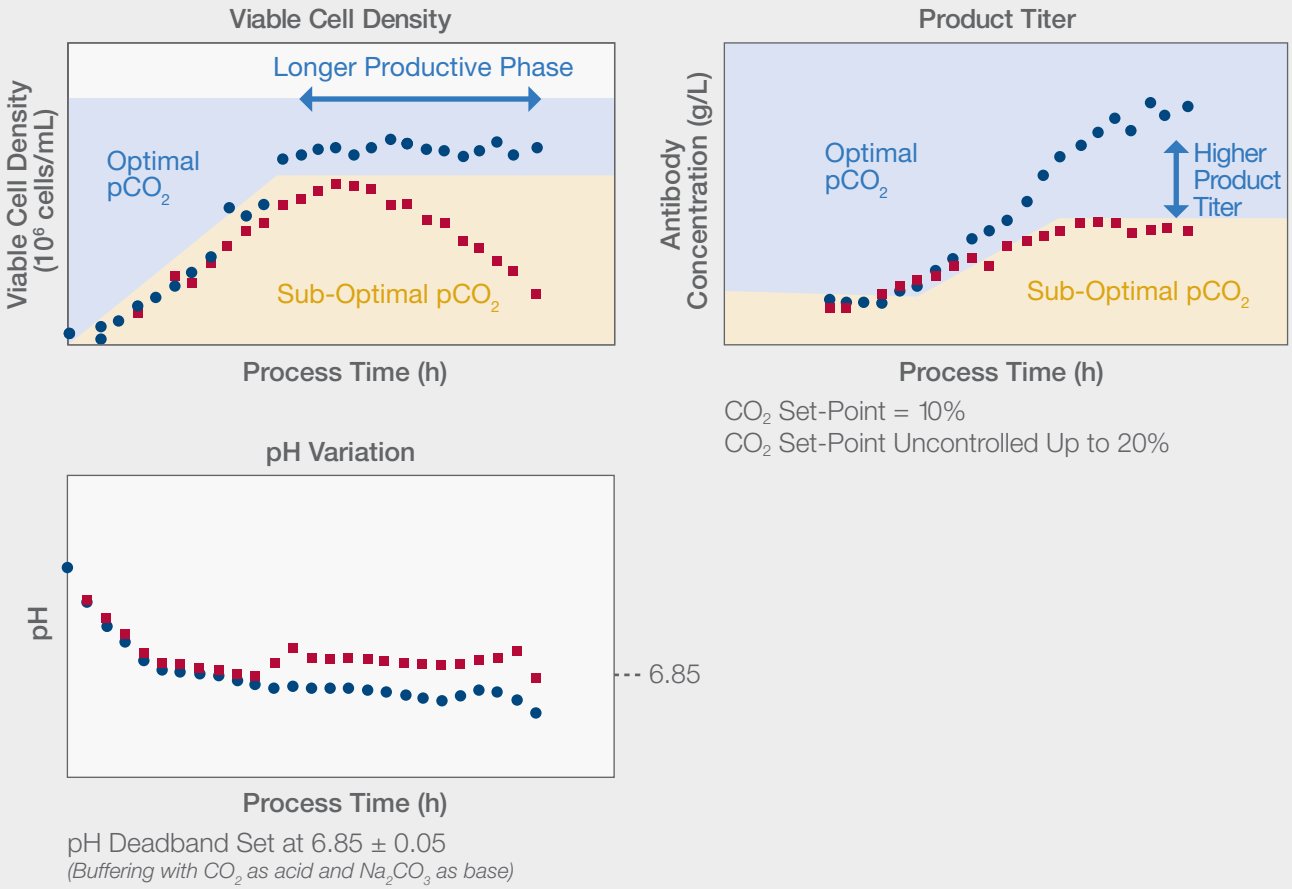
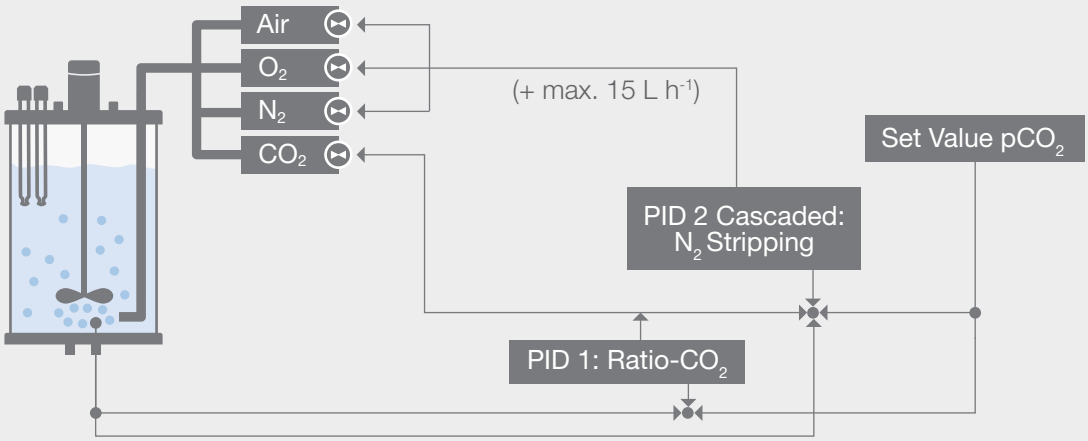



*Figure 13: $pCO_2$ impact in an example of CHO fed-batch process exemplified from the original joined research published by the Institute of Biochemical Engineering of University of Stuttgart and Boehringer Ingelheim Pharma GmBH.[9]*

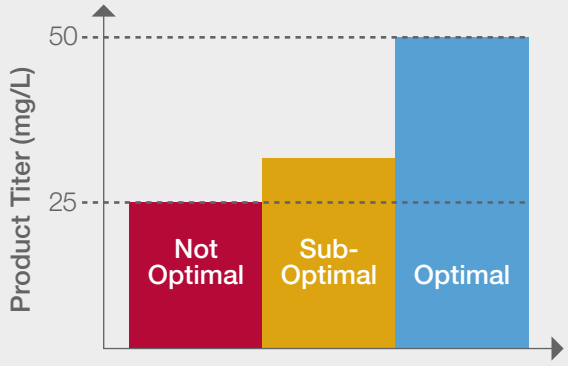
This shows that pH control alone is not sufficient for maximum process enhancement. When complemented by dedicated $CO_2$ measurement and control, productivity can be increased. As recognition increases for the importance of $CO_2$ control, companies are taking innovative steps to take advantage of this parameter in their own processes.

As previously mentioned, $CO_2$ should be controlled without the addition of acids and bases if possible to avoid increases in osmolality. One example of such a strategy is exclusively sparging and stripping $CO_2$. This method is described by Roche in a patent aimed to reach higher productivity for recombinant protein production through real-time, in-line $CO_2$ control.[22] Figure 14 illustrates this approach with a double cascade PID (Proportional Integral Derivative) controller based on in-line $pCO_2$ sensor feedback response to regulate the sparging/stripping of $CO_2$ by Microflow Controller (MFC) using $N_2$ as the stripping gas. Real-time $CO_2$ monitoring and control not only reduced process variability, but increased productivity in the form of higher protein titer.[22] This patent in particular describes reduced sensitivity of the cells to events such as lactate peaks after bolus introduction through $CO_2$ set-point control, ultimately leading to 50% higher protein titer than in control experiments. Other dissolved $CO_2$ control strategies implement in-line sensor feedback to control the motors responsible for the agitation speed in addition to the sparging/stripping strategy.[23]

**1** **Real-Time Dissolved $CO_2$ Measurement and Control**




**2** **Productivity Increase**




- **Not Optimal:** $pCO_2$ – Uncontrolled
- **Sub-Optimal:** $pCO_2$ – Controlled at 5%
- **Optimal:** $pCO_2$ – Controlled at 15%

Case control $pCO_2$ set-point at e.g. 15% with a Proportional Integral Derivative (PID) controller, based on a feedback response triggered by the signal of an in-line dissolved $CO_2$ sensor

**PID 1:**

- Add/slow-down $CO_2$ to keep the 15 ± 3% $pCO_2$
- Stop $CO_2$ addition when $pCO_2$ > 15 + 3%

**PID 2:**

- When $CO_2$ >> 15 + 3%, strip it with addition of $N_2$

*Figure 14: Increased product titer in CHO fed-batch process through $pCO_2$ in-situ monitoring and control. Exemplified from original source European Patent Office Application 09719289.2.*[22]

The automated monitoring and control of $CO_2$ with an optimized sparging/stripping strategy significantly benefited the bioprocess in each of these cases. When it was controlled, physically damaging concentrations of $DCO_2$ could be avoided for maximum viable cell density. Batch-to-batch reproducibility can be increased, especially in large volume or long duration processes. Some recent publications even highlight the possibility to use exclusively sparging gases to regulate pH (with no need for acids or bases) across all scales from laboratory to production.[24]

> **Productivity of a bioprocess can be increased with an optimized strategy for dissolved $CO_2$ real-time control.**

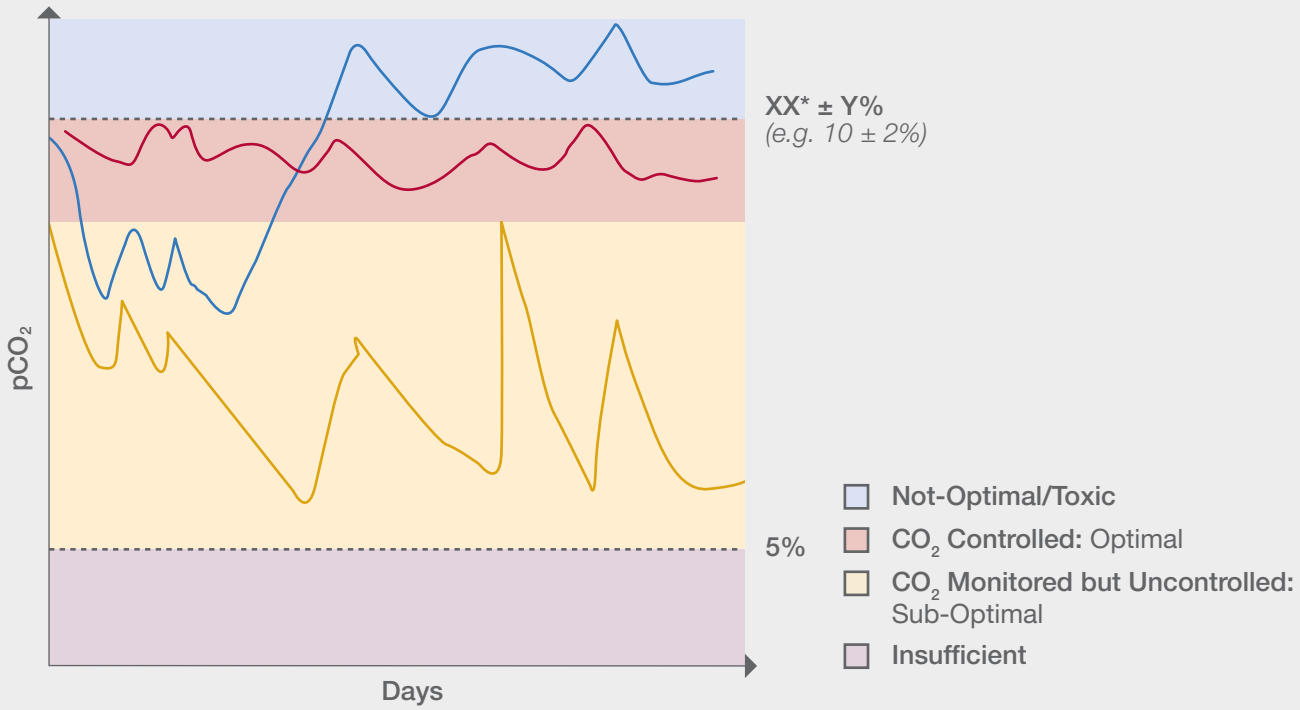



*Figure 15: Simplified example about how to exploit $pCO_2$ real-time control in order to maximize the bioprocess productivity.*

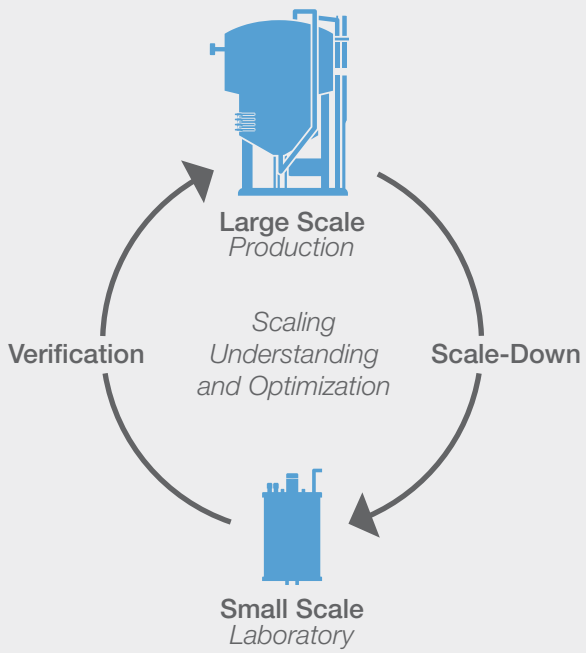
# 3.3 Development Productivity: Shorten Scale-Up/Scale-Down Iterations

Although the benefits to controlling $CO_2$ are clear, there are major challenges to keeping the $CO_2$ concentration consistent between the development scale and the final production scale.[25] Without real-time measurement and control of $DCO_2$ at the development scale, consistency is rarely achieved during scale-up/scale-down iterations between laboratory and production (Figure 16).[26] $CO_2$ is stripped more easily at laboratory scale due to the smaller surface-to-liquid ratio and hydrostatic pressure.[27] In small-scale bioreactors the majority of $DCO_2$ is stripped via surface aeration. In large-scale bioreactors, the liquid surface-to-volume ratio is decreased, thus requiring other strategies for $CO_2$ removal.[28] Without an adjusted strategy for the large scale, $CO_2$ will tend to accumulate, especially at the bottom of the bioreactor.

Therefore, the appropriate $K_LA$ for $CO_2$ has to be determined and its set-point monitored in real-time to ensure proper scalability without detrimental accumulation of carbon dioxide, as exemplified in Figure 17. These scale discrepancies can lead to significant changes in the bioprocess, such as changes to the lactate shift, and can ultimately lead to significant differences to the bioprocess KPIs (e.g. titer) and CQAs (e.g. glycosylation patterns).

By employing real-time monitoring and control of $CO_2$, processes at different scales have an additional CPP that can be kept consistent (or "correctly scaled") throughout the process (see Figure 18). This consistency reduces the extra time and cost for scale-up and scale-down experiments related to $CO_2$ and its many effects.

> **Utilizing real-time $CO_2$ control enables perfect replication of the optimal $CO_2$ profile from lab scale to production for maximized scale-up/scale-down efficiency.**




Troubleshooting often requires scaling down a process to find root issues. The cycling between scaling down and back-up again may have to be repeated numerous times until it is realized that $CO_2$ levels vary by scale.

*Figure 16: The traditional approach to align the performances between the scales is to iterate multiple scale-up/scale-down "test-and-see" experiments. This has a huge impact on the time-to-market, especially on increasingly needed products such as vaccines, mAbs, and cell therapies.*

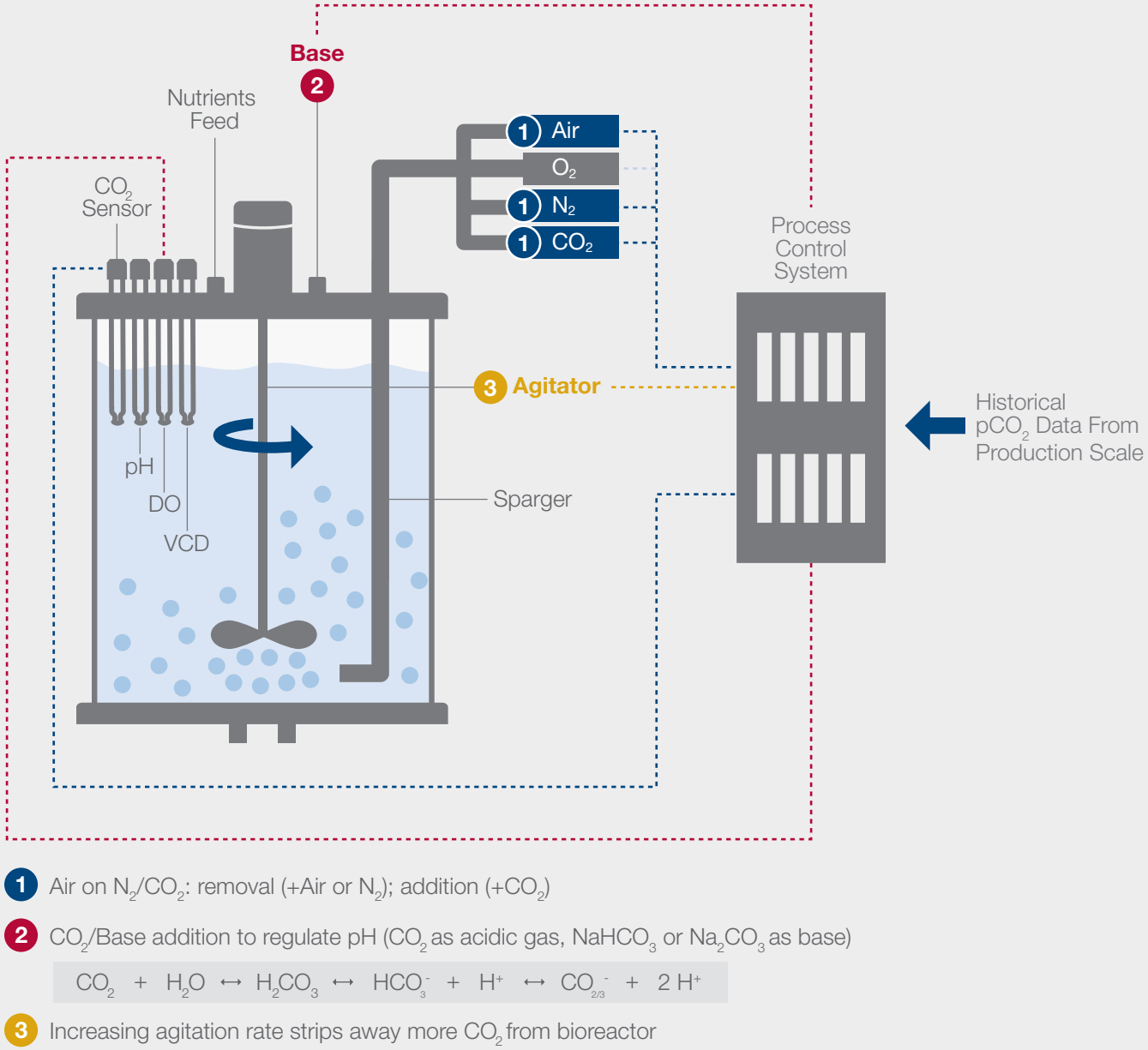



*Figure 17: Simplified example about how to set a laboratory bioreactor in order to mimic the production scale $pCO_2$ profile.*

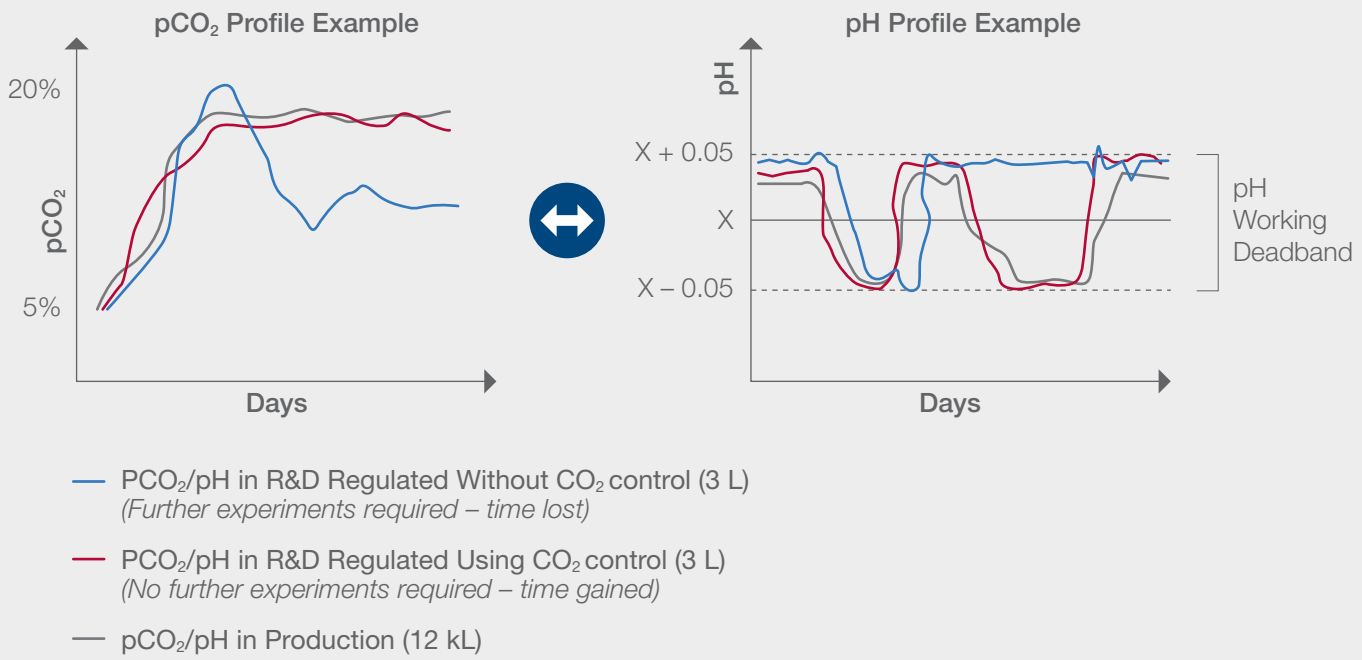



*Figure 18: Comparison of scale-down with and without $pCO_2$ real time-control. In the control case the laboratory scale bioreactor has been controlled in order to mimic the same $DCO_2$ profile of the production scale. Exemplified from original work presented at ESACT 2019 Congress.*[27]

# 4. Conclusion

$CO_2$ plays an important part of all bioprocesses. It is both a product and reactant of metabolic processes and is a component of the most commonly used buffer systems, such as the bicarbonate system. It has also been shown to be an important factor of the lactate consumption shift.

As a Critical Process Parameter (CPP) with so many important roles in the bioprocess, $DCO_2$ should have its own defined set-point and a control strategy to keep it at that point for each scale. Just like pH and DO, real-time monitoring and automated control of $CO_2$ are required for the optimal implementation of bioprocesses from R&D laboratories to the production floor. Through such an approach, bioproduction has been found to deliver higher viable cell density, a prolonged growth phase, and higher product yield with expected quality retained. A continuous monitoring and control of $CO_2$ throughout the entire process also enables reduction of scale-up/scale-down iterations to optimize the manufacturing process.

Fully attaining the benefits described in this white paper requires an accurate, precise, and preferably cost-effective technology for real-time $CO_2$ measurement. A review of commonly used off-line, at-line, and in-line technologies for $CO_2$ measurement will follow in the next part of the "**Dissolved $CO_2$" white paper series**.

# Glossary

Definitions in this glossary have been derived from Hamilton Whitepapers[A,B] or the PAT guidelines unless otherwise cited.

**Bioprocess Type—Batch**

The batch processes are often considered the first processes adopted by the biopharmaceutical industry, especially for fermentations. Microorganisms are added to culture media in the bioreactor, which has been pre-filled with nutrients like glucose, glutamine, other amino acids and minerals. The media remains the same during the entire process and is not supplemented, refilled or exchanged at any time. After an initial lag phase, the number of microorganisms increases sharply during the growth phase. Then, after a stationary phase of suspended population, the culture population drops off in a death phase. The cause for the population drop-off can be tied to the depletion of nutrient media and the accumulation of toxic substances.

**Bioprocess Type—Fed-Batch**

Fed-batch is the dominant bioprocessing method for both cell cultures, and microbial fermentations. The fed-batch process differs from the traditional batch process by adding nutrients in stages to maximize cell growth. The bioreactor is filled with a base amount of media to support initial cell growth. Feed media is added when needed to replace nutrients depleted by the increasing cell population. The cells and their product(s) remain in the bioreactor until the end of the run. With this setup it is possible to automatically regulate the addition of feed media according to nutrient levels or viable cell density.

**Bioprocess Type—Perfusion**

The term «continuous bioprocessing» refers mainly to perfusion technologies. The bioreactor runs at a fixed volume and fixed cell concentration for 30–90 days or longer depending on cell line. During this time the feed media is constantly refreshed and the secondary toxic metabolites eliminated while cells are simultaneously harvested for further processing. Perfusion technology is one of the newest methods for cell culture processes.

**Brønsted Acid/Base**

In the Brønsted–Lowry theory acids and bases are defined by the way they react with each other, which allows for greater generality. The definition is expressed in terms of an equilibrium expression:

$$\text{acid} + \text{base} \rightleftharpoons \text{conjugate base} + \text{conjugate acid}$$

$$HA + B \rightleftharpoons A^- + HB^+$$

With an acid, HA, the equation can be written symbolically as:

The equilibrium sign (⇌) is used because the reaction can occur in both forward and backward directions. The acid, HA, can lose a proton to become its conjugate base, A–. The base, B, can accept a proton to become its conjugate acid, $HB^+$. Most acid-base reactions are fast so that the components of the reaction are usually in dynamic equilibrium with each other.

**Carbon Evolution Rate (CER)**

It corresponds to the $CO_2$ produced by the cell culture and/or microbial fermentation (mol/l h). The CER can be calculated subtracting the inorganic carbon pool development over time (corresponding to the $NaHCO_3$ evolution over time), from the CTR.[29]

*CER = CTR – Inorganic Carbon Pool Over Time*

**Critical Process Parameter (CPP)**

Critical Process Parameter according to the PAT nomenclature. It is a parameter whose variability has an impact on a critical quality attribute (CQA) and, therefore, should be monitored or controlled to ensure the process obtains the desired quality.

*CPP Examples: pH, dissolved oxygen, and dissolved $CO_2$*

**Critical Quality Attribute (CQA)**

Critical Quality Attribute according to the PAT nomenclature. It is physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality.

*CQA Example: glycosylation patterns of monoclonal-antibodies. If the glycosylation patterns are not correct, then the proteins fold differently than expected, losing the therapeutic effect.*

**Carbon Transfer Rate (CTR)**

The carbon transfer rate corresponds to the transfer rate of $CO_2$ from gas to liquid phase in the bioreactor (mol/l h).

**Dissolved $CO_2$**

Dissolved $CO_2$ (or $DCO_2$) is a Critical Process Parameter in bioprocesses. A higher dissolved carbon dioxide level is toxic and can inhibit cell growth and reduce production of metabolites such as monoclonal antibodies (mAb). The dissolved $CO_2$ is commonly identified with the "Partial Pressure of $CO_2$" ($pCO_2$). The $pCO_2$ can be found expressed with different units in process analytics. To facilitate a comparison among suppliers and scientific literature a table with most common $pCO_2$ units conversion is presented.

| $pCO_2$ Units | mbar | kPA | mmHg | %Vol* |
|---|---|---|---|---|
| **mbar** | 1 | 0.1 | 0.750 | 0.1 |
| **kPa** | 10 | 1 | 7.50 | 1 |
| **mmHg** | 1.33 | 0.133 | 1 | 0.13 |
| **%Vol** | 10 | 1 | 7.5 | 1 |

**At temperature = 25°C | Atmospheric Pressure P = 1,013 mbar*

**Dissolved $O_2$**

Dissolved Oxygen (DO or $pO_2$) is a bioprocess Critical Process Parameter. Air or oxygen-enriched air is supplied to the bioreactor to support cell demand. Oxygen is used for cellular respiration and cellular growth. While important, DO can be controlled over a broader range than pH without too significantly impacting cell growth rates or product quality. Typical DO operating ranges for aerobic cultures lie between 30 to 40% air saturation. DO levels below this range will affect cell viability, whereas excessive DO levels can oxidize the end-product.

**European Society for Animal Cell Technology (ESACT)**

It is a Society which brings together scientists, engineers, and other specialists working with animal cells in order to promote communication of experiences between European and International investigators and progress the development of cell systems and productions derived from them.

**US Food and Drug Administration (FDA)**

The FDA is responsible for protecting the public health by ensuring the safety, efficacy, and security of human and veterinary drugs for the USA government.

**$K_LA$**

The $K_LA$ corresponds to the volumetric mass transfer coefficient for oxygen or $CO_2$ of a bioreactor and it is one of the dimensions which characterize it.[6]

**Key Performance Indicator (KPI)**

Key Performance Indicator, according to the PAT nomenclature. A KPI is a metric for the status of each production step. KPIs are related to CQAs and therefore influenced, as well, by the CPPs. As the CPPs remain within the pre-defined limits, the KPIs should indicate that each production step proceeds accordingly resulting, in the end, in a product having its CQAs within the appropriate limits, too.

*KPI Examples: viable cell density, culture viability, and product titer*

**Lactate Shift**

Lactate is strongly produced during a bioprocess exponential growth phase, while its net consumption is frequently observed when cells enter into the stationary phase. Such a metabolic shift from production to consumption is identified as “lactate shift” and it is desirable because it favors optimal process performance.[30]

**Minimal Essential Medium (MEM)**

Minimal Essential Medium (MEM) is a synthetic cell culture medium developed by Harry Eagle first published in 1959 in Science that can be used to maintain cells in tissue culture. It is based on 6 salts and glucose described in Earle's salts in 1934: (calcium chloride, potassium chloride, magnesium sulfate, sodium chloride, sodium phosphate, and sodium bicarbonate), supplemented with 13 essential amino acids, and 8 vitamins: thiamine (vitamin B1), riboflavin (vitamin B2), nicotinamide (vitamin B3), pantothenic acid (vitamin B5), pyrodoxine (vitamin B6), folic acid (vitamin B9), choline, and myo-inositol (originally known as vitamin B8).

**Dulbecco's Modified Eagle's Medium (DMEM)**

DMEM was originally suggested as Eagle's medium with a "Fourfold concentration of amino acids and vitamins" by Dulbecco and Vogt published in 1959. The commercial version of this medium has additional modifications detailed below.

**RPMI 1640 (RPMI Medium)**

RPMI 1640, also known as RPMI Medium, is a growth medium used in cell culture. RPMI 1640 was developed by George E. Moore, Robert E. Gerner, and H. Addison Franklin in 1966 at Roswell Park Memorial Institute, from where it derives its name. A modification of McCoy's 5A medium (or RPMI 1630), it was originally formulated to support lymphoblastoid cells in suspension cultures, but can also support a wide variety of adherent cells.

**Monitoring/Measuring At-Line**

Measurement where the sample is removed, isolated from, and analyzed in close proximity to the process stream.

**Monitoring/Measuring In-Line/In-Situ**

Measurement where the sample is not removed from the process stream and can be invasive or noninvasive

**Monitoring/Measuring On-Line**

Measurement where the sample is diverted from the manufacturing process, and may be returned to the process stream.

**Monitoring/Measuring Off-line**

The sample is taken out of the bioreactor in sterile conditions and analyzed in the lab after physical pretreatments (e.g. filtration and dilution).

**Osmolality**

Osmolality is a measure of the osmotic pressure of a solution, for example a complete cell culture medium. The osmolality of the culture medium should be similar to the osmolality of the natural environment of the cells, in vivo. The osmolality of cell culture media for most vertebrate cell lines is kept within a narrow range between 260 to 320 mOSM/kg.[31]

**Oxygen Transfer Rate (OTR)**

The oxygen transfer rate corresponds to the transfer rate of oxygen from gas to liquid phase in the bioreactor (mol/l h).

**Oxygen Uptake Rate (OUR)**

OUR corresponds to the oxygen consumed by the cell culture and/or microbial fermentation (mol/l h).

**Process Analytical Technology (PAT)**

The Process Analytical Technology guidance has been published in 2004 by the FDA. It is intended to describe a regulatory framework that will encourage the voluntary development and implementation of innovative pharmaceutical development, manufacturing, and quality assurance.

**Respiratory Quotient (RQ)**

Respiratory Quotient (RQ) is the the moles of carbon dioxide evolved per mole of oxygen consumed by the culture during the bioprocess. It is an indirect but a fairly rapid method of measurement to determine the lack of substrate in the growth medium.[32] It is also express as:[28]

$$RQ = CER/OUR$$

**Scale-Up/Scale-Down**

Scale-up and scale-down are therms referring to the transfer of the bioprocess from the R&D stage to the pilot or production stage (scale-up) or vice-versa (scale-down). The production/pilot bioreactor design is often much different than typically found in the laboratory. The larger bioreactor volume resulting from scale-up slows down the OTR and impacts the CER, further resulting, for example, in slower DO detection. If the PID control algorithms are set for the smaller scale, the response will be inaccurate. The mass transfer coefficient for oxygen, $K_LA$, must be kept constant in the scale up process to accurately predict OTR or CER. Frequent test runs need to be performed to adjust the control algorithms for dissolved oxygen.

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