Monitoring of Oxygen, pH, CO_2, and Biomass in Smart Single-Use Shake Flasks

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Shake flasks enjoy great popularity due to their ease-of-use and cost-effectiveness. Furthermore, using single-use shake flasks provides additional advantages over reusable glass versions, since they do not need to be cleaned or pre-sterilized. Despite their widespread use, there are, however, clear disadvantages associated with using shake flasks, which are primarily related to the lack of measurement and control capabilities. Therefore, this article discusses how optical sensors can be employed in single-use shake flasks to measure oxygen, carbon dioxide, pH, and biomass.

Keywords: Disposable cultivation systems, Erlenmeyer shake flasks, Online sensing, Optrodes, Orbitally shaken bioreactor

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1 Introduction

Shake flasks are the most commonly used cultivation systems in the development of biotechnological production processes. In addition to screening cell lines and process parameters, they are also used for the initial stages of inoculum production. They are easy to use, inexpensive, available in glass and plastic versions, both with and without baffles, and up to a volume of 5 L [1, 2]. However, standard shake flasks are not instrumented, which results in costly offline analysis and data evaluation during process development and optimization, thus complicating process analytical technology (PAT). It is therefore not surprising that work is being done to make shake flasks smarter to overcome these disadvantages.

There are several approaches to performing online measurements in shake flasks. In academia, modified glass shake flasks are often used in combination with traditional probes [3–6], self-made or modified fluorescence sensor spots and films [7–16], and bypass systems [7–9, 17, 18]. Some of these approaches can be applied without modifications to the flasks and thus are also suitable for single-use or disposable flasks. These include fluorescent nanoparticles [19, 20] or wirelessly connected sensor spheroids [21, 22]. Commercial products, in contrast, offer closed-loop solutions based on either glass or single-use shake flasks with off-gas analyzers and immobilized sensor spots, or reflectance measurements and corresponding software tools. Tab. 1 summarizes examples of commercial measurement systems in both glass and single-use shake flasks.

Off-gas analysis is a simple, non-invasive measurement method offered by several manufacturers. In HiTec Zang's respiration activity monitoring system (RAMOS) [6, 23–29], special glass shake flasks are actively gassed. At set intervals, the gassing is stopped and the change in oxygen concentration and total pressure is measured. The oxygen transfer rate (OTR) is then calculated from the change in oxygen concentration, and the carbon dioxide transfer rate (CTR) from the change in total pressure. This approach was further developed to create the transfer-rate online measurement (TOM) system from Kühner [30–32], in which an infrared carbon dioxide sensor is also integrated into the system so that the CTR can be calculated directly. Glass bottles with special lids and adapters for single-use shake flasks are also available for this system. A further system that operates in a similar way is BCPreFerm from BlueSens [33–35], which measures oxygen and carbon dioxide concentrations in modified glass flasks and can also be equipped to measure ethanol, methane and hydrogen.

Another non-invasive measurement method is the use of backscattered light, which can be employed to estimate biomass concentrations in microbial and plant cell suspension cultures. This approach is implemented in commercially available products from aquila biolabs (a scientific bioprocessing company) with the cell growth quantifier (CGQ) [32, 36–38] and from PreSens with the shake flask reader (SFR) vario [39–42]. Neither system requires any adaptations and can be used with both glass and single-use flasks.

In the commercial sector, sensor spots, which can be glued into glass flasks or purchased in pre-sterilized plastic Erlenmeyer flasks, are commonly used to measure dissolved

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Table 1. Overview of commercially available devices for measuring oxygen, CO_2, pH, backscattered light, and other parameters in shake flasks. For further information on the different methods for calculating OUR/OTR and CTR, please refer to the corresponding manufacturer's specifications and the references provided [48–50].

Manufacturer	Device name	Measuring phase	Single-Use	Oxygen	CO_2	pH	Backscatter	Other parameters measured	References
Hitec Zang	RAMOS	Gas	No [a),b)]	Yes [c)]	Yes [e)]	No	No	OTR [e)], CTR [e)], RQ [e)]	[6, 23–29]
Kuhner	TOM	Gas	Possible [a),b)]	Yes [c)]	Yes [c)]	No	No	OTR [e)], CTR [e)], RQ [e)]	[30–32]
BlueSens	BCPreFerm	Gas	No [a),b)]	Yes [c)]	Yes [c)]	No	No	OTR [e)], CTR [e)], RQ [e)], ethanol [c)], methane [c)], hydrogen [c)]	[33–35]
Aquila	CGQ	Liquid	Possible	No	No	No	Yes	Growth rate [e)]	[32, 36–38]
PreSens	SFR	Liquid	Possible	Yes [d)]	No	Yes [d)]	No	OUR [e)]	[26, 43, 44]
PreSens	SFR vario	Liquid	Possible	Yes [d)]	Yes [d)]	Yes [d)]	Yes	OUR [e)], pressure, temperature, shaking rate	[39–42]
C-CIT	CITSens Memo	Liquid	Yes [b)]	No	No	No	No	Glucose, lactate	[45–47]

a) Modified glass flask; b) modified cap; c) off-gas analyzer; d) sensor spot; e) calculated values.

oxygen concentrations, carbon dioxide concentrations, and pH values. Examples of such devices include the SFR [26, 43, 44] and SFR vario [39–42] from PreSens. Furthermore, the CITSens MeMo from C-CIT [45–47] also enables glucose and lactate concentrations in suspensions to be measured. In this case, the sensor is attached to the lid of a single-use flask and suspended in the culture.

Application examples and measurement details for the use of single-use shake flasks for liquid measurements with sensor spots and backscattered light are explained in Sect. 3.

3 Sensor Properties and Use

3.1 Measurement Principles, Ranges, and Specifications

Oxygen concentration, CO_2 concentration, pH, and biomass are determined optically. However, while biomass is measured by the increasing reflectance of the microbes themselves, the other parameters are detected by changes in the fluorescence of optrodes, also called sensor spots, integrated in the flask.

Depending on the rotation speed, flask volume, flask type, and media viscosity, the reflectance signal will differ. Therefore, biomass sensors are coupled with an acceleration sensor to adjust to the media sickle travelling in the flask [40]. Instead of measuring a complete revolution, only a defined segment is selected where the liquid sickle is presumed to be. The light pulse always covers the same angular segment resulting in a smoother signal. In contrast to absorbance measurements, which are only precise at low biomass levels, reflectance signals offer a wide range of measurements [39] but lack accuracy for very small biomass concentrations. The smallest assessable optical density (OD) may be 0.2 under optimal conditions but in many cases even an OD of 1 cannot be determined accurately because additional reflections in the moving system interfere with the biomass signal.

Polymers with integrated fluorescent indicator dyes that change their fluorescence are used to measure O_2, CO_2, and pH value. LEDs are used to excite the indicator and its emissions are detected by photodiodes. The oxygen sensor emits fluorescence that is dependent on the oxygen concentration. If the indicator is in its excited state and encounters an oxygen molecule, the energy is transferred to this molecule via non-radiative transfer, reducing or quenching the fluorescence signal. The grade of quenching correlates to the oxygen partial pressure of the analyte in the matrix, which is in dynamic equilibrium with the oxygen in the culture broth [51]. For pH and CO_2 sensors, pH-sensitive dyes are used. For CO_2 measurements, the pH indicator is shielded by a hydrophobic layer so that only CO_2 molecules can penetrate and consequently change the pH in the area surrounding the indicator. Tab. 2 provides an overview of sensor specifications.

3.2 Application Examples

The most typical application for online sensors is in process monitoring, for example to avoid oxygen limitations, to react to critical pH values or to determine the end of the growth phase. However, sensor technology can also be used abiotically before an experiment to determine k_La values. To ensure comparability, the approach proposed in the guidlines "Recommendations for process engineering characterisation of single-use bioreactors and mixing systems by using experimental methods (2nd edition)" from the

Table 2. Measurement range, resolution, and response time of different sensing principles.

	O_2	pH	CO_2	Biomass
Measurement range	0–100 % O_2	Neutral version: 5.5–8.0 pH Acidic version: 4.0–7.5 pH	1–25 % CO_2 at atmospheric pressure (1013.15 hPa)	OD 1–80
Resolution	± 0.01 % O_2 at 0.21 % O_2	± 0.01 pH at pH = 7	± 0.06 % at 2 % CO_2	Depending on culture
Response time $t_{63\%}$ [a)] [s]	< 15	< 60	< 90	N.A.

a) Calculated from $t_{90\%}$ response times.

DECHEMA Single-Use Technology expert group was used in this study [52]. Very good reproducibility of aeration and degassing measurements was shown (Fig. 1a), allowing the corresponding k_La value to be precisely determined (Fig. 1b). For 250-mL and 500-mL shake flasks, k_La values of 8 to 111 h^{-1} and 5 to 80 h^{-1} were determined, respectively (Fig. 1c,d).

When determining k_La values, the response time of the O_2 sensor spot must be taken into account, allowing the maximum measurable k_La value to be estimated [52].

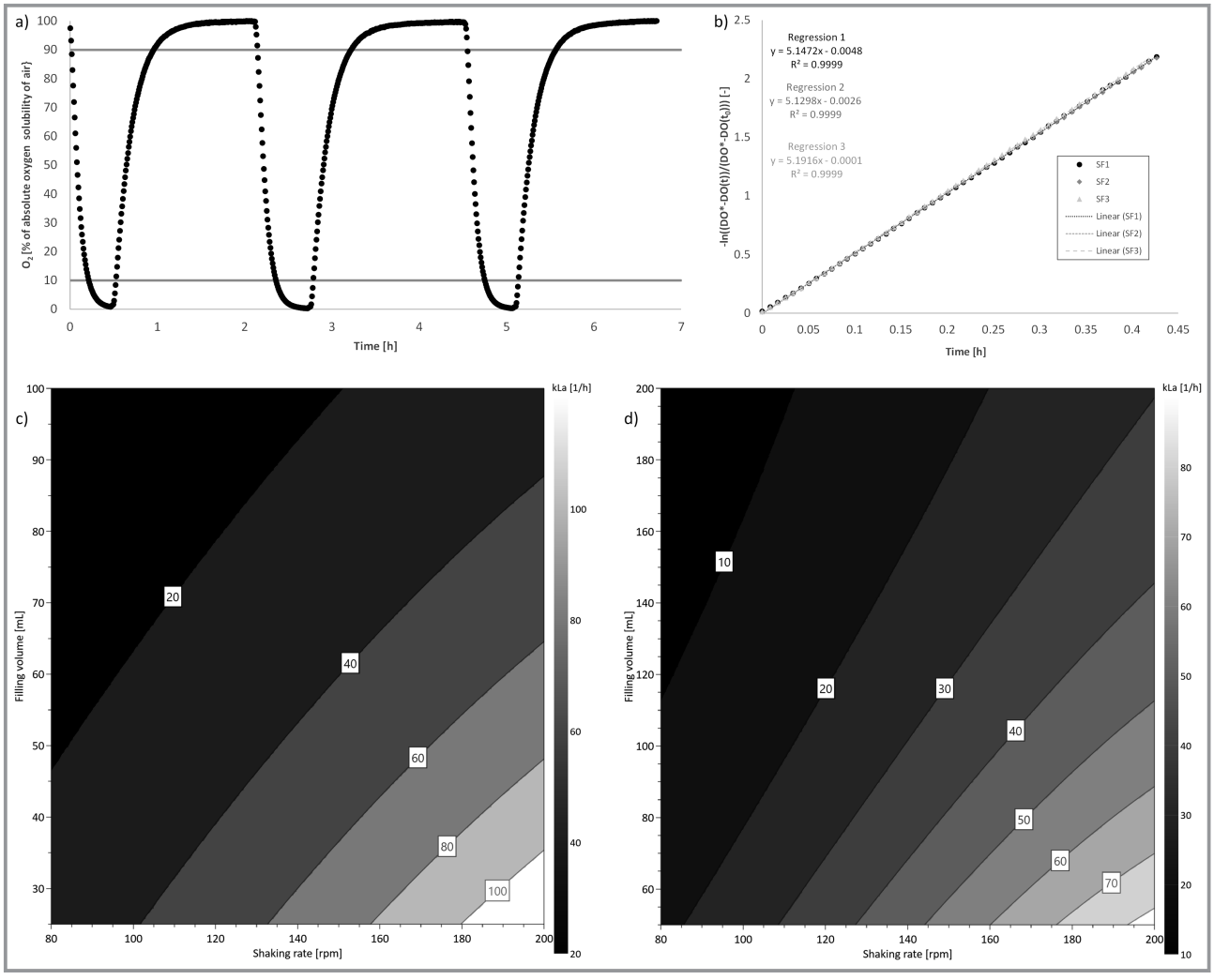

Figure 1. Determination of k_La values in single-use shake flasks. a) Example measurements for 500-mL shake flasks with 200 mL filling volume, 80 rpm shaking rate and 50 mm shaking amplitude. The horizontal lines indicate the evaluation area between 10 and 90 %; b) evaluation of the experiment based on the recommendations by the DECHEMA "Single-Use Technologies" expert group, resulting in a k_La value of 5.156 ± 0.032 h^{-1}; c) k_La contour plot for 250-mL shake flasks dependent on the shaking rate (80 to 200 rpm) and filling volume (10 to 40 %) at 50 mm shaking amplitude and d) contour plot for 500-mL shake flask with the same parameters as in c).

According to a criterion established by van't Riet [53], k_La values up to 257 h^{-1} can be measured with a response time $t_{63\%}$ of 14 s, while stricter criteria set by Zlokarnik [54] and Garcia-Ochoa [55] reduce this value to maxima of 51 h^{-1} and 26 h^{-1}, respectively. This information should therefore be considered when comparing literature values, which are heavily dependent on the measurement method and the sensors used, especially for higher k_La values. The k_La value can then be used to calculate the OTR, which can be used for online process characterization, as shown in the example *S. cerevisiae* cultivation below.

The true power of online measurements in shake flasks is only revealed by multiple measurement signals. One reason for this is that not every measurement signal is suitable for every organism-media combination [56]. Furthermore, multiple signals can be used if the signal-to-noise ratio of one signal is too high or other limitations occur. An example *S. cerevisiae* cultivation is shown in Fig. 2. While the biomass signal based on backscattered light qualitatively describes the biomass concentration curve well, due to the insufficient culture density within the first two hours, the initial biomass increase is unfortunately not measurable. Furthermore, the signal noise is also increased, making it impossible to calculate the growth rate with this signal alone. During the first two hours of cultivation in particular, the biomass signal is not at all meaningful, and during the first six hours, the calculated OUR provides much more accurate information, clearly reflecting the exponential growth phase when glucose is used as carbon source. The metabolic switch from ethanol formation to consumption after six hours can also be easily seen, which is further reflected by the decrease in CO_2 formation and the slowing of the decrease in pH. However, the usefulness of the OUR decreases sharply as soon oxygen limitation starts to occur after seven hours when metabolic activity can be better monitored by the CO_2 signal and the pH. As a result, the biomass signal now provides the user with more information, making the linear growth of the culture during this phase apparent. The combination of all these signals thus allows the cultivation to be described without additional offline measurements.

3.3 Prediction and Evaluation Based on Online Monitoring in Shake Flasks

The use of optical sensors in shake flasks provides for even more interesting applications than the examples listed above. One such application could be the prediction of cultivation events. The approach proposed by Pretzner et al. and verified using example *E. coli* cultivations uses a particle filter to predict critical events such as the time at which oxygen limitation occurs or when the cultivation ends [41]. This use of smart shake flasks with a combination of sensors also makes it possible to determine key performance indicators (KPIs), such as the specific growth rate, biomass yield, and cell-specific oxygen demand for different organism media combinations [56]. It has been shown that this approach enables KPIs to be determined for microorganisms (using *E. coli* and *S. cerevisiae* as examples), plant cells

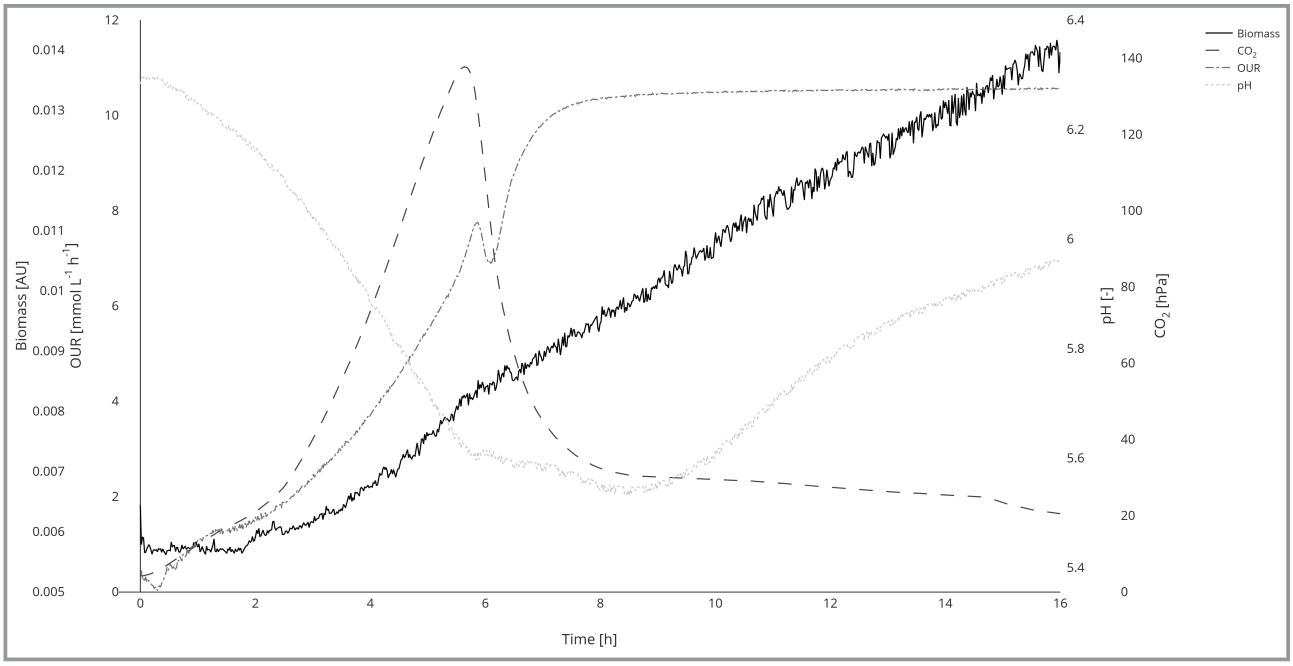

Figure 2. Online measurement of a *S. cerevisiae* strain H1022 cultivated in YPD medium (500-mL Corning Erlenmeyer flask with 100 mL filling volume, 30 °C, 180 rpm, 50 mm shaking amplitude). All parameters (biomass, CO_2, pH, and O_2) were measured simultaneously using an experimental version of the SFR, the latter was used in combination with the determined k_La values to calculate the OUR.

(*Vitis vinifera*) and animal cells (High Five, CHO and HEK). A graphical representation of the comparison between a conventional black-box approach and an intelligent approach can be found in Fig. 3.

4 Conclusion

In this short communication we explain the concepts underpinning smart shake flasks and how they can be used. Their non-invasive integrated sensor technology for biomass, oxygen, pH, and CO_2 can be used for process engineering characterization of shaken bioreactors and to provide online information about the metabolic status of a cultivation, such as specific growth rate, biomass yield, oxygen limitations and substrate limitations. Since sampling is not required, any disturbances to the culture are avoided, and because the different sensors provide signals independently of each other, they can compensate for any deficiencies in the data from other sensors, thus providing a more complete picture of the cultivation and enabling extended analyses to be performed that cannot be carried out using individual parameters. This even makes it possible to predict cultivation events such as the time of harvest, limitations, and end of the exponential phase.

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Abbreviations

CTR	Carbon dioxide transfer rate
CGQ	Cell growth quantifier
KPI	Key performance indicator
OD	Optical density
OUR	Oxygen uptake rate
OTR	Oxygen transfer rate
PAT	Process analytical technology
RAMOS	Respiration activity monitoring system
RQ	Respiratory quotient
SFR	Shake flask reader
TOM	Transfer-rate online measurement

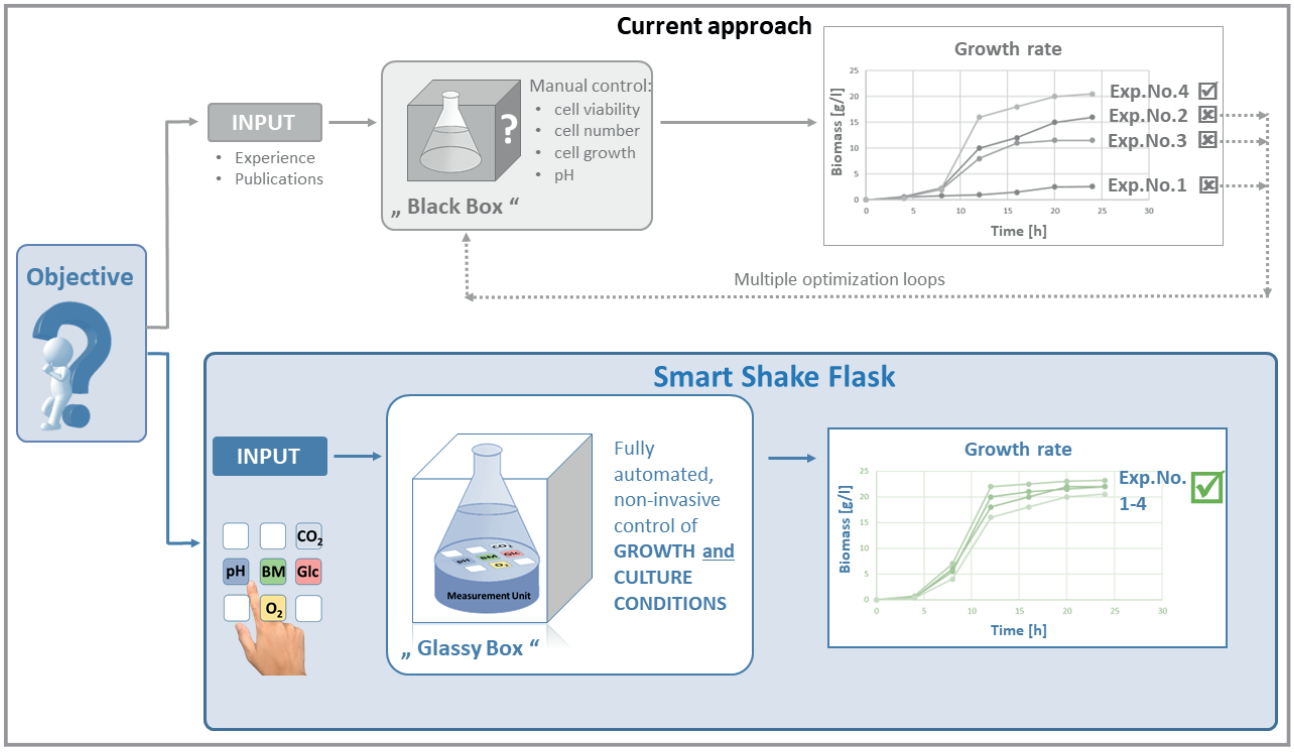

Figure 3. Comparison between the state-of-the-art method for shake flask cultivations and the smart flask approach. This concept increases the reproducibility and stability of shake flask experiments while reducing the amount of work and resources required. Determining critical events and KPIs for different cell and media combinations can be performed with an initial offline measurement and then based on online-data alone.

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