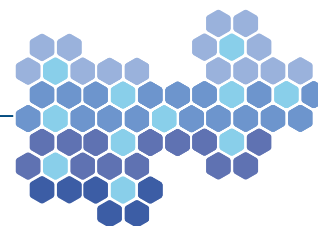

Non-Invasive Optical Dissolved Oxygen Quantification in Small-Scale Bioreactors

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Non-invasive quantification of dissolved oxygen using fluorescence-based optical sensors can be implemented in a vast variety of small-scale bioreactors, offering several advantages over conventional electro-chemical quantification methods.

Small-scale bioreactors such as microtitre plates and shake flasks, or small-scale continuous cultivation systems, are extensively used in industry and academia for both microbial biotechnology and cell culture technology since they offer the advantage of easy and relatively inexpensive parallelisation. However, small-scale bioreactors often lack the possibility of dissolved oxygen (DO) quantification in the reaction mixture. No – or only very little – attention is paid to the oxygen supply of the growing culture when using these cultivation systems since classical electro-chemical methods are usually not applicable in such small devices. Hence, oxygen limitation is one of the major problems in small-scale aerobic cultivation and is often neglected or simply overlooked, which could cause severe drawbacks with regard to scale up or reproducibility. Therefore, a reliable method for the determination of DO concentration in small-scale cultivation systems is needed.

Small chemical optical oxygen sensors – applying oxygen sensitive fluorophores – allow non-invasive measurement and meet the demand for such small-scale cultivation systems.

Fluorescence-Based Measurement

The application of fluorescence-based DO measurement has been studied extensively since the mid 1980s (1). Fluorescence methods are often superior compared with other optical methods and conventional amperometric quantification due to their increased sensitivity. Another advantage compared with classical electro-chemical quantification methods is the reduced size of the sensor that can easily be mounted in small-scale cultivation vessels. Quantification is carried out by means of fluorescence quenching. In order to compensate for problems like light scattering, fluctuations in light intensity and detector sensitivity, or the intrinsic fluorescence of the sample, a second oxygen insensitive fluorescence dye can be used as an internal standard (2), or alternatively fluorescent decay time can be measured. As decay times of the applied sensing molecules are in the microsecond range, they do not interfere with natural fluorescence.

Keywords

Dissolved oxygen quantification

Fluorescence-based dissolved oxygen measurement

Small-scale bioreactors

Shake flasks

Small-scale continuous cultivation systems

Dissolved oxygen concentration during shake flask cultivations was determined with a shake flask reader (SFR) (Presens GmbH, Regensburg, Germany) allowing parallel cultivation of up to nine shake flasks and wireless data transfer via Bluetooth; this reader can be mounted in conventional shakers and also permits optical pH measurement. Measurement of DO concentration in small-scale continuous cultivations carried out in 10mL test tubes was performed applying a four-channel fibre optic transmitter; the sensors were connected to the transmitter via optical fibres that allowed parallel measurement in up to four bioreactors.

Aerobic Shake Flask Cultivations

Changes in cellular metabolism, which took place during growth of L-lysine producing *Corynebacterium glutamicum* ATCC 13287 and ATCC 21526 on glucose in a medium also containing citrate and threonine, could be quantified applying the SFR for online DO monitoring. Both strains exhibited a typical two-phase profile: the first characteristic peak is linked to the depletion of citrate which is preferentially consumed by the cells; the second peak was characteristic for the complete uptake of threonine as well, and for the beginning of L-lysine production by the auxotrophic strains investigated. To ensure reproducible results of duplicate cultivations, shake flasks with similar k_La values were used (the k_La is a specific coefficient which quantifies oxygen mass transfer in a given cultivation system). DO concentrations exhibited standard deviations of three per cent. The volumetric oxygen transfer coefficient k_La

Figure 1: Chemical optical sensors integrated in a shake flask; the fluorescent dyes are excited with light of a certain wavelength and the sensor response is read out by the shake flask reader

was determined using a dynamic method described previously (3). It was shown that online DO quantification was suited to reflecting the different phases of cellular metabolism once the profile of substrates and products was known – which can be beneficial during medium/ growth parameter optimisation (4).

Another example of DO monitoring in aerobic shake flask cultivation involved the growth of *Saccharomyces cerevisiae* (baker's yeast) in a medium containing different carbohydrates – namely glucose, galactose and fructose. The specific oxygen uptake rates of exponentially growing cells during growth on different sugars were calculated using a stoichiometric model of the cellular metabolism, and k_La determination of the shake flasks was carried out applying the method referred to above (3). Online measurement yielded comparable specific oxygen uptake rates on glucose and fructose of 9mg $O_2g^{-1}h^{-1}$, as well as a six-fold increase in oxygen uptake to 60mg $O_2g^{-1}h^{-1}$ during growth on galactose (4).

Small-Scale Continuous Cultivation Systems

Small-scale continuous cultivation of *Schizzosacharomyces pombe* was performed in 10mL test tubes placed in a circulating water bath at 30°C; the test tubes were equipped with fluorescence sensors for DO quantification which was carried out in four parallel reactors. Since disturbance of the metabolic steady state due to sampling throughout the entire cultivation process is not desirable in such small devices, non-invasive methods for monitoring the metabolic state of the culture have to be applied. Online measurement is a suitable way of determining achievement of the metabolic steady state as depicted in Figure 2. Initially, batch growth of the culture is shown in the figure, while the transition to continuous cultivation and the achievement of the metabolic steady state is indicated by constant DO concentrations. Parallel determination of cell dry weight confirmed these findings and supported use of the method to estimate the point in time at which a microbial culture reaches steady state metabolism.

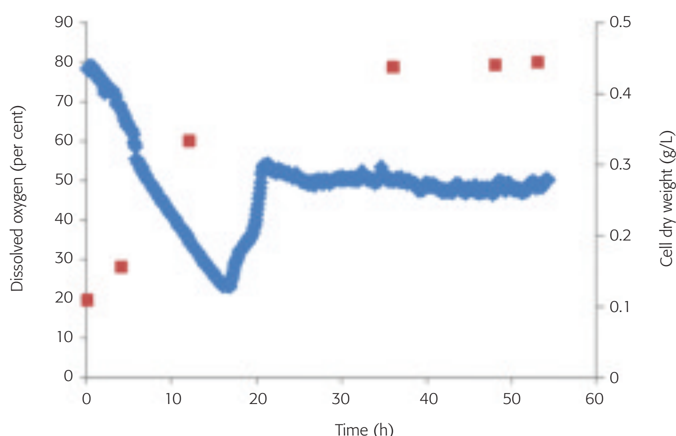


Figure 2: Dissolved oxygen (DO) and cell dry weight course during the batch start-up and the stationary phase of a continuous cultivation using *Schizzosaccharomyces pombe*. DO concentration is shown in blue and cell dry weight in red

Another important fact is the oxygen transfer to the liquid phase of the test tubes used for continuous cultivation. A comparison of DO concentration in stirred tubes and bubble reactors showed clearly that oxygen transfer in bubble tubes is not sufficient to meet the oxygen demands of the microorganisms.

DO Measurement in Microtitre Plates

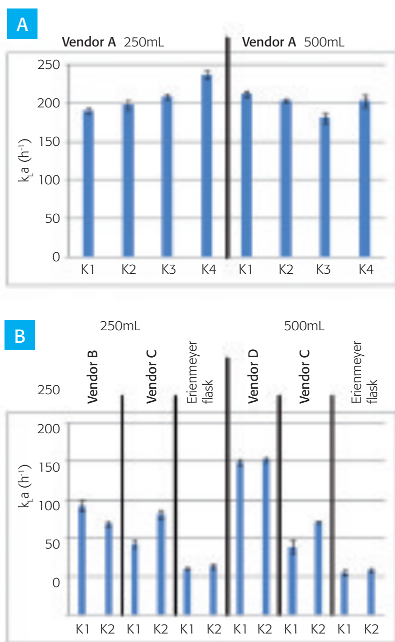
The use of microtitre plates enables a high level of parallelisation of experiments, making them useful in high throughput screening procedures. Using commercial microtitre plate fluorescence readers, John *et al* demonstrated that volumetric oxygen transfer coefficients comparable with the lower range of stirred tank reactors could be achieved in 96-well microtitre plates (5). *C glutamicum* ATCC 21253 exhibited the same growth behaviour as shake flask or stirred bioreactor cultivations with regard to biomass concentration and specific growth rate.

Application of high throughput DO concentration for screening purposes was described by Velagapudi *et al* (6). 27 deletion mutants of *S cerevisiae*, applying glucose and galactose as a carbon source, were screened for various cultivation parameters. Overall growth on glucose was predominantly fermentative, characterised by high ethanol yields and low oxygen uptake rates, whereas growth on galactose showed an increased activity of the respirative metabolism causing reduced ethanol formation and increased oxygen uptake by the different deletion mutants. The specific oxygen uptake rate during growth on glucose increased with increasing specific growth rates; by comparison, the oxygen uptake varied more strongly during growth on galactose, with some mutants being highly respirative whereas others almost completely lacked cellular respiration.

Reproducible Geometry of Shake Flasks

Since reproducibility is a basic requirement that has to be fulfilled by the cultivation system of choice, different shake flasks were investigated with regard to the reproducibility of their geometry. This was performed by determining the k_La value of the different flask types, since the volumetric oxygen transfer coefficient is strongly dependent on the geometry of the flasks. Online measurement of DO concentration is quite a feasible method for this task, and k_La values could be determined precisely – showing differences among shake flasks from the same lot, and even more significant differences

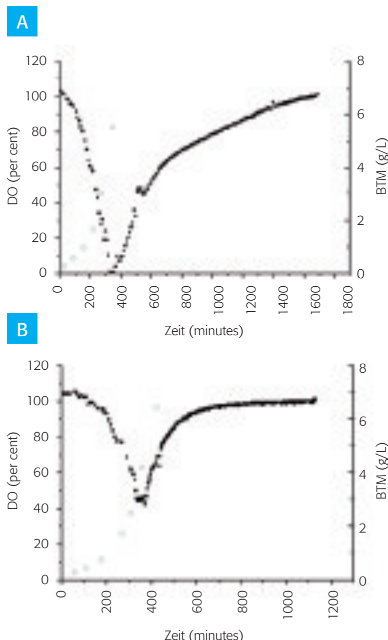
Figure 3: A) Volumetric oxygen transfer coefficients (k_La) of fully automated manufactured 250mL and 500mL baffled glass shake flasks. **B)** Volumetric oxygen transfer coefficients (k_La) of handmade baffled glass (vendor B and vendor C) and polypropylene (vendor D) shake flasks. Unbaffled glass flasks are shown for comparison



among shake flasks from different vendors. Figure 3A shows the k_La values of four shake flasks considered to possess identical geometries (vendor A). The variations in k_La values of up to 10 per cent for both the 250mL and 500mL flasks, clearly indicates that slight differences in flask geometry can occur during the production process, and this can have a major impact on oxygen transfer from the gas to the liquid phase.

The k_La values of shake flasks – including unbaffled shake flasks – obtained from different vendors are compared in Figure 3B. Shake flasks obtained from vendor C showed differences of up to 25 per cent in the determined k_La value, whereas the deviation of shake flasks obtained from vendor B was comparable with that of those obtained from vendor A. Only shake flasks obtained from vendor D and unbaffled flasks showed comparable k_La values – thus suggesting an identical geometry of the tested shake flasks. According to these findings, it would seem crucial to account for flask geometry and oxygen transfer potential when reproducibility of cultivations is necessary.

Figure 4: A) Cultivation profile of C glutamicum grown on BHI++ medium using fully automated manufactured baffled shake flasks (250mL, vendor A). **B)** Cultivation profile of C glutamicum grown on BHI++ medium using handmade baffled shake flasks (250mL, vendor B)



This becomes even more important when switching to biological experiments. Cultivation profiles of aerobic *C glutamicum* ATCC 13032 in brain heart infusion (BHI) medium at 30°C and 200rpm are shown in Figures 4A and B. Cultivations carried out in shake flasks obtained from vendor A (Figure 3A) showed no oxygen limitation throughout the entire cultivation, whereas cultivations carried out in shake flasks purchased form vendor B exhibited a clear oxygen limitation towards the end of the cultivation.

Conclusion

The method of non-invasive quantification of dissolved oxygen using chemical optical sensors can be implemented in a vast variety of small-scale bioreactors. During high throughput screening procedures, quantification of DO concentration can contribute additional information on oxygen-limiting conditions during the process. Moreover, application of method in shake flasks could support online process control and monitoring, and thus advance the application of shake flasks instead of stirred bioreactors for standard purposes.

Besides biological applications, online DO quantification is useful for determining the oxygen transfer characteristics of small-scale bioreactors. Knowing these characteristics, it is possible to determine oxygen uptake rates in small-scale cultivations and highly parallelised experimental setups.

References

1. Wolfbeis OS and Caroline FM, Long wavelength fluorescence indicators for the determination of oxygen partial pressures, *Anal Chem Acta* 160, pp301-304, 1984
2. Klimant I and Wolfbeis OS, Oxygen-sensitive luminescent materials based on silicone-soluble ruthenium diimine complexes, *Anal Chem* 67, pp3,160-3,166
3. Dunn IJ, Heinzle E, Ingham J and Prenosil JE, *Biological reaction engineering. Principles, applications and modeling with PC simulation*, Weinheim: VCH, 1992
4. Schneider K, Schutz V, John GT and Heinzle E, Optical device for parallel online measurement of dissolved oxygen and pH in shake flask cultures, *Bioprocess Biosyst Eng* 33, pp541-547, 2010
5. John GT, Klimant I, Wittmann C and Heinzle E, Integrated optical sensing of dissolved oxygen in microtiter plates: a novel tool for microbial cultivation, *Biotechnol Bioeng* pp829-836, 2003
6. Velagapudi VR, Wittmann C, Lengauer T *et al*, Metabolic Screening of Saccharomyces cerevisiae Single Knockout Strains Reveals Unexpected Mobilization of Metabolic Potential, *Process Biochem* 41, pp2,170-2,179, 2006

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