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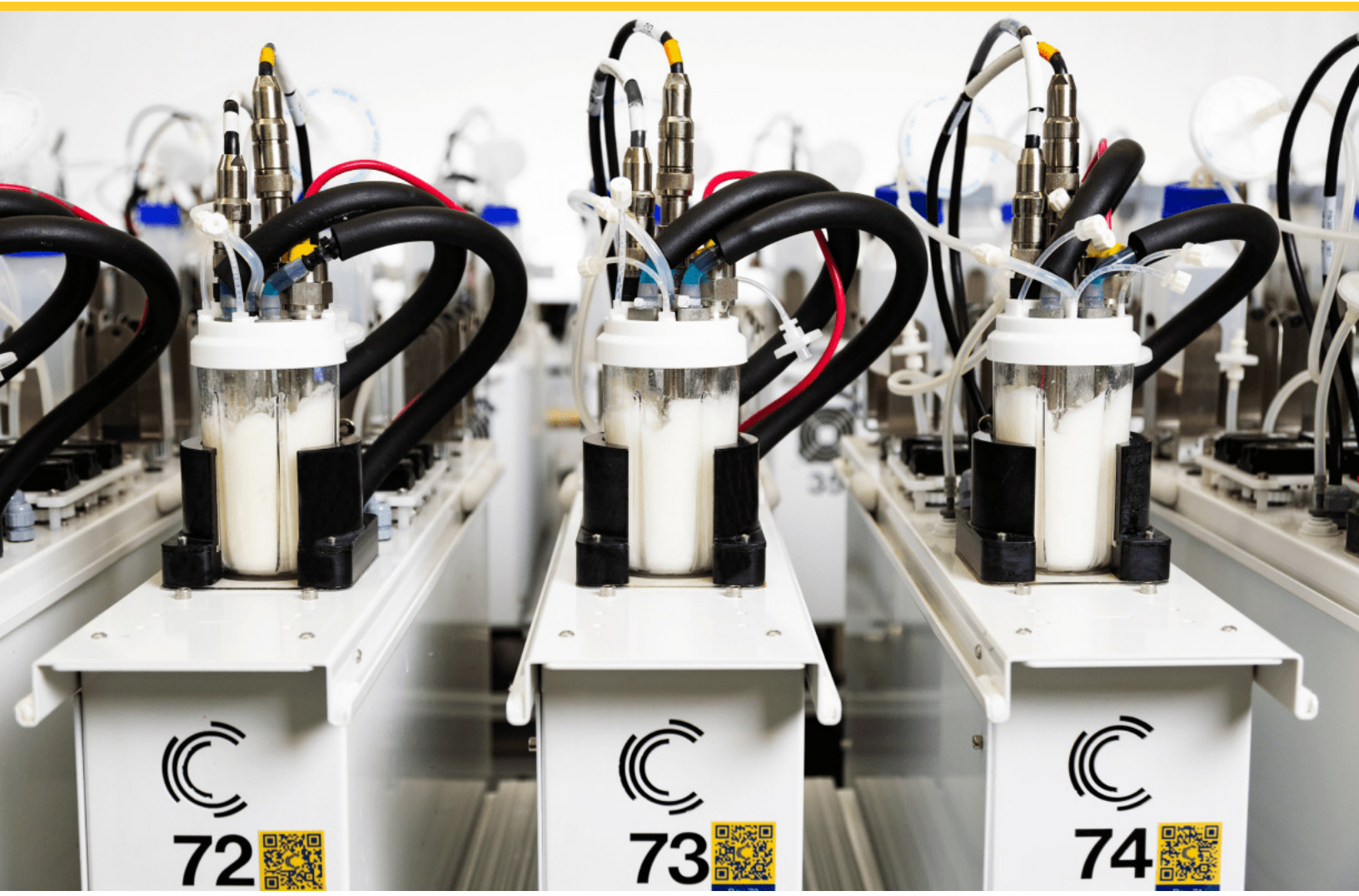

ENSURING DATA QUALITY THROUGH MASS BALANCE CALCULATIONS IN CULTURE BIOSCIENCES' CLOUD BIOREACTORS

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The Importance of Mass Balance Calculations

In order to make improvements to a microbial strain and fermentation process, it is important to have a thorough understanding of carbon fluxes within a bioreactor. While volumetric measurements can be informational, such as titer in g/L, they do not provide information around the total amount of product made or total loss of carbon to byproducts such as CO_2. Without calculation of mass balances and use of mass-based measurements, it is difficult to gain more than a cursory estimate of strain performance and product fluxes.

Measurements taken throughout a fermentation to accurately track total mass additions and losses enable the calculation of mass-based metrics from the fermentation. Culture Biosciences' automated cloud bioreactors are equipped with hardware and software features that allow for accurate tracking of mass (both liquid and gas), which provides a more comprehensive assessment of strain performance and opportunities for both strain and process improvements.

INTRODUCTION

To truly understand both strain performance and the impact of bioprocess adjustments it is critical to know the amount, kinetics, and efficiency of molecule production; namely, titer, rate, and yield. A comprehensive understanding of these metrics allows for an assessment of the commercial feasibility of a process and informs where improvements to a microbial strain and fermentation process should be directed.

Throughout a microbial fermentation process, there are numerous inputs and outputs that together contribute to performance. A fermentation may begin with a batch medium, into which microbes are inoculated and start to grow. Once the initial carbon source is exhausted, cells may be maintained in a nutrient-limited state with the addition of various feeds. The pH is maintained with the addition of acid or base, and mass may be removed as samples to take measurements or as part of a continuous process. In addition, other factors such as liquid evaporation and uptake or evolution of gases may impact mass entering or leaving the reactor. All of these factors impact the total mass in the reactor, which in turn determines the total amount of product in the system. Inputs and outputs to a fermentation are described in Figure 1.

Measurements Required for Calculation of a Mass Balance

Figure 1: *There are many inputs and outputs in a fermentation process which must be tracked and measured in order to calculate accurate metrics from the fermentation.*

For all fermentations run at Culture Biosciences, the following inputs and outputs are always measured:

- **O_2 Delivered:** Each vessel is fitted with four Mass Flow Controllers (MFCs), which measure the amount of each gas (Air, N_2, CO_2, O_2) delivered.
- **Initial Process Mass:** The initial mass of each fermentation is measured prior to the initiation of each fermentation.
- **Manual Additions:** The mass of all manual additions, such as inoculum and addition of inducers are measured.
- **Mass Pumped:** Scales on up to 5 feed bottles allow for the mass added (accounting for evaporation from bottles and tubing) to each vessel throughout the fermentation to be measured. Any mass pumped out of the reactor is also accounted for.
- **Vessel Evaporation:** The amount of evaporation over the course of the fermentation is continuously estimated in real time by custom software.
- **CO_2 Evolved and O_2 in the Off-Gas:** Individual custom-built off-gas sensors fitted to each reactor allow for continuous measurement of off-gas composition.
- **Manual Removals:** The mass of all removals, such as samples, are measured.
- **Final Vessel Mass:** The final process mass of each fermentation is measured.

Accounting for all of these inputs and outputs is necessary to accurately measure the total amount of product in the reactor. Without an accurate measurement of total product by mass (in contrast to a volumetric measurement of titer, which does not account for reactor volume), calculated performance metrics will be inaccurate. This, in turn, could lead to an inaccurate estimate of strain performance, which at its worst could lead to the development of a fermentation process that is not commercially viable.

Measurement of all these inputs and outputs requires installation of additional equipment that is not typically included in off-the-shelf fermentation systems. For example,

to measure gas leaving the reactor, each vessel must be equipped with off-gas sensors that log measurements for O_2 and CO_2 in the off-gas. Continuous off-gas sensors typically cost about $8,000 each. Even with auxiliary hardware installed, advanced software infrastructure is then required to aggregate data, and personnel time is needed to analyze it to calculate mass balances. Many labs do not make these investments, preventing calculation of full mass balances thus complicating development and optimization of commercially viable bioprocesses.

Culture's cloud-based bioreactor platform has been developed from the outset with the importance of mass-based measurements in mind. Culture's bioreactors have a 250mL working volume, and have features included in many similarly sized reactors. A distinguishing feature of Culture's system is that each reactor is equipped with hardware to accurately measure every input and output for every fermentation process. Custom control software enables all inputs to the reactors to be delivered accurately and all manual additions or removals to be tracked. Together, these features allow mass balances to be calculated across a range of organisms and fermentation processes, giving customers high quality mass-based data measurements. These comprehensive datasets can then be used to calculate mass-based metrics that reflect the true performance of a microbe within a fermentation.

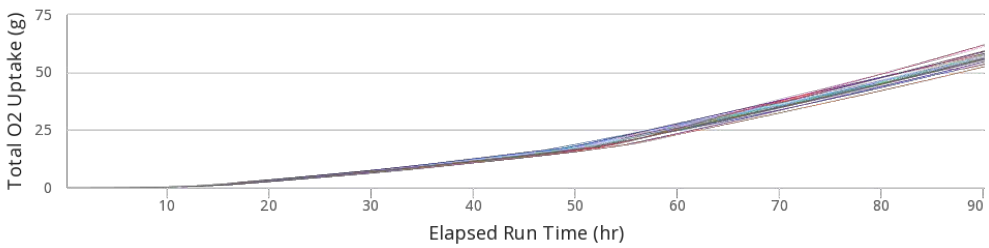
MATERIALS AND METHODS: A SUMMARY OF CULTURE'S TRACKING INFRASTRUCTURE FOR MASS BALANCES

Gas Delivery and Evolution

Each of Culture's bioreactors are fitted with four mass flow controllers (MFCs) capable of delivering between 0 - 45 mmol/min of gas. These, as with many of Culture's measurements, are reported online as a volumetric measurement (in this case SCCM), but are converted to mass for mass balance calculations. Each flow controller can be calibrated for a variety of different gases (O_2, CO_2, N_2, etc.) so that all input gases into the fermenter are accurately measured. Each bioreactor is also equipped with off-gas sensors measuring O_2 and CO_2 exiting the reactor. Together, by tracking both gas in and gas out, these measurements allow oxygen uptake (g) and carbon dioxide (g) evolved from cells to be calculated for each fermentation run. This can be observed in Figure 2, where the cumulative amount of O_2 taken up and CO_2 evolved are recorded over the course of a run and the final values are used in the mass balance calculations. As seen in this graph, the values vary significantly between conditions or strains, and are required for accurate mass balance calculations.

Measurement of Gas Uptake and Evolution

(a) Total Oxygen Uptake



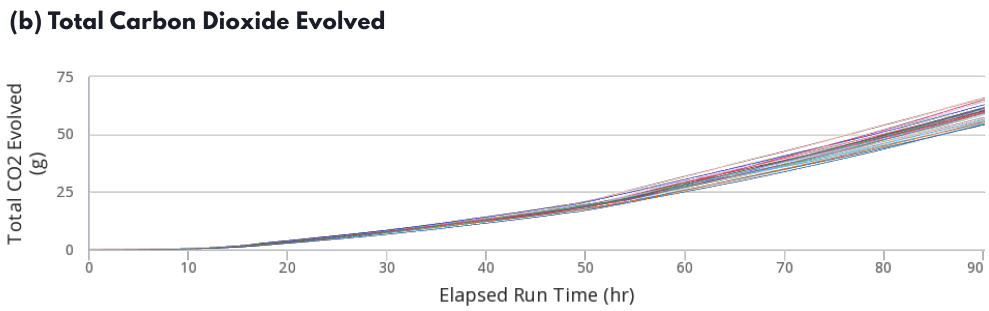


Figure 2: *Measurements of flow rates and off-gas concentrations enable calculation of the mass of (a) oxygen taken up and (b) carbon dioxide evolved by organisms in the reactor over the duration of the cultivation.*

Evaporation Studies

Fermentation processes rely on aeration and agitation in order to efficiently mix the fermentation broth and transfer oxygen into solution. These, combined with the temperatures required for optimal growth and production by different organisms, can result in the saturation of air exiting the reactor with evaporated liquid. Despite the use of condensers to condense liquid vapor so that it does not exit the reactor, significant vessel mass can still be lost to evaporation. Failure to account for this loss results in an inaccurate assessment of the mass, and therefore total product, in the fermentation vessel.

Accounting for the loss of liquid to evaporation can improve the estimation of reactor broth mass and total product throughout the fermentation. However, a number of reactor specific conditions such as condenser temperature and vessel back pressure can impact evaporation. To account for these, evaporation under a wide variety of conditions in Culture's bioreactors was empirically determined.

Vessels were filled with 200mL of PBS and were incubated over a range of temperatures from 4 - 37°C, airflows of 2.25 - 13.5mmol/min, and agitation ranging from 750 - 3500rpm. The mass evaporated was measured over six days of incubation and the experimentally derived values were used to build a response surface methodology model of evaporation.

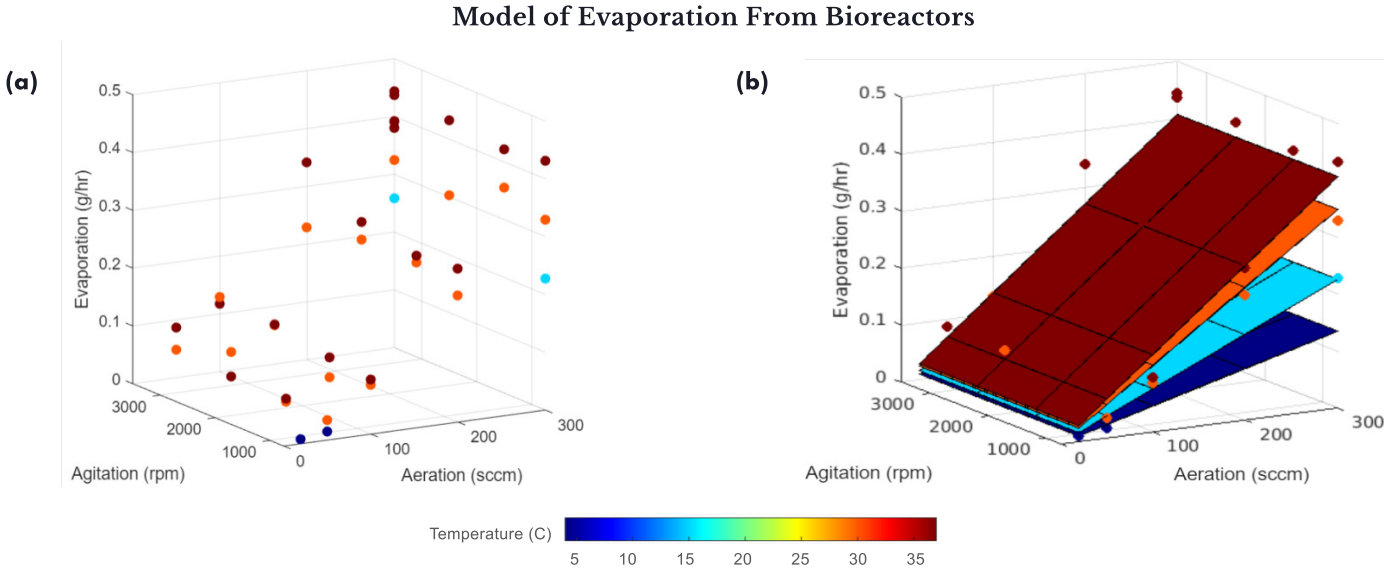


Figure 3: *(a) Evaporation rates were measured in Culture's reactors under a number of different conditions (b) and results were used to calculate a RSM of evaporation to be incorporated into experiments.*

The model generated can be described by the following formula:

Evaporation = A(total gas flow)*(temperature) + B*(agitation) + C*(total gas flow)*

Where A, B, and C are coefficients derived from the experiment data, and result in a predictive model with an R^2 of 0.968. Each of the coefficients quantifies the relative impact of each factor in the model. In Culture's reactors, as can be seen from the RSM model in Figure 3b, the primary factor impacting evaporation from the reactor is the total gas flow through the reactor. Temperature has a large impact over a wide range of temperatures, but a much more modest impact under biologically relevant conditions for most organisms, and interacts with the gas flow rates to impact evaporation. Agitation had the lowest impact of factors tested on evaporation.

This model is then applied to online data in real time (Figure 4), providing a more accurate estimate of the broth mass at any given time point. While this model does not account for changes in evaporation due to changes in volume, type of liquid media, or the presence of microorganisms in the broth compared with the 200mL of PBS in the reactor used in each condition, it does provide a better estimate of vessel mass at a given time point than without an evaporation correction factor.

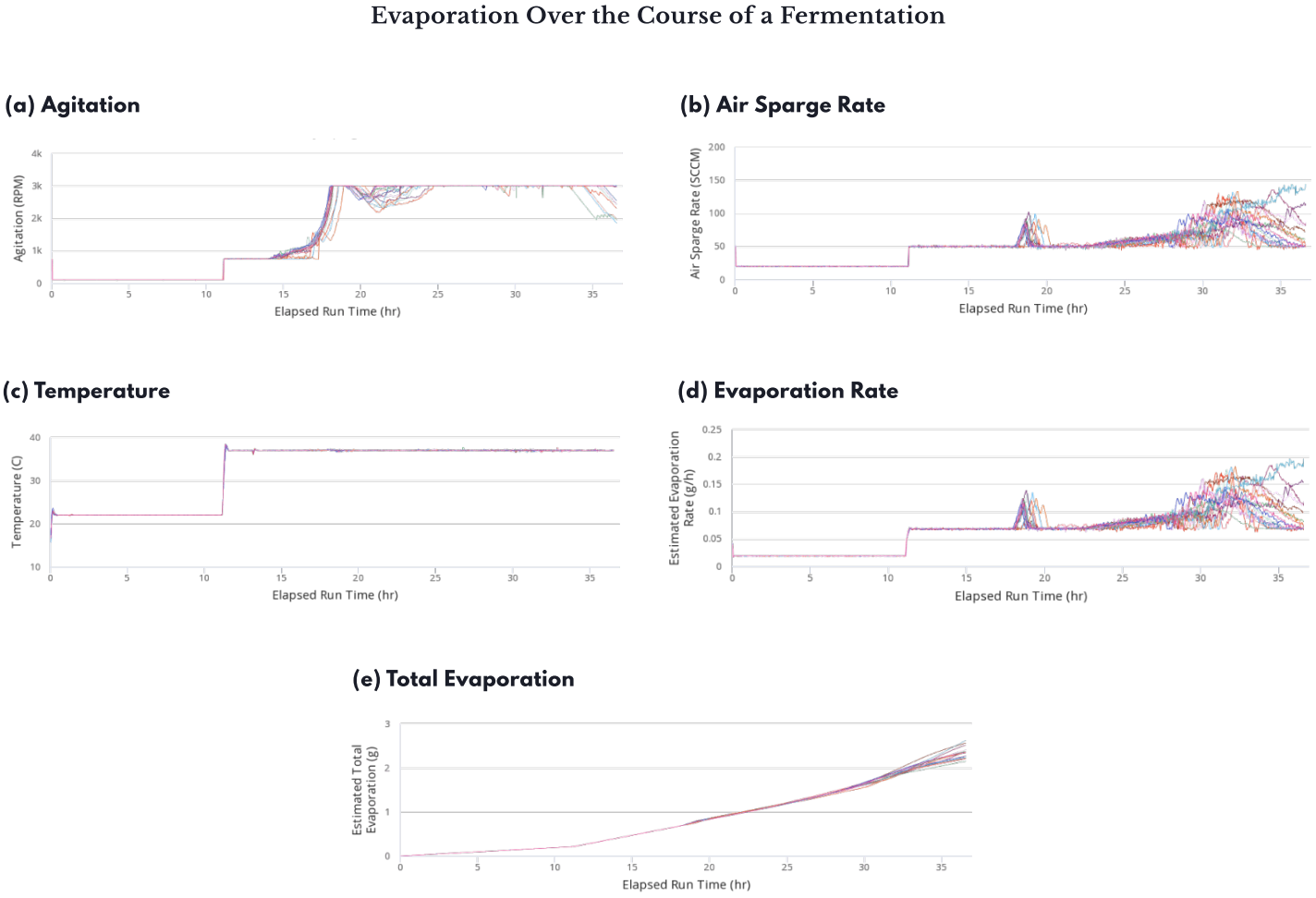


Figure 4: *Evaporation corrections are applied to a fermentation run based on agitation (a), airflow (b) and process temperature (c). When changes are made to the setpoints that impact evaporation, the corresponding changes in evaporation rates are applied within the run (d) and incorporated into the total evaporation over the course of the fermentation run (e).*

Changes in setpoints (Figures 4 a, b, c) impact evaporation throughout the fermentation (Figure 4d), which are reflected in changes in slope of the total vessel evaporation line (Figure 4e). This mass of evaporation is then applied to correct the fermentation broth mass to enable accurate calculation of the broth mass at a given time point. In this example, evaporation was even estimated during a cooled hold before inoculation over the first portion of the experiment.

In an analysis of over 500 fermentation runs (Figure 5a), ranging from 22 hours to 120 hours in length and using various feeding strategies, the median calculated evaporation from the vessels was 4.95% of the total final broth mass, representing a significant amount of the total broth volume. However, depending on the conditions of the process, evaporation can account for a much larger proportion of the total broth. Failure to account for this loss in analytical measurements leads to an overestimate of the mass in the reactor, impacting mass balance closure (Figure 5b) and increasing the potential to yield misleading results.

Vessel Mass Lost to Evaporation and Impact on Mass Balance

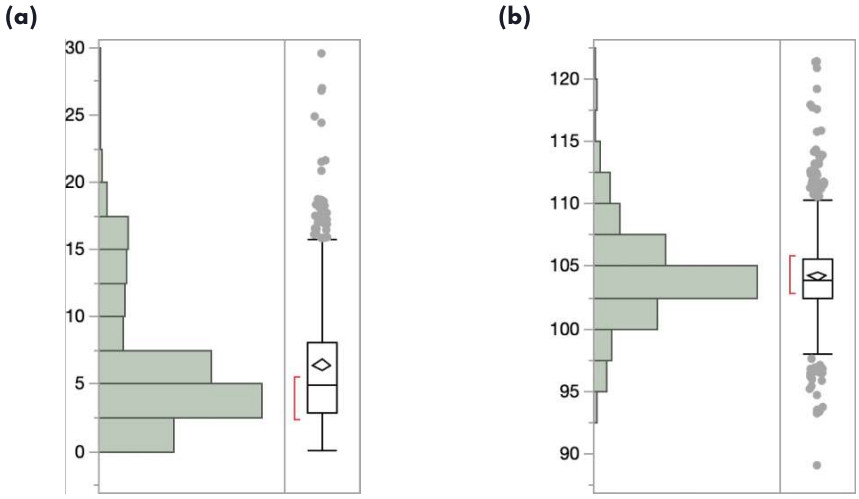


Figure 5: *(a) Evaporation from fermentation vessels as a percentage of total final broth mass was calculated from over 500 individual fermentation runs and a variety of fermentation processes. (b) Evaporation accounts for an average of 4.95% of the vessel mass and failure to account for this loss results in an inaccurate mass balance.*

Fluid Delivery Into Fermentation Vessels

A common method of fluid delivery to reactors is through the use of peristaltic pumps. These pumps provide a simple and economical method for delivery of feeds, acid, and base over a wide range of flow rates. They can also be programmed to change pumping rates over the course of a fermentation.

However, delivery of feeds via a peristaltic pump is prone to error, with actual feed rates often deviating significantly from the setpoint. Feed rates can be influenced by factors as nuanced as how a feed line is threaded through the pump by an individual operator or the length or age of the pump tubing. In addition, unless pumps are regularly calibrated with the relevant solutions they are pumping, deviation from the setpoint can increase over time between calibrations. It is not uncommon for feed quantities delivered via a peristaltic pump to accumulate errors of 5 - 10% from setpoint over the course of a fermentation. These deviations can significantly change results by impacting derived calculations calculated on a volumetric basis such as yield, or by altering strain physiology itself.

A more precise manner of delivering feeds to a bioreactor is by feed weight. Traditionally, scales are expensive and require a large lab footprint, limiting the feasibility of this approach for many labs. In contrast, each of Culture's tanks is equipped with individual load cells for each feed, and can accommodate up to five individual feeds. This capability, described below, typically results in feed delivery within +/- 3% of the intended setpoint over the course of the fermentation, with real-time feed correction based on weight measurements.

Each feed rate is programmed within a fermentation protocol using the peristaltic pumps fitted to the reactor. The load cell measures the mass of each feed bottle and compares that to the predicted mass based on the commanded pump flow rate. Polypropylene feed bottles were selected to deliver feeds, as bottles with the smallest mass serves to minimize errors in mass measurements. A custom developed control algorithm determines whether the actual feed delivered deviates from the intended flow rate based on the bottle weight and inputted liquid density, and provides feedback to adjust the pump speed to match the intended flow rate accordingly. This can be visualized in Figure 6, where a constant flow rate setpoint was programmed into the reactors (Figure 6a), but the actual feed rates are constantly being adjusted based on feedback from the load cells in order to achieve the intended setpoint (Figure 6b).

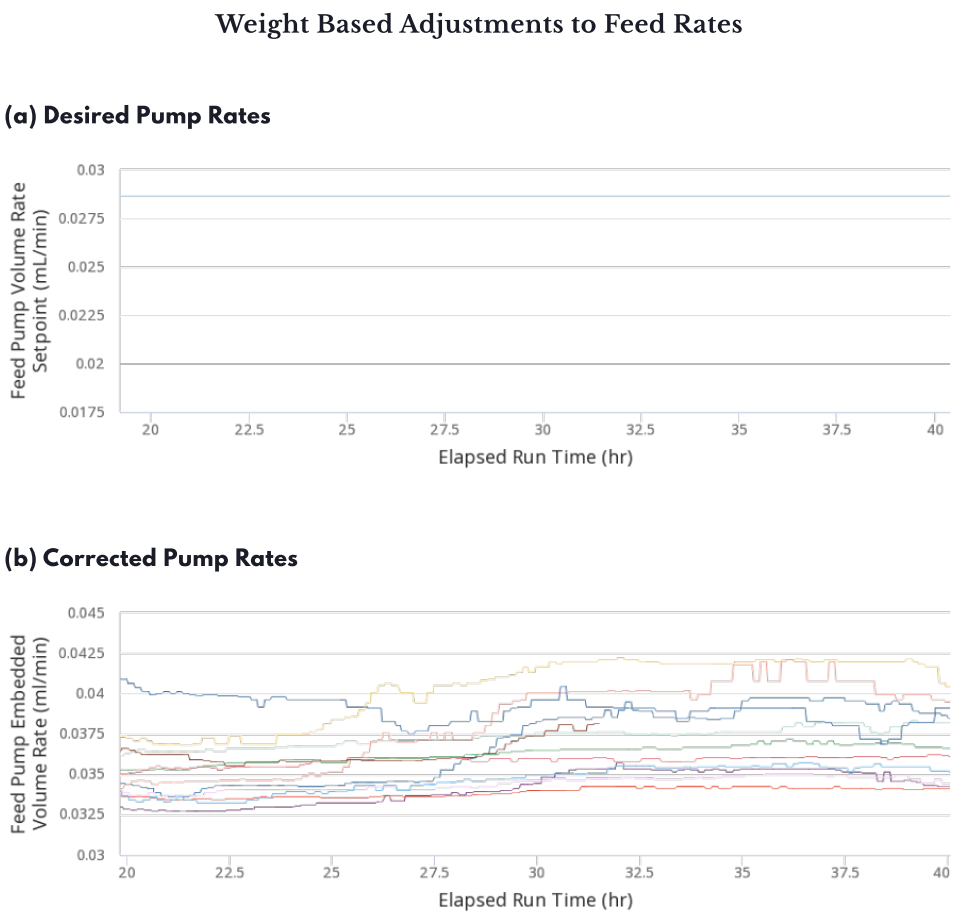


Figure 6: *Weight based adjustments to feed rates. Feed pump setpoints are programmed into each reactor (a), and pump rates are continually adjusted based on feedback from load cells under each feed bottle (b).*

To further improve liquid delivery, additional features have been incorporated into the control system in order to ensure world class precision in liquid delivery to the reactors. The feed control algorithm contains custom logic to reject false signals from environmental scale noise as well as accidental bumps. The algorithm also calculates fluid delivery deviations based on an array of continuously calculated scale deltas. This minimizes any errors that could occur from manual adjustments that were made after the scales were tared, and also allows the algorithm to adapt to any continuous changes in the hardware such as the peristaltic tube slowly loosening or wearing down.

Like the fermentation vessel, liquid additions to the vessel may evaporate. This is especially relevant for commonly used volatile liquids such as ammonium hydroxide and methanol. To this end, similar studies to the one described above were performed to empirically determine the evaporation rate of commonly used liquids from liquid delivery bottles. As the liquid type and density are inputted into each fermentation recipe, these evaporation rates are also incorporated into the mass-based feed algorithms so evaporated liquid is accounted for in load cell signal outputs, further increasing precision.

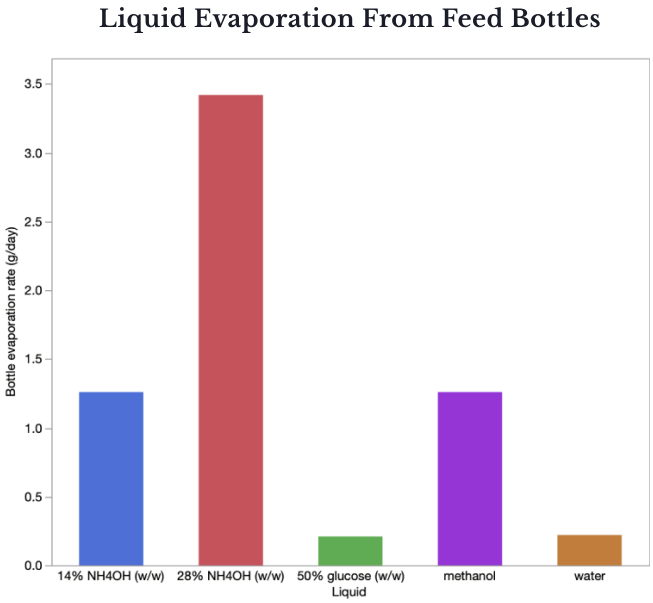


Figure 7: *Evaporation from feed bottles can account for a significant portion of the feed and varies widely based on the liquid type. The evaporation rate of commonly utilized liquids was quantified by measuring the loss of mass over a period of time.*

Weight based additions (with mechanisms to protect against and account for load cell disturbances during setup and the run), as well as accounting for liquid class and evaporation in the fermentation recipe together allow for accurate liquid delivery, typically within +/- 3 % of intended delivery rates. This not only results in accurate estimation of liquid delivery, but minimizes physiological variability attributed to imprecise feed delivery.

Manual Removals and Additions

Throughout a fermentation, samples are routinely removed from the vessel to take intermediate measurements that provide a more comprehensive understanding of strain performance. Over the course of a cultivation, this volume can represent a significant proportion of the total fermentation broth. These masses, along with any manual additions, must be accounted for in order to account for mass entering and exiting the fermentation.

Culture tracks all sample removal and manual additions to the reactor and these amounts are accounted for in mass balance calculations. Additionally, these manipulations are tracked to maintain an accurate volume estimate throughout the fermentation. Consequently this enables other variables and calculations, such as feed rates or off-gas rates, to be consistently adjusted based on an accurate tank volume at any given time.

RESULTS AND DISCUSSION

Together, the features described above allow all of the inputs and outputs from the reactor to be measured. There are a number of different mass balance calculation methods that can be used in order to track inputs and outputs from the reactor, or to estimate the final broth mass, which is then compared with the actual.

Culture's method tracks all mass added to and leaving the reactor. With these values, a mass balance for each fermentation run can be calculated using the following formula:

$$\% \text{ Mass balance} = (\Sigma \text{mass out} \div \Sigma \text{mass in}) \times 100$$

where

$$\Sigma \text{ mass in} = \text{Initial process broth mass (g)} + \text{net manual additions (g)} + \text{total mass pumped (g)} + \text{oxygen uptake (g)}$$

$$\Sigma \text{ mass out} = \text{final process broth mass (g)} + \text{net manual removals (g)} + \text{Carbon dioxide evolved (g)} + \text{evaporation (g)}$$

In an analysis of over 500 runs, the mass balance closure across multiple organisms and fermentation processes ranging from 22 - 120 hours were analyzed. The distribution of mass closure across these runs is shown in Figure 8a, below. From these data, the mass balance closure was between 97% and 103% in over 75% of the runs (Figure 8b).

Together, these results demonstrate the ability of Culture Biosciences' cloud bioreactors to deliver the highest quality fermentation data. Throughout a run, customers can visualize a live mass estimate of each reactor (Figure 9), which incorporates all the calculations described above.

As it is unfeasible to place 250ml reactors on load cells in order to have an accurate real-time process mass, it is challenging to approximate the mass of the reactor at any given time during the fermentation. Standard workflows often only measure broth mass at the conclusion of the fermentation, leaving midpoint measurements without an associated broth mass. In contrast, the mass estimate provides insight into the mass of the reactor at any point during the run. In turn, this allows midrun measurements to be combined with an estimated real-time broth mass in order to have a more accurate estimation of total product in the reactor. This enables customers to do more with their fermentation data; instead of relying on only endpoint measurements based on an accurate mass, they can have greater insight into the entire duration of their fermentations.

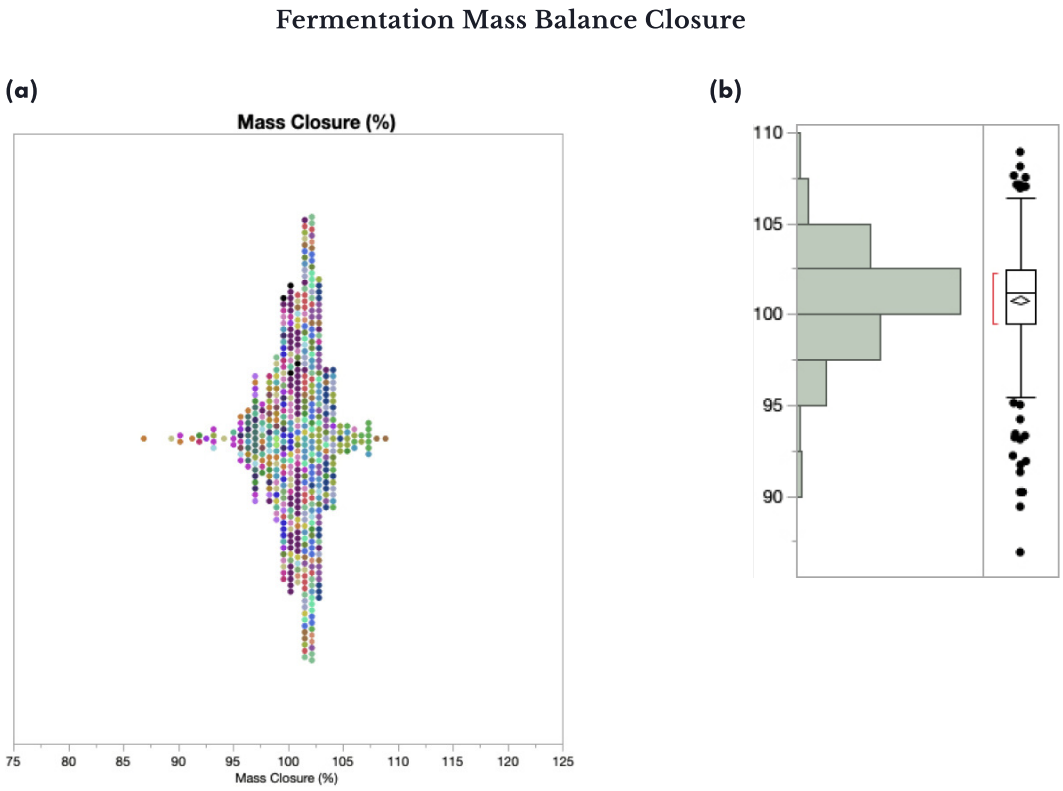


Figure 8: *Mass balances were calculated from over 500 microbial fermentation runs. (a) Percentage mass balance closure was calculated using the % Mass Balance equation described above. Colors denote different fermentation experiments and processes. (b) A distribution of % Mass Closure from these runs demonstrates that in excess of 75% of runs fall between 97% and 103% mass closure.*

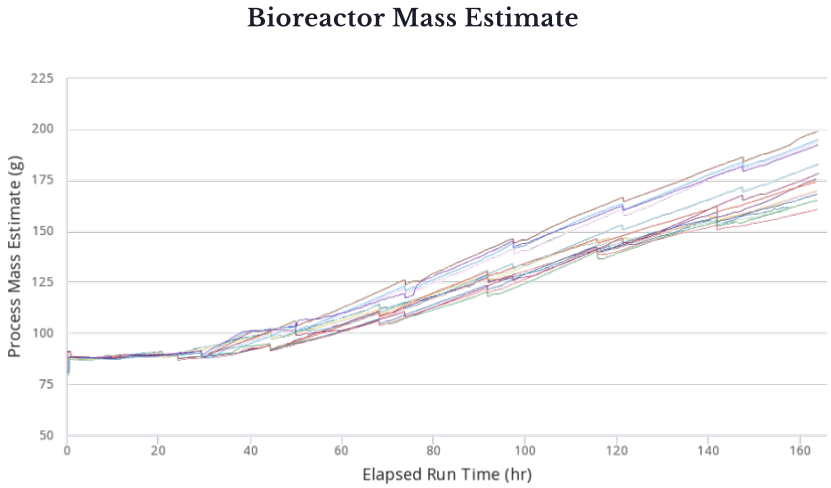
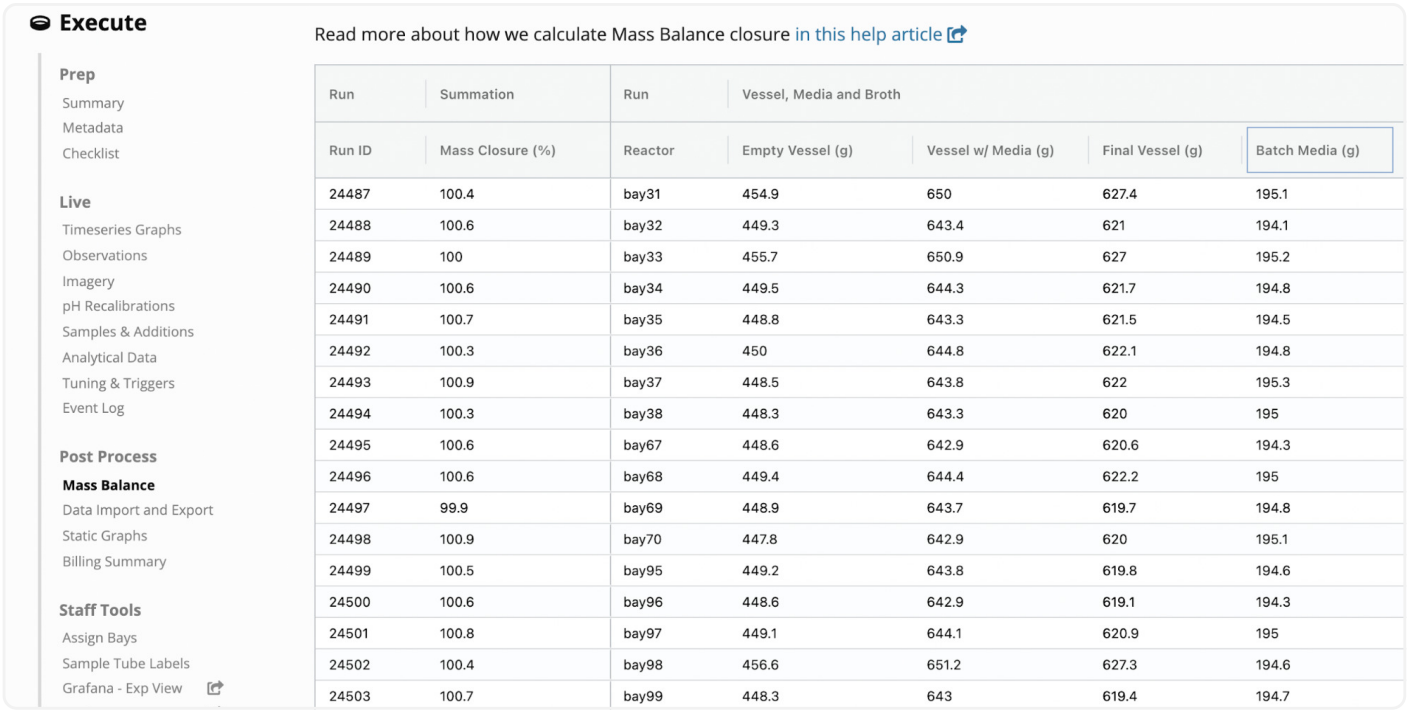


Figure 9: *In these representative plots, an estimate of the mass of each reactor can be visualized over the entire fermentation run. Manual additions and removals, such as inoculation and samples can be easily visualized, as well as changes in the reactor mass due to evaporation or addition of feeds.*

Mass Balance Closure Data



Run	Summation	Run	Vessel, Media and Broth			
Run ID	Mass Closure (%)	Reactor	Empty Vessel (g)	Vessel w/ Media (g)	Final Vessel (g)	Batch Media (g)
24487	100.4	bay31	454.9	650	627.4	195.1
24488	100.6	bay32	449.3	643.4	621	194.1
24489	100	bay33	455.7	650.9	627	195.2
24490	100.6	bay34	449.5	644.3	621.7	194.8
24491	100.7	bay35	448.8	643.3	621.5	194.5
24492	100.3	bay36	450	644.8	622.1	194.8
24493	100.9	bay37	448.5	643.8	622	195.3
24494	100.3	bay38	448.3	643.3	620	195
24495	100.6	bay67	448.6	642.9	620.6	194.3
24496	100.6	bay68	449.4	644.4	622.2	195
24497	99.9	bay69	448.9	643.7	619.7	194.8
24498	100.9	bay70	447.8	642.9	620	195.1
24499	100.5	bay95	449.2	643.8	619.8	194.6
24500	100.6	bay96	448.6	642.9	619.1	194.3
24501	100.8	bay97	449.1	644.1	620.9	195
24502	100.4	bay98	456.6	651.2	627.3	194.6
24503	100.7	bay99	448.3	643	619.4	194.7

Figure 10: *At the conclusion of each run, mass balance data for each reactor is published in Culture's custom data analysis tool, Console.*

At the conclusion of each run, mass balance data for each run is easily accessible through Culture's data analysis tool, Console (Figure 10). This transparency enables customers to have confidence in the quality of data provided for each run.

Conversely, mass balances can be used as a simple diagnostic tool; runs that fall outside expected mass balance metrics can be highlighted for quality investigations. This level of visibility for quality control purposes is a feature that allows Culture to provide high quality data and reduce release of results that may be inaccurate and therefore confounding to customers.

Combining this data with additional analytical measurements based on the mass of whole cell broth, performed either by Culture or by customers, allows for calculation of carbon balances and fluxes, which can provide additional insight into opportunities for strain and process optimization.

High-throughput fermentation, high-quality insights.

CONTACT US

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