Application note

Gas analysis mass spectrometer applications in fermentation and cell culture processes

Prima BT and Prima PRO Process Mass Spectrometers

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Introduction

The word 'biotechnology' was first used by Karl Ereky, a Hungarian agricultural engineer, in 1919.[1] The fermentation process plays a key role in biotechnology, producing a wide range of key products in a variety of industries:

- **Pharmaceuticals:** antibiotics, vaccines, prophylactics, hormones such as human insulin
- **Bioenergy:** bio-alcohol fuels based on low value, non-food based feedstocks
- **Biomaterials:** energy efficient, biodegradable plastics
- **Animal nutrition:** feed supplements, amino acids

In recent years the use of on-line Process Analytical Technology (PAT) has become a high-profile endeavor in the biotechnology industry. Yet, many fermentation scientists have been using Thermo Scientific™ process mass spectrometers as PAT tools since the early 1980s to monitor the composition of gas streams into and out of fermentors and bioreactors with speed and precision. Other important fermentation products include industrial enzymes, food additives and vitamins.

Fermentation

Fermentation is the term used by microbiologists to describe generating a product by means of the mass culture of a microorganism. This product can either be the cell itself (biomass production), the microorganism's own metabolite or a foreign product. Microorganisms that carry out their metabolism using oxygen are referred to as aerobic microorganisms. Some microorganisms can substitute nitrate or sulfate for oxygen and therefore grow in the absence of oxygen. These microorganisms are referred to as anaerobic.

Types of fermentation process

There are three variations of the fermentation process:

- **Batch fermentation**
  A sterilized nutrient solution in the fermentor is inoculated with microorganisms and incubation is allowed to proceed. During the course of the fermentation, oxygen is added (in case of aerobic microorganisms) along with acid or base to control the pH. The composition of the culture medium, the biomass concentration and the metabolite concentration generally change constantly as a result of cell activity.
- **Fed-batch fermentation**
  This is an enhancement of the closed batch process, where substrate is added in increments as the fermentation progresses.
- **Continuous fermentation**
  An open system is set up and sterile nutrient solution is added to the bioreactor continuously. An equivalent amount of converted nutrient solution with microorganisms is simultaneously harvested off the system.

Historically, fermentors and bioreactors were designed to be used repeatedly, with the vessel being sterilized carefully between batches to avoid possible contamination. More recently 'single use' vessels have become common, removing the need for costly and time consuming sterilization schedules. Figure 1 shows a 50 litre Thermo Scientific HyPerforma™ Single-Use Bioreactor (S.U.B.) which is used in mammalian cell culture fermentation. The complete line of HyPerforma S.U.B.s includes 50, 100, 250, 500, 1,000 and 2,000 litre sizes that helps ensure consistent scalability while providing a wide range of application usages. Additionally, Thermo Fisher Scientific supplies single-use fermentors (S.U.F.) of 30 and 300 litre sizes for use in bacterial or fungal fermentations.

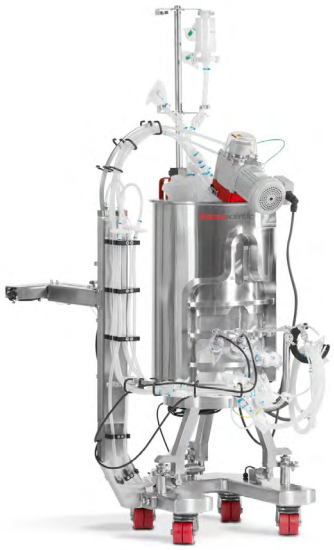

Figure 1: 50 L Thermo Scientific HyPerforma Single-Use Bioreactor (S.U.B.).

Three types of host organism are available for fermentation processes:

- **Bacteria: Lactic acid bacteria, *E. coli* and *Bacillus***
  These offer rapid growth, high cell densities and cheap substrates. They are relatively easy to cultivate and scale up but many are considered to be unsafe as pathogens. Product recovery can be much more difficult.
- **Microbial: Yeasts and molds such as *Saccharomyces cerevisiae* (Brewers' yeast)**
  These have been used for many years and are considered safe, making product approval less difficult. They can generate relatively cheap, high product volumes.
- **Mammalian: Chinese Hamster Ovary (CHO) cells, hybridomas**
  These produce highly dedicated products in very specific and active forms—vaccines, monoclonal antibodies, interferons and recombinant therapeutic proteins. Production routes are more complex than bacterial and microbial processes and the cell lines are less robust and more sensitive to shear and stress.

Thermo Scientific gas analysis mass spectrometers have been used successfully for over 30 years on bacterial and microbial processes. More recently, there has been increasing interest in mammalian cell culture because they offer the prospect of radical advances in vaccines, monoclonal antibodies and gene therapy. Although the requirements of mammalian cell fermentation processes provide challenges to the off-gas analyzer, our mass spectrometers have already proved invaluable in improving understanding and increasing yields of mammalian cell cultures.

The need for gas analysis

In any fermentation it is essential to monitor the state of the culture, since its health determines the conversion rate of nutrients, the formation of unwanted byproducts and, in the worst case, the onset of poisoning. Analysis of the respiratory gases being fed into and removed from the fermentor is an ideal way of characterizing the fermentation. It is noninvasive and enables monitoring of the physiological state of the fermentation, including growth kinetics and substrate consumption. It also helps determine the optimum point to halt the process for maximum yield.

Why use mass spectrometry for gas analysis?

Many fermentations are characterized by small changes in oxygen and carbon dioxide concentrations at critical phases of the fermentation, for example, during the lag phase when the microorganisms exist in equilibrium with the nutrients. It is vital that the method used for measuring off gas is capable of fast, precise analysis. The speed of MS makes it ideal for the fermentation application but speed must not be at the expense of precision. It is equally important that precise data is acquired; otherwise small changes in concentration will be lost.

Unfortunately, it is not uncommon to assume that the measurement of oxygen and carbon dioxide in the off gas is all that is required when making the first steps towards process control, and that sufficient accuracy can be achieved by discrete measurement technology. Both of these assumptions are false. Firstly, the sparge gas is always variable due to external biology—there are humans and animals undergoing respiration, consuming O_2 and generating CO_2, but during daylight hours there is also plant photosynthesis consuming CO_2 and generating O_2.

Secondly, the ubiquitous twin-tower desiccant dryer systems will either absorb or regurgitate CO_2 depending on where they are in the regeneration cycle.

These effects are illustrated in Figure 2 which shows an example of the day and night variations in sparge gas levels of CO_2 and O_2 measured with a Thermo Scientific Prima PRO Process Mass Spectrometer over 24 hours.

Only accurate comparison of sparge gas and effluent gas can provide accurate pre-screening for possible contamination. Accurate comparison is also required in order to calculate real-time information regarding culture respiration and the availability of nutrients.

Advantages of magnetic sector MS

Two types of MS have been used to monitor fermentation processes: magnetic sector, where charged particles are separated in a variable magnetic field, and quadrupole, where charged particles are separated in a variable RF field. Thermo Fisher Scientific manufactures both quadrupole and magnetic sector mass spectrometers; over thirty years of industrial experience have shown the magnetic sector based analyzer offers the best performance for fermentation off-gas analysis.[2,3]

Key advantages of magnetic sector analyzers include improved precision, accuracy, long intervals between calibrations and resistance to contamination. Typically, analytical precision is between 2 and 10 times better than a quadrupole analyzer, depending on the gases analyzed and complexity of the mixture.

The signal intensity at any specific mass position on a magnetic sector analyzer appears as a flat top peak. This means that any small drift in the mass scale will not result in a change in signal intensity. This is not the case with quadrupole mass spectrometers that provide rounded peaks. The magnetic sector analyzers used in the Thermo Scientific Prima family of mass spectrometers are laminated, so they scan at speeds equivalent to that of quadrupole analyzers, offering the unique combination of rapid analysis and high stability. This allows the fast and extremely stable analysis of an unlimited number of user-defined gases.

The excellent long term stability provided by magnetic sector MS is illustrated in Table 1. A Thermo Scientific Prima BT Bench Top Gas Analysis Mass Spectrometer was configured to analyze nitrogen, oxygen, argon and carbon dioxide in a cylinder of compressed air continuously, without interruption or recalibration, for seven days. The analysis cycle time was 5 seconds to measure these four components. Day-to-day mean values for nitrogen and oxygen varied by 0.005 %mol or less, day-to-day mean values for carbon dioxide varied by 1 ppm or less. Figure 3 shows graphical displays of the four gas readings, taken from the Thermo Scientific GasWorks® Software Data Review Plus module. This long term stability is only available from a magnetic sector MS—quadrupole mass spectrometers require frequent calibration to correct for their inherent drift.

Rapid multi-stream sampling

If the MS is to monitor multiple bioreactors then a fast, reliable means of switching between streams is required. Solenoid valve manifolds have too much dead volume and rotary valves suffer from poor reliability so Thermo Fisher Scientific developed the unique Rapid Multi-Stream Sampler (RMS). It offers an unmatched combination of sampling speed and reliability and allows sample selection from up to 64 streams. Stream settling times are application dependent and completely user configurable. The RMS includes digital sample flow recording for every selected stream. This can be used to trigger an alarm if the sample flow drops, for example if a filter in the sample conditioning system becomes blocked. The RMS is heated to ensure fast response to even the most 'sticky' of volatiles.

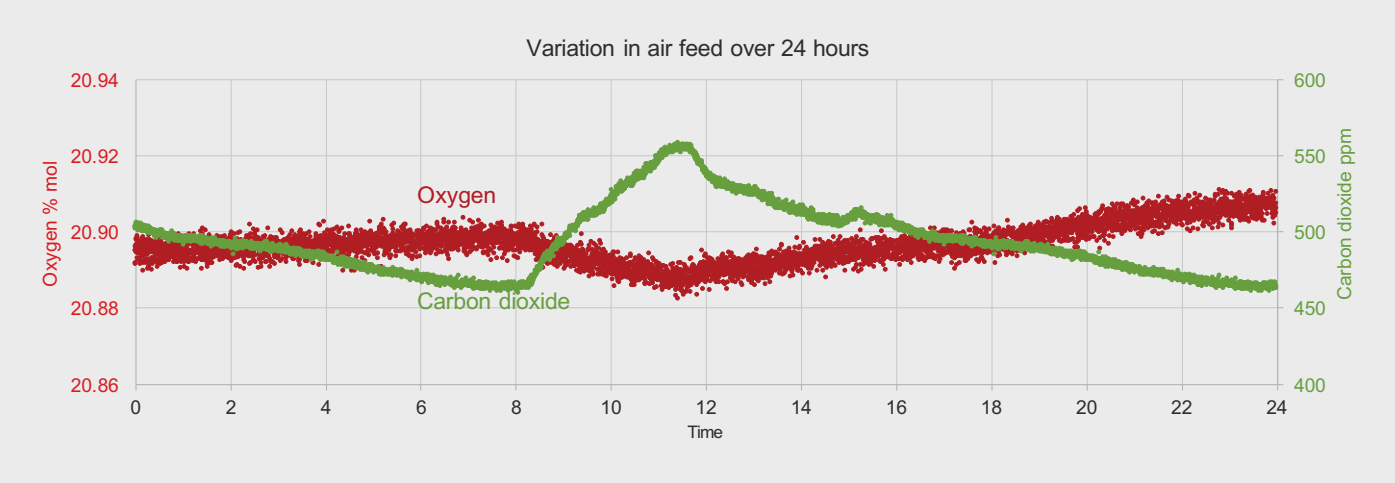

Figure 2: Example of day and night variations in sparge gas levels of CO_2 and O_2 measured with Prima PRO over 24 hours.

	Nitrogen %mol		Oxygen %mol		Argon %mol		Carbon dioxide ppm	
	Mean	Std dev	Mean	Std dev	Mean	Std dev	Mean	Std dev
Day 1	78.0807	0.0028	20.9459	0.0026	0.9337	0.0003	396.84	1.31
Day 2	78.0767	0.0023	20.9494	0.0023	0.9342	0.0003	397.46	1.25
Day 3	78.0761	0.0024	20.9500	0.0023	0.9342	0.0003	397.34	1.28
Day 4	78.0798	0.0023	20.9469	0.0023	0.9337	0.0003	396.31	1.31
Day 5	78.0777	0.0030	20.9487	0.0028	0.9339	0.0003	396.76	1.34
Day 6	78.0741	0.0023	20.9518	0.0022	0.9344	0.0003	397.47	1.27
Day 7	78.0750	0.0023	20.9512	0.0022	0.9342	0.0003	397.23	1.30

Table 1: Table 1 Analysis of compressed air cylinder over 7 days by Prima BT magnetic sector MS.

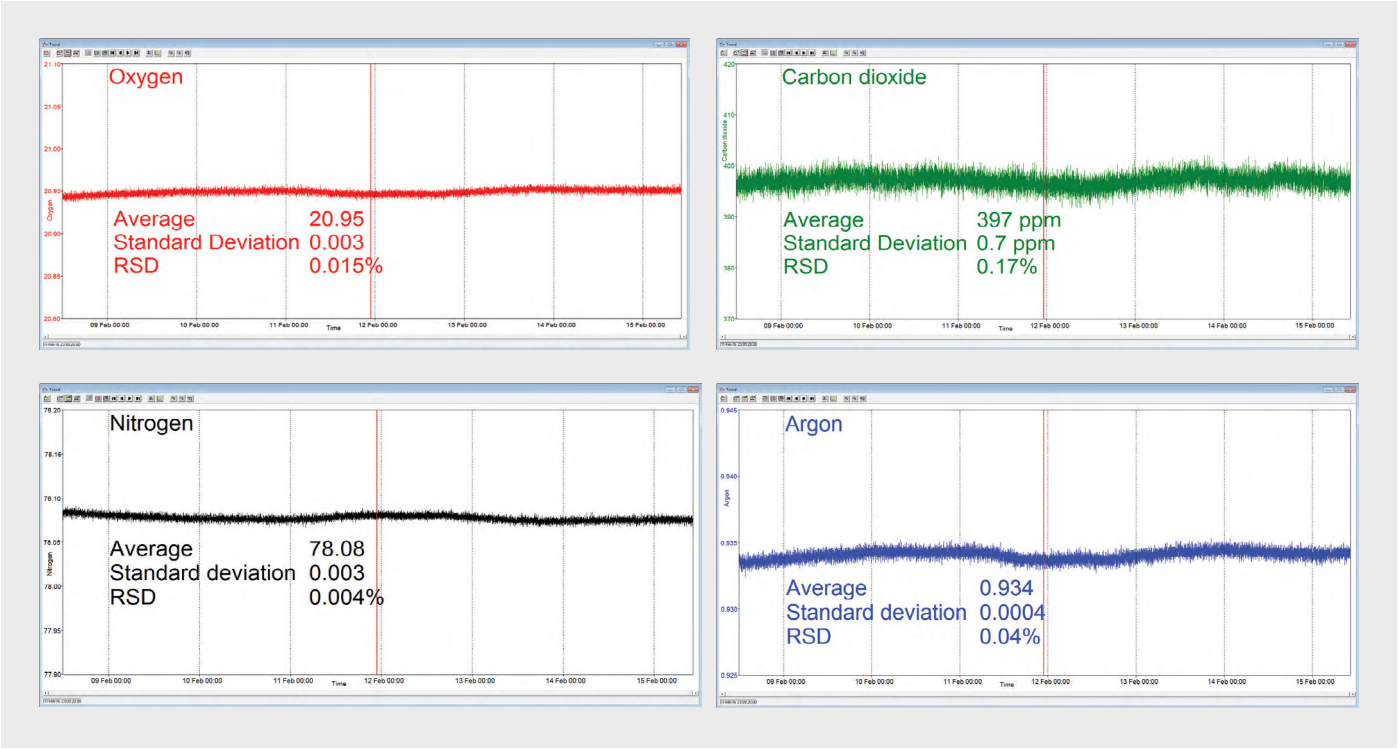

Figure 3: Long term stability data from Prima BT magnetic sector MS.

CER (CO_2 Evolution Rate)	=	(%Volume of CO_2 out × flow out)	–	(%Volume CO_2 in × Flow in)
OUR (O_2 Uptake Rate)	=	(%Volume of O_2 in × flow in)	–	(%Volume O_2 out x Flow out)
RQ (Respiratory Quotient)	=	CER/OUR		

Table 2: Respiratory quotient for fermentation off-gas analysis.

Respiratory quotient

Respiration is the process whereby an organism oxidizes food to produce energy. An important control parameter in the fermentation process is therefore the Respiratory Quotient (RQ). This is the ratio of the Carbon Dioxide Evolution Rate (CER) to the Oxygen Uptake Rate (OUR). The full calculation of RQ is shown in Table 2.

A high RQ means that high levels of CO_2 are being produced and the metabolism is therefore operating at high efficiency. The RQ is also invaluable in identifying and controlling the carbon source being metabolized. For example, if glucose (or other carbohydrates) are the carbon source, then RQ will theoretically be 1.

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$$

$$RQ = \frac{6CO_2}{6O_2} = 1$$

However this equation only relates to combustion, it does not cover the production of biomass or product formation. So for example the RQ for glucose is unlikely to be 1.00, it may actually be something like 1.04.

Accurate determination of RQ relies on determination of the ratio of the flows in and out of the bioreactor. This ratio is easily determined by a scanning MS, which can measure N_2 and Ar in addition to O_2 and CO_2. At least one of these two gases will be inert to the process so it can be used effectively to correct for the humidity change that occurs when the dry air feed gas is bubbled through the fermentor liquid. Without this correction, errors are introduced into the head-space data due to dilution by the additional water vapor.[4] The calculation for RQ using nitrogen as the flow correction is shown in Table 3. The Thermo Scientific GasWorks Software calculates RQ as a standard feature for the fermentation application.

=	{CO_2out	x	(N_2in / N_2out)}	–	CO_2in
	O_2in	–	{O_2out	x	(N_2in / N_2out)}

Table 3: Respiratory quotient calculated by MS

Figure 4 shows data from an *E. coli* fed batch fermentation. Our MS measures oxygen, carbon dioxide, nitrogen and argon in both inlet and outlet, and calculates RQ. At temperature T°, a reagent is introduced to induce cells to change from multiplying to expressing the desired product.

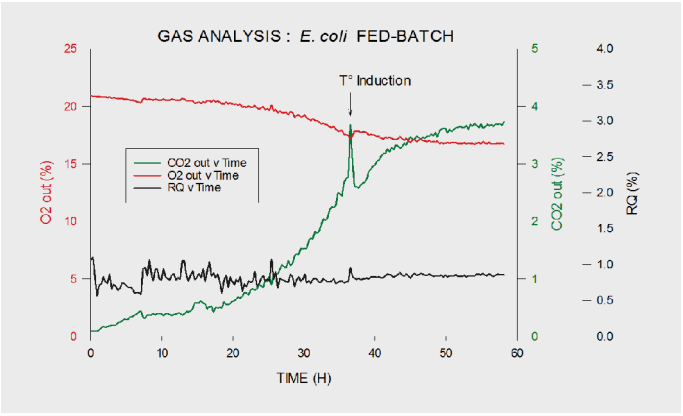

Figure 4 Off-gas and RQ data generated by MS from *E. coli* fed-batch fermentation.

Analysis of volatiles

The respiratory gases are not the only species of interest in the off gas. Volatile organics such as methanol, ethanol, ethyl acetate and even hydrogen sulfide can be found at ppm levels in the head space and their analysis can yield vital information on the well-being of the fermentation. However, their analysis provides certain technical problems that must be overcome if the analytical data is to be meaningful.

For example the measurement of trace levels of methanol and ethanol require the measurement of the CH_2OH+ fragment at mass 31. However we need to consider the presence of a very large peak at mass 32 from the percentage levels of O_2 in the vent gas. We need to correct for the tail from the 32 peak if we are to make an accurate measurement of trace alcohol levels.

The intensity of the tail from O_2 at mass 31 compared with the intensity of the peak at mass 32 is around 0.02 %. When the concentration of O_2 is around 20 % this means the signal at mass 31 is equivalent to around 40 ppm. During calibration this interference is recorded so that subsequent analysis is properly corrected. On a quadrupole MS this interference level is much greater and also variable, resulting in excessive uncertainty in low level ethanol measurement. Effectively a low level ethanol signal gets 'buried' in the noise from the oxygen peak. With a magnetic sector instrument the measurement is very reproducible and methanol and ethanol can be measured with a precision down to 10 ppm.

Figure 5 shows data from an *S. cerevisiae* fed batch fermentation. Our MS measures oxygen, carbon dioxide, nitrogen and argon in the inlet; it also measures ppm levels of ethanol in the head space in the outlet, and calculates RQ. Ethanol is consumed as the initial carbon source, then switched over to glucose to express the desired product. It is worth noting that the precision is lower at the very start of the fermentation since the volume of oxygen consumption is extremely low and the signal to noise ratio is correspondingly low. This period is termed the lag phase during which the cell count is extremely low. Once the organisms begin to multiply, the precision quickly improves.

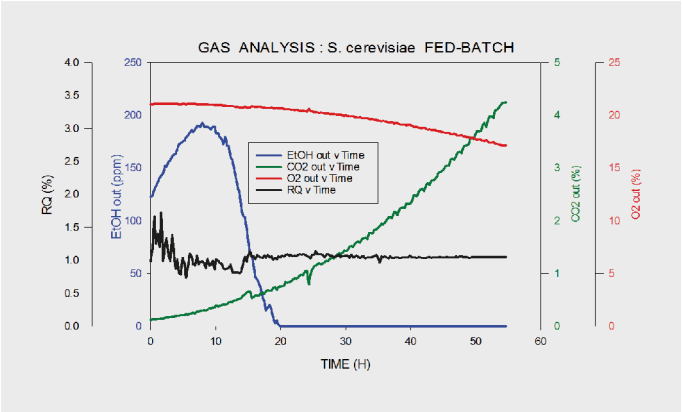

Figure 5: Off-gas and RQ data generated by MS from *S. cerevisiae* fed-batch fermentation.

The standard performance specifications for our Prima PRO magnetic sector MS is shown in Table 4. Precision is the standard deviation observed over 16 hours. Note the extremely high precision—0.05 % relative over 16 hours for oxygen. The analysis time including stream switching time is 30 seconds per stream for all 6 components. This reduces to just 10 seconds per stream if methanol and ethanol are omitted from the analysis.

Component	Concentration range %mol	Precision of analysis by Prima PRO (single standard deviation) ≤
Nitrogen	0 – 100	0.005 %mol
Oxygen	0 – 100	0.005 %mol
Argon	0 – 1	0.001 %mol
Carbon dioxide	0 – 10	0.1 % relative or 0.0003 %mol*
Methanol	0 – 1	2 % relative or 0.001 %mol*
Ethanol	0 – 1	2 % relative or 0.001 %mol*

* Whichever is greater

Table 4: Example of standard performance specification for Prima PRO Process MS.

Mammalian cell culture

In microbial and bacterial fermentations, the feed gas composition is relatively constant—either air or air enriched with oxygen. In mammalian cell fermentations, the feed gas composition is a frequently changing mixture of several compounds (e.g., nitrogen, oxygen and carbon dioxide). The feed gas concentration ranges vary dramatically—for example carbon dioxide can vary from tens of parts per million to tens of percent. If an overlay gas is used this introduces further complications because the overall inlet gas composition is a combination of sparge gas and overlay gas. Also the use of sodium bicarbonate as a pH buffer can contribute to the carbon dioxide level in the outlet.

These factors all contribute to a challenging set of requirements for the off-gas analyzer if it is to provide meaningful on-line data to complement off-line data such as viable cell count and metabolite concentration data. Figure 6 illustrates how the magnetic sector's speed and precision are able to provide this valuable data. A Prima BT monitored two 5 litre bioreactors in which modified Chinese Hamster Ovary (CHO) cells were used to express monoclonal antibodies.

The MS analyzed inlet and outlet gases for O_2, CO_2, N_2 and Ar on both bioreactors. Besides the on-line MS measurements, cell count (both total and viable), lactate levels and glucose levels were analyzed off-line. For each bioreactor there was just one inlet—the sparge gas, which was a combination of O_2, CO_2, N_2 and Air. Oxygen feed was used to control the dissolved oxygen and carbon dioxide was used to control pH. MS cycle time was 15 seconds per point including settling time, and concentrations were averaged over 99 readings to reduce noise caused by perturbations in sparge gas concentrations. As there is little activity at the beginning of the run, Figure 6 displays data from 100 hours onwards.

Although both bioreactors were controlled by the same controller, Bio 1 exhibits a very different inlet CO_2 profile as significant quantities of CO_2 were injected to adjust pH. The more stable CO_2 profile in Bio 2 led to a higher Viable Cell Count and an extended culture duration; during subsequent runs the control strategy was modified, leading to further extensions in culture durations and increased VCC levels.

Scale up from laboratory to bulk production

The manufacturing process typically begins with cell cultures grown in the laboratory. Then, during the scale-up process, cells are sequentially transferred to larger and larger fermentors, eventually into production vessels that can hold up to 20,000 litres of growth media and cells. It is vital to maintain the precise environment that specific cells need to remain healthy and grow—this requires precise off-gas analytical data through every stage of the scale up process, from laboratory to pilot plant to bulk production. In some cases one mass spectrometer fitted with a suitable RMS multi-stream inlet can monitor all the fermentors, in other cases separate MS analyzers have to be used in the laboratory and on the plant. It is critical that results from the two analyzer platforms correlate to ensure a smooth transition through the various stages of scale up.

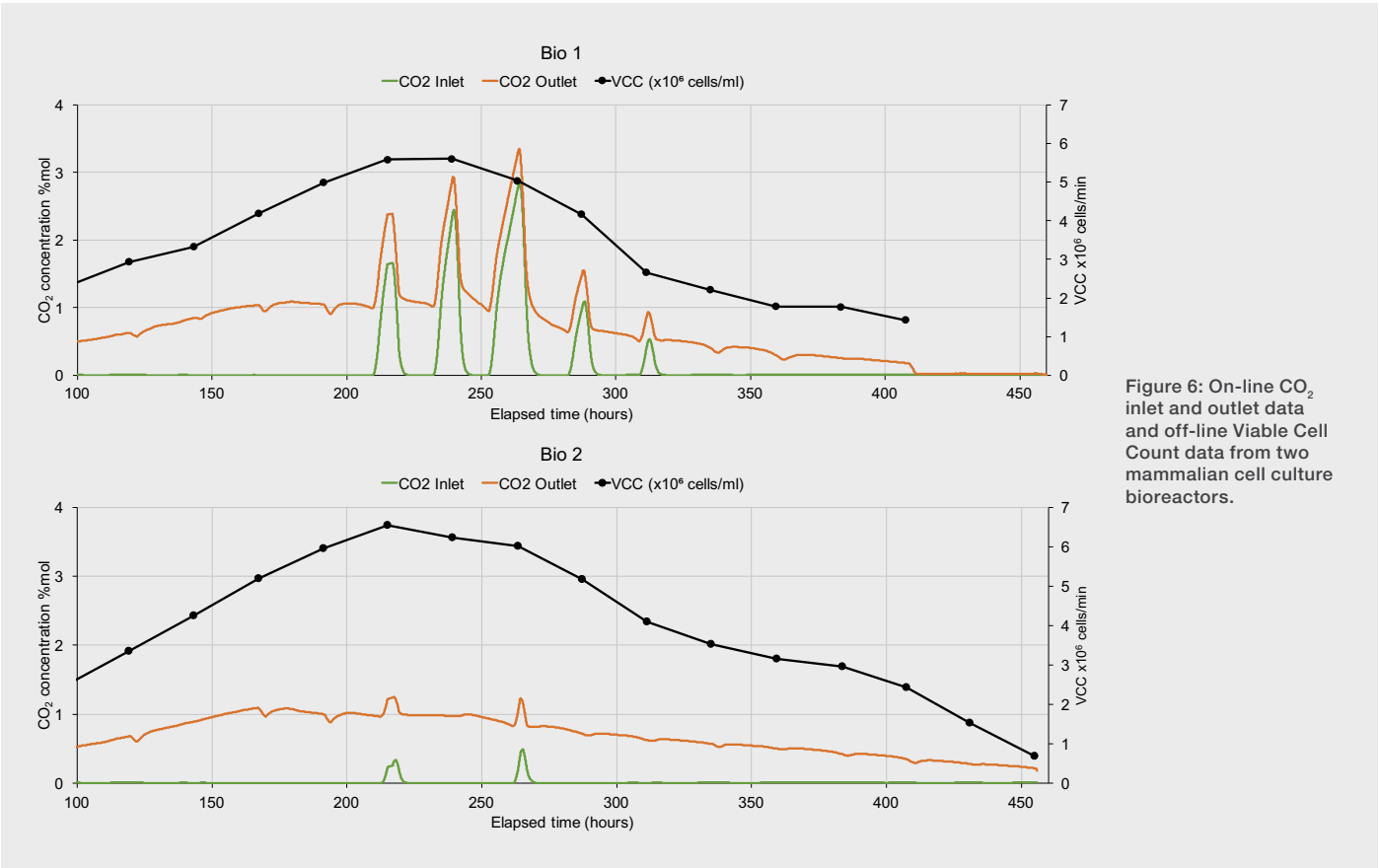

Figure 6: On-line CO_2 inlet and outlet data and off-line Viable Cell Count data from two mammalian cell culture bioreactors.

Figure 7 shows an example of a mass spectrometer suitable for fermentation process development, the Prima BT, Figure 8 shows an example of a mass spectrometer suitable for production process monitoring, the Prima PRO. Both system share Thermo Fisher Scientific's magnetic sector analyzer for high precision multicomponent gas analysis and both offer the uniquely reliable Rapid Multi-Stream Sampler.

Summary

Magnetic sector mass spectrometers have demonstrated the highest levels of precision for fermentation off-gas analysis and have been successfully monitoring fermentor off-gas at many of the world's leading biotechnology and pharmaceutical companies for many years. By combining high speed with excellent stability, the magnetic sector analyzer lends itself ideally to this demanding application.

Thermo Fisher Scientific's gas analysis MS product range, Prima BT and Prima PRO, provide fast, precise off-gas analysis through every stage of the fermentation and cell culture processes from laboratory to pilot plant to bulk production. Prima PRO is equipped to monitor 60+ bioreactors without compromising sterility. Prima BT provides a bench-top solution for smaller scale bioreactors, being configured with 15 sample and 6 calibration ports. The highly precise and complete gas composition measurements provided by both models are easily incorporated into the APC system. Significant improvements in process control can be achieved very quickly—usually within a day or two of start-up.

Figure 7: Prima BT Bench Top Mass Spectrometer

Figure 8: Prima PRO Process Mass Spectrometer

References

1. M.G. Fári, U.P. Kralovánszky, The founding father of biotechnology: Károly (Karl) Ereky. Int. J. Hortic. Sci., Vol 12, n.1, p.9-12, 2006.
2. David Pollard, Jens Christensen, Vent Gas Analysis, Encyclopedia of Industrial Biotechnology: Bioprocess, Bioseparation, and Cell Technology 2010, John Wiley & Sons.
3. Joseph S. Alford, Bioprocess control: Advances and challenges, Computers & Chemical Engineering Volume 30, September 2006.
4. P.C. van der Aar, A.H. Stouthamer, H.W. van Verseveld, Possible misconceptions about O_2 consumption and CO_2 production measurements in stirred microbial cultures, Journal of Microbiological Methods Volume 9, Issue 4, June 1989.

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