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Device for sterile online measurement of the oxygen transfer rate in shaking flasks

Tibor Anderlei*, Jochen Büchs

Department of Biochemical Engineering, Aachen University of Technology, Worringerweg 1, D-52056 Aachen, Germany

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Abstract

The oxygen transfer rate (OTR) is the most suitable measurable parameter to quantify the physiological state of a culture of aerobic microorganisms since most metabolic activities depend on oxygen consumption. Online measurement of the oxygen transfer rate in stirred bioreactors is state of the art although technically difficult. However, the online determination of the oxygen transfer rate in shaking bioreactors under sterile conditions has not been possible until recently. A newly developed measuring device eliminates this deficit. Extremely useful information about cultivating conditions and the physiological state of microorganisms can be gained in early stages of research and bioprocess development from many reactors operated in parallel. © 2000 Elsevier Science S.A. All rights reserved.

Keywords: Shaking bioreactor; Oxygen transfer; Bioprocess monitoring; Respiration; Bioreactor; Fermentation

1. Introduction

Shaking flasks were introduced into biotechnology in the beginning of this century. In 1933 Kluyver and Perquin started the first submersed fermentation of a fungus in a shaking flask [1]. Nowadays shaking flasks have become irreplaceable in applications that require a high number of experiments, for example screening for efficient strains or optimisation of media. Although shaking bioreactors are frequently used, the online measuring possibilities are very limited. This deficit can be reduced by a measuring device, which determines the oxygen transfer rate (OTR) of a microbial culture online under sterile conditions. Connecting every shaking flask to an online measuring device, for example during screening for an efficient strain, will eliminate the biggest advantage of shaken bioreactors: saving time through easy handling. Therefore, the strategy for mass screening should be to choose several interesting screening conditions (representatives of the main variations) and carry out parallel fermentations in the online measuring device. To correctly follow this strategy the fermentation conditions of the online measurement have to be equivalent to those in a normal shaking flask (for example flask with cotton closure). To fulfil this demand a special measuring method and a flask were developed. The results of the development and some online data of exemplary fermentations will be presented in this contribution.

2. Results and discussion

2.1. Measuring method and device

The structure of the online measuring device for the oxygen transfer rate is shown in principle in Fig. 1a. During fermentation a measuring cycle is continually repeated. This measuring cycle is separated into a measuring and a rinsing phase (Fig. 1b). During the rinsing phase gas with a calculated composition flows through the measuring flask.

To protect the measuring flask against contamination two sterile filters (F1, F2) are installed at the inlet and the outlet. The partial pressure of oxygen in the headspace of the measuring flask is detected by an oxygen gas sensor. At the beginning of the measuring phase the inlet (V1) and outlet valves (V2) of the measuring flask are closed. The continuing respiration activities of the microorganisms subsequently lead to a decrease of the partial pressure of oxygen in the headspace of the measuring flask (Fig. 1b). A computer calculates the oxygen transfer rate (OTR) of the particular measuring flask from the change of the partial pressure (Fig. 1c). After the measuring phase the valves (V1, V2) are opened again and “fresh” gas can flow through the flask. A newly developed calibration strategy ensures the compensa-

* Corresponding author. Tel.: +49-241-80-7179; fax: +49-241-888-8265.
E-mail address: anderlei@biovt.rwth-aachen.de (T. Anderlei).

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Nomenclature

n_{O_2}	moles of oxygen (mol)
OTR	oxygen transfer rate (mol l^{-1} h^{-1})
Δp_{O_2}	difference of oxygen partial pressure (bar)
R	gas constant (bar l mol^{-1} K^{-1})
T	temperature (K)
Δt	time of the measuring phase (h)
V_g	gas volume (l)
V_l	liquid volume (l)

tion of the drift of the oxygen sensors. With this device it is possible to operate up to 12 fermentations in parallel.

The main goal during the development of the measuring device (Fig. 1a) was to achieve equivalent fermentation conditions in the measuring shaking flask (Fig. 2) as in the normal shaking flask. Therefore, equal hydrodynamic conditions of the liquid and of the headspace gas composition have to be created in the measuring flask. To reach this goal the lower part of a measuring flask, in which the liquid is rotating, is not modified and shaped like the normal shaking flask. Furthermore the oxygen sensor does not touch the liquid and therefore does not disturb the flow of the liquid. To achieve equal conditions of the headspace gas composition a mathematical model was developed [2]. The model is able to calculate the gas composition in the headspace of a normal shaking flask. With this information the computer connected to the measuring device (Fig. 1a) controls a gas mixing system. The gas mixing system generates a gas equivalent to the headspace gas composition in the normal shaking flask. This gas mixture is fed to the measuring flask.

2.2. Fermentations

All fermentations were performed with an online measuring device for the determination of the oxygen transfer rate using a measuring flask (Fig. 2) corresponding to the 250 ml normal shaking flask.

2.2.1. Yeast Saccharomyces cerevisiae

The yeast *Saccharomyces cerevisiae* is possibly one of the most widely investigated microorganisms. This is a consequence not only of its importance in industry but also of its distinct glucose metabolism [3]. The fermentation of the yeast was carried out in a complex medium with 1% glucose, 0.5% peptone and 0.5% yeast extract.

Fig. 3 shows the development of the oxygen transfer rate and of the glucose concentration during a fermentation in a shaking flask. Without any lag phase the yeast starts to consume oxygen indicated by the oxygen transfer rate level of 0.005 mol l^{-1} h^{-1} at the beginning. After 7 h the nutrient glucose (the only carbon source) is exhausted. But the following increase of the oxygen transfer rate indicates that the

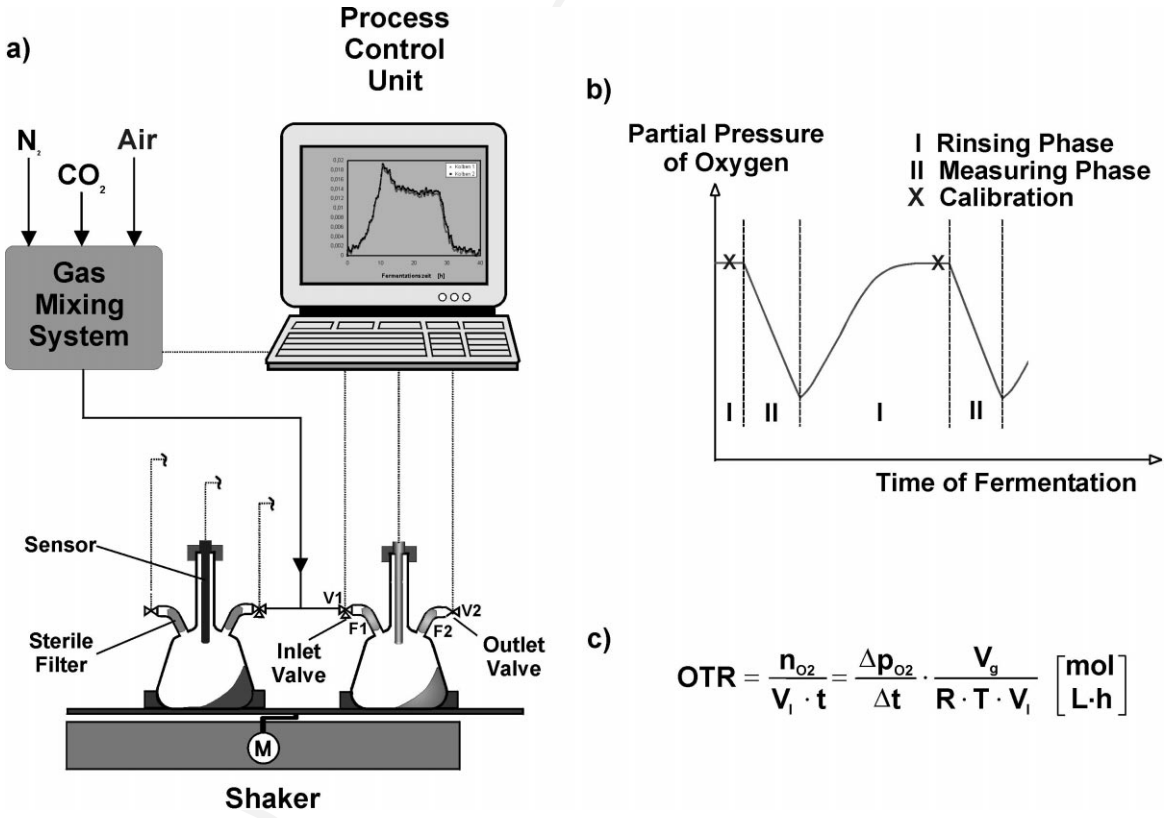

Fig. 1. (a) Principle of the measuring device. (b) Partial pressure of oxygen during a measuring cycle. (c) Equation to determine the oxygen transfer rate.

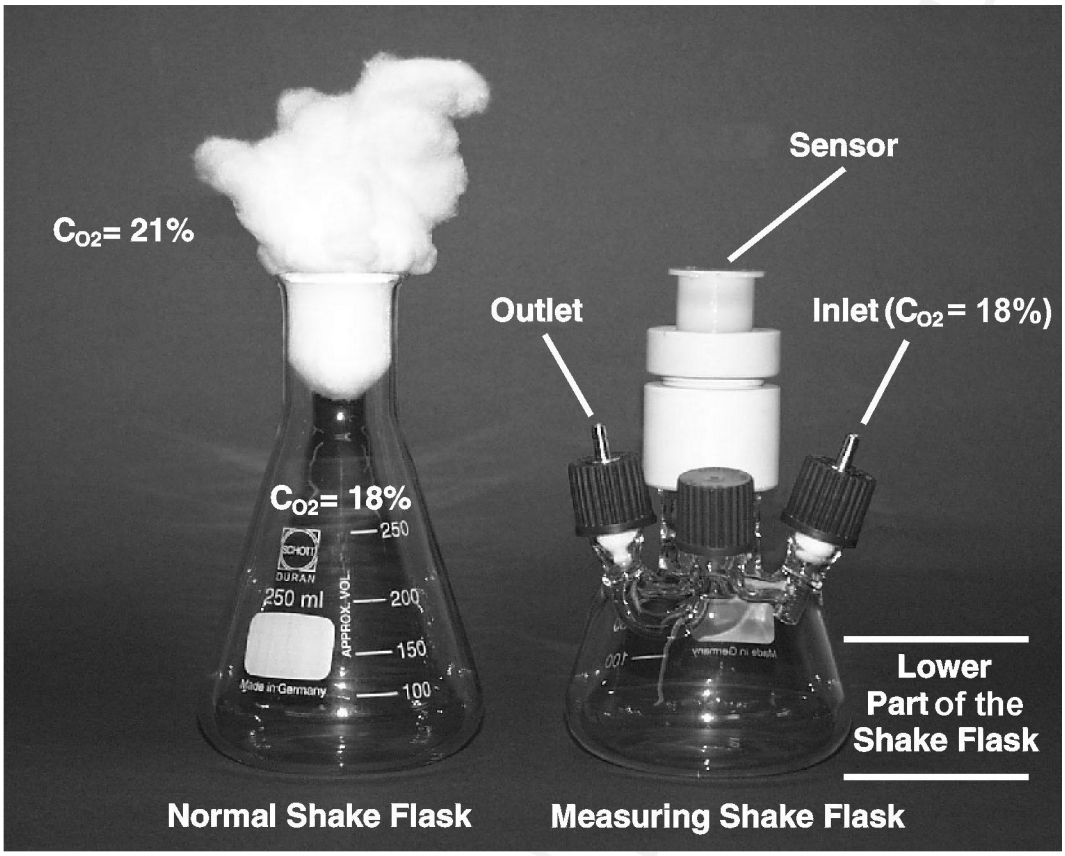

Fig. 2. Comparison of the normal and the measuring shaking flask.

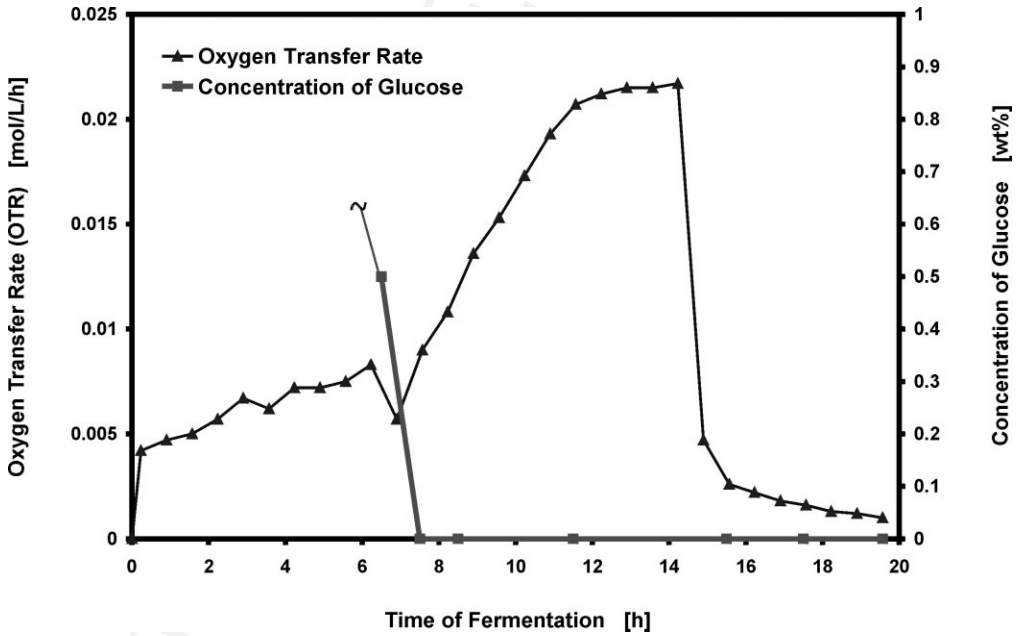

Fig. 3. Oxygen transfer rate and glucose concentration of a fermentation of the yeast *Saccharomyces cerevisiae*, shaking conditions of the rotary shaker: shaking frequency 200 rpm, shaking diameter 50 mm, fermentation temperature 30°C, filling volume 25 ml.

yeast consumes another carbon source. The well-known explanation of this phenomenon is the Crabtree effect [3]. Because of the Crabtree effect the yeast produces ethanol under aerobic conditions from glucose in the first phase of the fermentation. Ethanol is the nutrient of the second growth phase. After 15 h all carbon sources are exhausted and the oxygen transfer rate rapidly declines. This steep decrease at the end of the fermentation is nearly always an indication for a complete exhaustion of an essential substrate. Similar data of a batch fermentation of the yeast *S. cerevisiae* in a stirred bioreactor were obtained by Käppeli [3] with an online exhaust gas analysis.

2.2.2. Fungus Botrytis cinerea

In co-operation with the company BASF AG (Ludwigshafen, Germany) the fungus *Botrytis cinerea* was investigated in order to create an optimal medium. Hundred millilitres of the basic medium was filled in the measuring shaking flask. 10^3 spores ml^{-1} of the fungus were used as inoculum.

In Fig. 4 two fermentations are shown with different media. The fermentation with the basic medium (straight line) starts after 20 h with a relatively low oxygen consumption. The oxygen consumption significantly increases at about 60 h. Then the oxygen transfer rate suddenly levels off after 70 h and begins to decrease very slowly. This shape of the oxygen transfer rate curve indicates a substrate limitation. To prove the assumption of a substrate limitation several fermentations were carried out (data not shown). The dotted line represents a fermentation with the basic medium enriched with magnesium sulphate. The dotted line reaches a higher oxygen transfer rate than the straight line. This higher oxygen transfer rate verifies the assumption of a substrate limitation. However, the slow decrease of the oxygen transfer rate at the end of the fermentation indicates that a limitation by another substrate is still present.

2.2.3. Yeast Hansenula polymorpha

In co-operation with the company Rhein Biotech GmbH (Düsseldorf, Germany) an investigation of their screening conditions for the recombinant strain of the yeast *Hansenula polymorpha* was conducted. Rhein Biotech uses the yeast as a host cell for the production of heterologous proteins [4]. The medium was a defined synthetic medium with glycerol as the only carbon source.

In Fig. 5 the development of the oxygen transfer rate of three experiments with an uracil auxotroph mutant of the yeast *H. polymorpha* is presented. The concentration of uracil and the liquid volume in the measuring shaking flasks were varied. The curve with the diamonds shows the experimental data of the fermentation with the original medium used by the company. As explained in the previous example (see *B. cinerea*) the shape of the curve between 12 and 30 h indicates a substrate limitation. After 30 h the oxygen transfer rate drops down, because an essential substrate, here the carbon source glycerol, is completely exhausted. The fermentation represented by the line with the squares was carried out with a double concentration of uracil. The oxygen transfer rate starts to rise in the same way as in the case of the standard conditions, but reaches a higher level. This fact

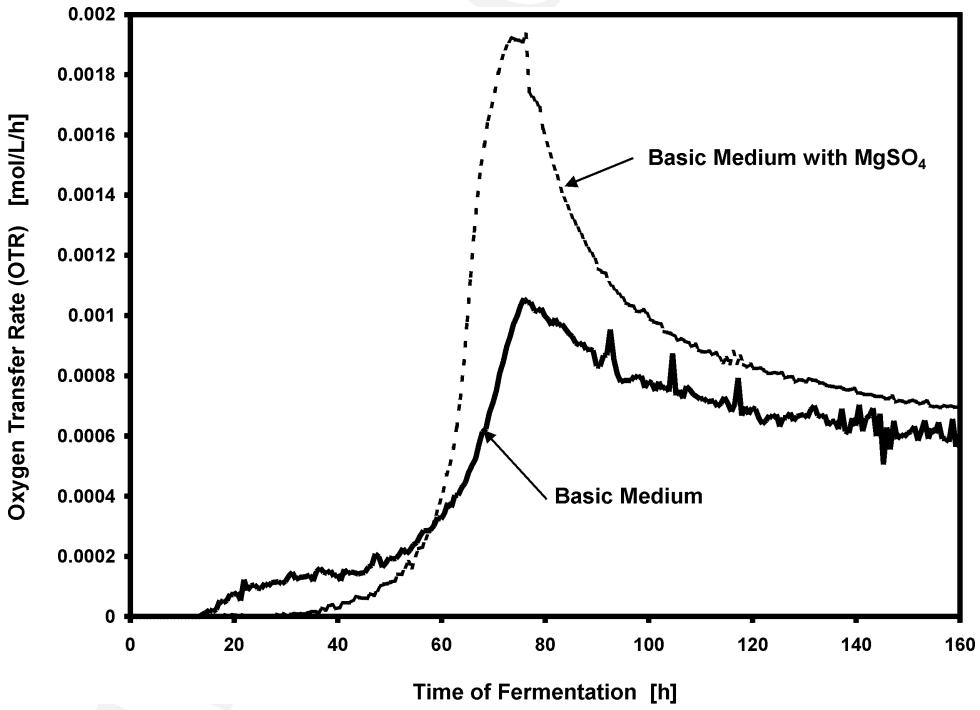

Fig. 4. Oxygen transfer rate of a fermentation of the fungus *Botrytis cinerea*, shaking conditions of the rotary shaker: shaking frequency 180 rpm, shaking diameter 25 mm, fermentation temperature 18°C, filling volume 100 ml.

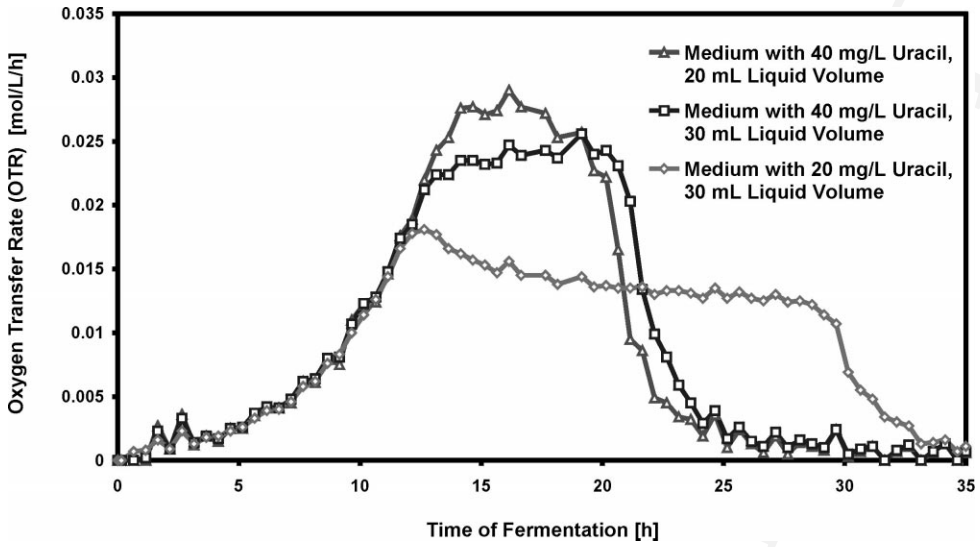

Fig. 5. Oxygen transfer rate of a fermentation of an uracil auxotroph mutant of the yeast *Hansenula polymorpha*, shaking conditions of a rotary shaker: shaking frequency 300 rpm, shaking diameter 25 mm, fermentation temperature 37°C.

proves that uracil was the limiting substrate. Between 12 and 21 h the oxygen transfer rate stays at the same level before it levels off. The plateau between 12 and 21 h is a characteristic sign of an oxygen limitation. To overcome this limitation there are different ways. One easy way is to lower the liquid volume in the shaking flask. The result is presented by the line with the triangles. The reduced liquid volume leads to a higher maximum oxygen transfer capacity of the shaking flask. This supports the idea of an oxygen limitation.

3. Conclusion

With the newly invented device for online measurement of the oxygen transfer rate under sterile conditions a lot of information about the culture and the fermentation conditions can be gained as shown in the three examples. A short summary about different biological phenomena and how they affect the shape of the curve of the oxygen transfer rate is presented in Fig. 6. These graphs are of course

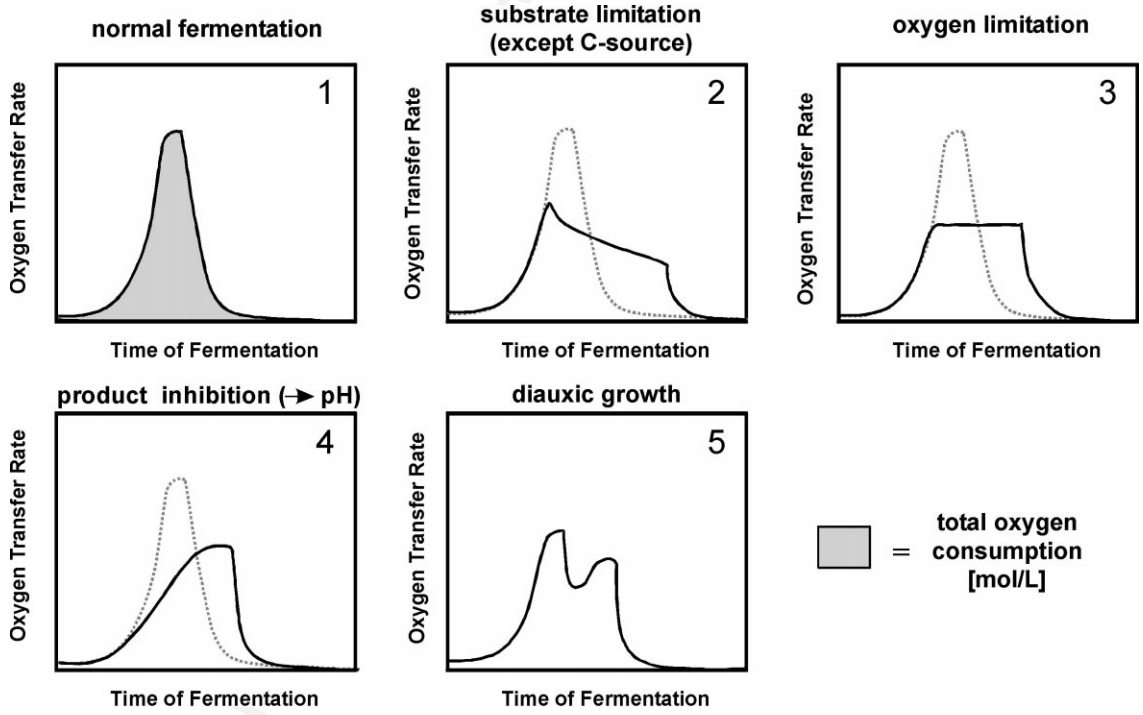

Fig. 6. Selection of typical biological phenomena and their effects on the oxygen transfer rate.

simplified and show only how one effect influences the oxygen transfer rate. In real examples two or more influences may be superimposed. However, as the experiments have shown, the shape of the oxygen transfer rate curve always indicates whether the fermentation conditions limit the growth of the microorganisms or not. Furthermore, the online measured oxygen transfer rate gives very valuable information for engineers, who have to scale up a bioprocess from the shaking flask to the stirred bioreactor. For this reason the device will support the screening of media or strains and should be part of the scale-up procedure in research institutes or industry.

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