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Online respiration activity measurement (OTR, CTR, RQ) in shake flasks

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Abstract

Online measurement of respiration activity (including oxygen transfer rate (OTR), carbon dioxide transfer rate (CTR), respiratory quotient (RQ)) of microbial cultures in stirred bioreactors with exhaust gas analysis has been state of the art for years. As much more experiments are conducted in shaking bioreactors compared to stirred bioreactors, Anderlei and Büchs [Biochem. Eng. J. 7 (2001) 157] developed a measuring device (OTR-Device) for online determination of the oxygen transfer rate in shake flasks under sterile conditions. In this paper, an extension of the OTR-Device, termed respiration activity monitoring system (RAMOS) is described, which allows additional measurement of the carbon dioxide transfer rate and the respiratory quotient in shaking bioreactors. Fermentations of the yeasts *Saccharomyces cerevisiae* and *Pichia stipitis* carried out with RAMOS are presented. These measurements show very clearly the differences in respiration activities between the Crabtree-positive yeast *S. cerevisiae* and the Crabtree-negative yeast *P. stipitis*. Furthermore, a fermentation of the bacterium *Corynebacterium glutamicum* is presented, showing the influence of an oxygen limitation on the metabolic activities of the culture. Also, a fermentation of a hybridoma cell line was carried out with RAMOS to elucidate the measuring sensitivity of the system. The new device provides the most important and characteristic parameters (OTR, CTR, RQ) representing biological cultures online, enabling users to draw conclusions on metabolisms of microorganisms already in shaking bioreactors.

Keywords: Shake flask; Oxygen transfer rate; Carbon dioxide transfer rate; Respiratory quotient; Bioprocess monitoring; Bioreactor; Crabtree; Hybridoma; *Saccharomyces cerevisiae*; *Corynebacterium glutamicum*

1. Introduction

Shake flasks have become irreplaceable in microbial applications requiring extensive experimentation, for example, in screening for efficient strains or in optimising culture media. A major disadvantage of shake flasks as an experimental system is the lack of online monitoring and control [2,3]. Therefore, a tool (the OTR-Device) was developed by Anderlei and Büchs [1] to enable online measurement of the oxygen transfer rate (OTR) in shaking bioreactors under sterile conditions. The oxygen transfer rate is a commonly employed parameter for quantifying the physiological state of an aerobic culture, since most metabolic activities depend on oxygen consumption. Substrate or oxygen limitations, product inhibitions, diauxic growth and other biological phenomena may be uncovered based on the course of the oxygen transfer rate during fermentation [1].

The carbon dioxide transfer rate (CTR) and the respiratory quotient (RQ) in shaking bioreactors provide further information, used in predicting biomass concentration, evaluating energetic efficiencies of growth, monitoring growth yields and controlling fed batch processes [4,5]. Therefore, the measurement of the carbon dioxide transfer rate was implemented in the OTR-Device [1]. The new measuring system (termed respiration activity monitoring system (RAMOS)), which combines online oxygen and carbon dioxide measurement in shaking bioreactors, is introduced and results obtained from initial studies with two types of yeast, a bacterial culture and an animal cell culture, are discussed in this paper.

2. Material and method

2.1. Measuring method and device

2.1.1. Assembly

Fig. 3 illustrates the set-up of a RAMOS device with eight measuring flasks and six normal shake flasks running in

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Nomenclature

CTR	carbon dioxide transfer rate (mol/l h)
OTR	oxygen transfer rate (mol/l h)
R	gas constant (bar l/mol/K)
RQ	respiratory quotient
T	temperature (K)
V_G	gas volume (l)
V_L	liquid volume (l)
Δp_{O_2}	difference of oxygen partial pressure (bar)
Δp_{CO_2}	difference of carbon dioxide partial pressure (bar)
Δt	time of the measuring phase (h)

parallel for sampling. For further information on the general set-up of RAMOS, refer to literature on the oxygen transfer rate measuring device presented by Anderlei and Büchs [1].

2.1.2. Measuring method

During fermentation, a measuring cycle is continuously repeated. This measuring cycle is comprised of a measuring phase and a rinsing phase (see Fig. 1b). During the rinsing phase, air is flushed through the measuring flask at a specific flow rate (see below). At the beginning of the measuring phase, inlet and outlet valves of the measuring flask (see Fig. 1a) are closed. Respiration of the microorganisms leads to a decrease in the partial pressure of oxygen and to an increase in the carbon dioxide partial pressure in the headspace of the measuring flask, as illustrated in Fig. 1b. The partial pressures are monitored by an oxygen sensor and a differential pressure sensor. Assuming linear changes in the measuring phase, a computer calculates the oxygen transfer rate and the carbon dioxide transfer rate and thus the respiratory quotient, as shown in the simplified Eqs. (1)–(3)

$$\mathrm{OTR} = \frac{\Delta p_{O_2}}{\Delta t}\frac{V_G}{RTV_L} \quad (1)$$

$$\mathrm{CTR} = \frac{\Delta p_{CO_2}}{\Delta t}\frac{V_G}{RTV_L} \quad (2)$$

$$\mathrm{RQ} = \frac{\mathrm{CTR}}{\mathrm{OTR}} \quad (3)$$

After the measuring phase, the valves are opened again, and the next measuring cycle starts. Before each measuring phase, the sensors are calibrated using the known steady state gas composition to compensate for signal drift, as indicated in Fig. 1b.

The gas concentration in the headspace of a normal flask equipped with a sterile barrier, e.g. a cotton plug, is calculated using the method of Mrotzek et al. [8]. The air flow rate through the measuring flask during the rinsing phase is adjusted such that the gas concentration in the headspace of the measuring flask is equivalent to that in the normal shake flask, as illustrated in Fig. 2. The establishment of equivalent fermentation conditions in the measuring flask and the normal shake flask with reference to the hydrodynamics and the gas concentration in the headspace is required for the comparison of the systems. Thus, results determined with RAMOS can be transferred to the normal shake flasks running in parallel to the measuring flasks.

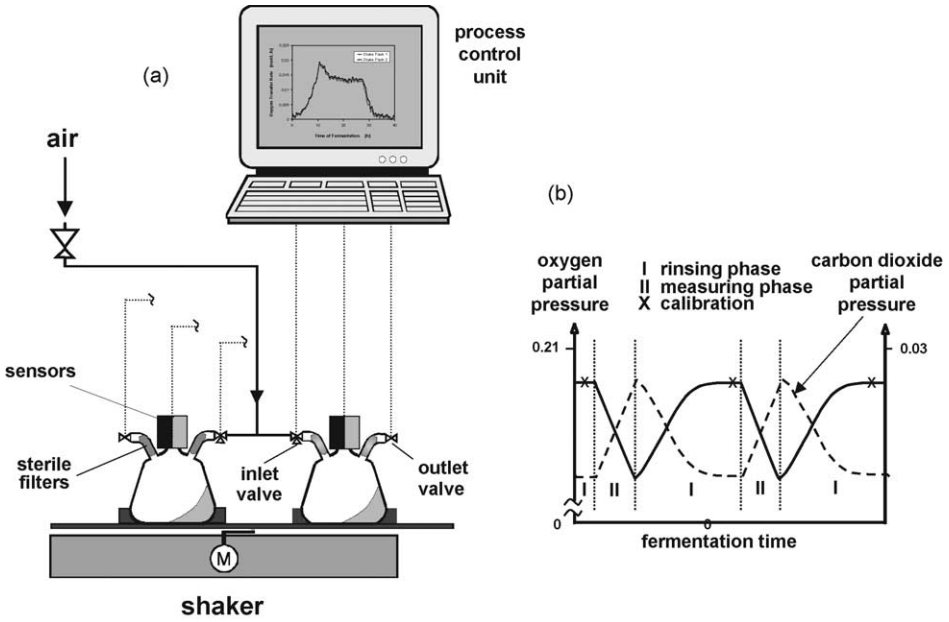

Fig. 1. (a) Principle and general set-up of RAMOS. (b) Partial pressure of oxygen and carbon dioxide during a measuring cycle: (—) oxygen partial pressure and (- - -) carbon dioxide partial pressure.

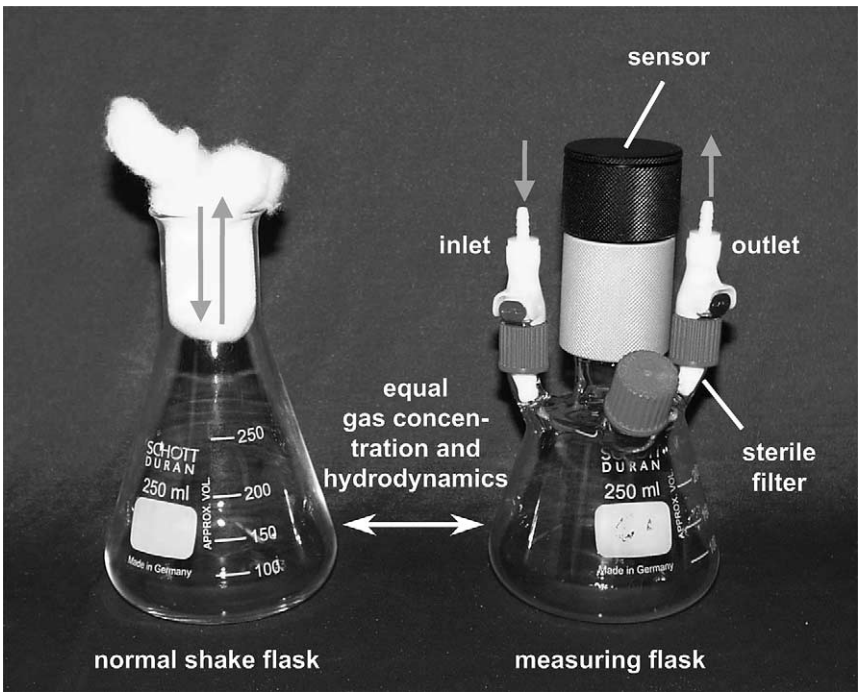

Fig. 2. Comparison of the normal shake flask to the measuring flask used for RAMOS.

2.2. Biological systems

2.2.1. Yeast Pichia stipitis and Saccharomyces cerevisiae

The Crabtree-negative yeast *P. stipitis* (CBS 5774) and the Crabtree-positive yeast *S. cerevisiae* (DSM 70449) were cultivated. Both fermentations were performed with RAMOS to determine oxygen and carbon dioxide transfer rates using measuring flasks corresponding to a normal 250 ml shake flask, as shown in Fig. 2. Pre-culture conditions and medium composition for *S. cerevisiae* and *P. stipitis* were identical. Pre-culture was carried out in a 250 ml shake flask filled with 50 ml complex medium comprised of 10 g/l peptone, 10 g/l yeast extract and 20 g/l glucose. Pre-cultures were cultivated at 30 °C, at 200 rpm (rotary shaking) on a shaker (Kühner AG, Birsfelden, Switzerland) with 50 mm shaking diameter. After the optical density (600 nm) of the pre-culture reached 13, the cell broth was used for inoculation. One hundred millilitres of main-culture medium was inoculated with 3 ml pre-culture. Then, the medium of the main culture was distributed among the single flasks (measuring and normal shake flasks) to guarantee absolute comparability. The fermentation conditions of the yeasts *S. cerevisiae* during the main culture were: 25 ml liquid volume, 200 rpm shaking frequency, 50 mm shaking diameter, and 30 °C incubation temperature. The fermentation conditions of the yeast *P. stipitis* during the main culture were: 10 ml liquid volume, 100 rpm shaking frequency, 50 mm shaking diameter, and 30 °C incubation temperature.

2.2.2. Bacteria Corynebacterium glutamicum

The fermentation of *C. glutamicum* (ATCC 13032) was performed with RAMOS using measuring flasks corresponding to a normal 250 ml shake flask, as shown in Fig. 2. Pre-culture was carried out in a normal 250 ml shake flask filled with 20 ml complex medium comprised of 10 g/l peptone, 10 g/l yeast extract, 2.5 g/l NaCl, and 20 g/l glucose. Pre-cultures were cultivated at 30 °C, at 200 rpm (rotary shaking) on a shaker (Kühner AG, Birsfelden, Switzerland) with 50 mm shaking diameter. After the optical density (600 nm) of the pre-culture reached 10, the cell broth was used for inoculation. Three hundred and sixty millilitres of main-culture medium was inoculated with 20 ml pre-culture. Then, the medium of the main culture was distributed among the single flasks (measuring and normal shake flasks). The main-culture medium contained per litre: 30 g glucose, 20 g $(NH_4)_2SO_4$, 0.5 g $NaH_2PO_4{\cdot}H_2O$, 0.5 g K_2HPO_4, 0.25 g $MgSO_4{\cdot}7H_2O$, 0.001 g $FeSO_4{\cdot}7H_2O$, 0.001 g $MnSO_4{\cdot}4H_2O$, 0.005 g $ZnSO_4{\cdot}7H_2O$, 0.0005 g $CuSO_4{\cdot}5H_2O$, 0.002 g $CaCO_3$, 0.0002 g biotin, 0.0001 g thiamine-di-chloride. The fermentation conditions of *C. glutamicum* during the main culture were: 10–30 ml liquid volume, 200 rpm shaking frequency, 50 mm shaking diameter, and 30 °C incubation temperature.

2.2.3. Hybridoma cells

An industrial hybridoma cell line was investigated producing the antibody IgG1. Measuring flasks corresponding to a normal 250 ml shake flask were used. Pre-culture was carried out in a normal spinner flask following

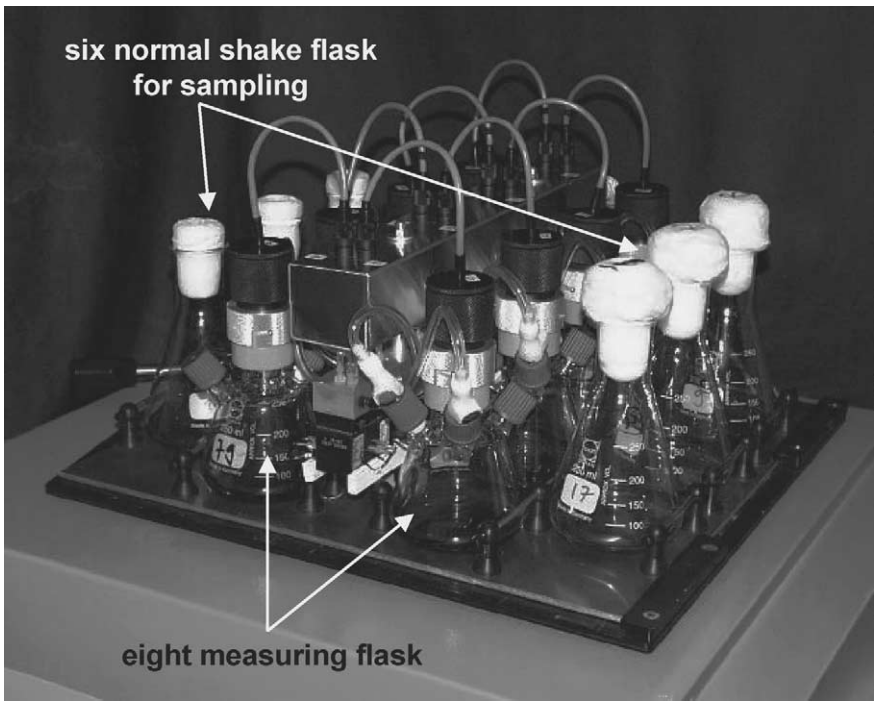

Fig. 3. Set-up of RAMOS: eight measuring flasks and six normal shake flasks.

four phases. In the first phase, 20 ml inoculum with 0.97×10^6 cells/ml (viability 90%) was diluted with 20 ml PRO-CHO 4 CDM (Biowhittaker, 12-029Q). Twenty-four hours later (1.05×10^6 cells/ml, viability of 99%), the culture was diluted again with 62 ml PRO-CHO 4 CDM (liquid volume: 100 ml). At hour 72, the cell count results in 1.01×10^6 cells/ml with a viability of 77%. Thereupon, the culture was supplemented with 100 ml PRO-CHO 4 CDM and with 5 ml foetal bovine serum (Life Technologies, 10270–106), to increase the viability of the culture. At hour 96, 100 ml of the culture (1.51×10^6 cells/ml, viability of 87%) was mixed with 200 ml PRO-CHO 4 CDM (liquid volume: 300 ml). After 120 h, the pre-culture (1.07×10^6 cells/ml, viability of 89%) was ready for inoculation. In the main culture, the filling volume of the measuring flasks was 100 ml. Two different media were used. Medium 1 was prorated in two measuring flask with each containing: 20 ml pre-culture, 80 ml PRO-CHO 4 CDM (Biowhittaker, 12-029Q), and 1 ml foetal bovine serum (Life Technologies, 10270–106). Medium 2 was prorated in two measuring flask with each: 101 ml inoculated medium 1 supplemented with 1 ml Opti Mab Comp A (Life Technologies, 11908–035) and 200 μl high density lipid concentrate (500×, Life Technologies, 01-0305A). The fermentation conditions of the hybridoma cells during the main culture were: 100 rpm shaking frequency, 50 mm shaking diameter, and 37 °C incubation temperature. The inlet air was mixed with carbon dioxide to obtain a concentration of 5% CO_2. At hour 70, the measuring flasks were taken to a laminar flow bench for sampling.

2.3. Analytical methods

During the fermentation, samples were taken from normal shake flasks (250 ml, narrow necked, cotton plug) running in parallel to the measuring flasks as illustrated in Fig. 3. To determine ethanol and glucose concentration, supernatant of the samples were analysed by high-pressure liquid chromatography (column: organic acid resin (#7907–99), pressure: 60 bar, temperature: 30 °C, detector: RI, liquid phase: 1 mM H_2SO_4, liquid flow rate: 0.6 ml/min, Dionex, Idstein, Germany). Furthermore, the optical density was determined with a photometer (UVIKON 922, Kontron Instruments, Germany) at a wave length of 600 nm. The cell count of the hybridoma cells was evaluated with a hemocytometer (Typ Neubauer improved, Germany). The viability of the cells was defined by dying with erythrosine-red.

3. Results and discussion

On-line measurements of the oxygen transfer rate in shake flasks with animal cells, plant cells, fungi, yeast cells and bacteria have now been carried out with RAMOS [1,9–11]. In order to demonstrate the additional information content of the carbon dioxide transfer rate, two yeast cultures and a bacterial culture showing distinct carbon metabolisms are presented in this paper. We also carried out a fermentation of a hybridoma cell line to elucidate the sensitivity of the oxygen transfer rate measurement of RAMOS.

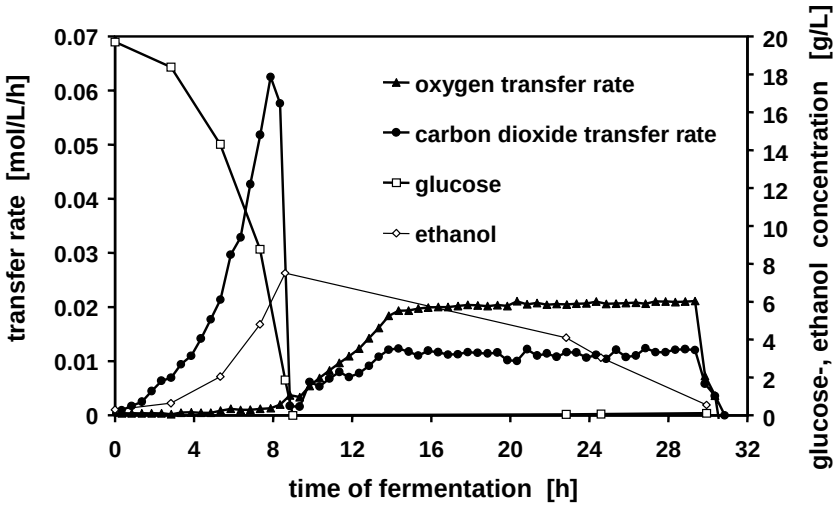

Fig. 4. Oxygen and carbon dioxide transfer rates, glucose and ethanol concentration from the off-line analysis of a fermentation of the yeast *Saccharomyces cerevisiae*. Shaking conditions of the rotary shaker: 200 rpm shaking frequency, 50 mm shaking diameter, 30 °C fermentation temperature, and 25 ml filling volume.

3.1. *Saccharomyces cerevisiae*

S. cerevisiae was selected because it is the best investigated yeast, due to its high industrial relevance (Käppli, 1986). Fig. 4 shows the results of a fermentation carried out with RAMOS. During the first 8 h, the oxygen transfer is low. Thereafter, the oxygen transfer rate increases to 0.02 mol/l h and remains at this level until hour 30, when it drops.

By examining the course of the oxygen transfer rate during fermentation, only information concerning the maximum oxygen transfer rate and the time of carbon source exhaustion is obtained [1]. The plateau of the oxygen transfer rate between hours 13 and 30 represents the maximum oxygen transfer rate capacity of the system, which can also be calculated [9]. This implies that the fermentation of the yeast is oxygen-limited in this phase. The sharp drop in the oxygen transfer rate at hour 30 indicates that the carbon source has been completely exhausted.

In Fig. 4, additionally the course of the carbon dioxide transfer rate is shown. The carbon dioxide transfer rate rises exponentially during the first 8 h. During this phase, the yeast uses the respiro-fermentative glucose metabolism, because of the Crabtree-effect [6], and produces carbon dioxide and ethanol. After 8 h, the carbon dioxide transfer rate decreases steeply, which agrees very well with the exhaustion of the initial carbon source glucose (Fig. 4). Subsequently, the carbon dioxide transfer rate rises in parallel with the oxygen transfer rate until hour 11, before it levels off and remains at a steady level below the oxygen transfer rate. In this phase, the respiratory quotient (not shown) is around 0.5, indicating the consumption of a carbon source which is more reduced than glucose. In this case, the reduced carbon source is the ethanol produced in the first fermentation phase (see Fig. 4). Similar data from a *S. cerevisiae* batch fermentation in a stirred bioreactor (10 l) were obtained by Fiechter [7] using an online exhaust gas analysis. In our case, these results were obtained from flasks with only 25 ml filling volume.

3.2. *Pichia stipitis*

The yeast *P. stipitis* was chosen as a second model microorganism because it is Crabtree negative, in contrast to *S. cerevisiae*. Fig. 5a shows data on the oxygen and carbon dioxide transfer rates and the respiratory quotient from the fermentation of *P. stipitis*. The oxygen transfer rate and the carbon dioxide transfer rate rise during the first 5 h, with a respiratory quotient of about one. These transfer rates contrast those of the Crabtree-positive yeast *S. cerevisiae*, shown in Fig. 4. After 5 h, the oxygen transfer rate levels off and plateaus at approximately 0.02 mol/l h until hour 21, indicating an oxygen limitation of the microbial culture [1]. As can be calculated from Maier et al. [9], the operating conditions of the above described *Saccharomyces* fermentation (200 rpm, 25 ml filling volume) and the *Pichia* fermentation (100 rpm, 10 ml filling volume) result in nearly equivalent maximum oxygen transfer capacities. In Figs. 4 and 5, very similar levels of the OTR plateaus are observed. While the oxygen transfer rate in Fig. 5 levels off (hour 5), the carbon dioxide transfer rate keeps on rising exponentially, as does the respiratory quotient. Subsequently, both signals (CTR, RQ) drop after hour 10, indicating the depletion of the initial carbon source glucose. It can be deduced from this data that during this time period (hours 5–10) the yeast *P. stipitis* consumes glucose through its aerobic and anaerobic pathways, producing carbon dioxide and ethanol. This agrees with off-line measurements illustrated in Fig. 5b. As in the fermentation of the yeast *S. cerevisiae*, the carbon dioxide declines (here, after hour 12) below the oxygen transfer rate resulting in a respiratory quotient below one. This behaviour demonstrates that *P. stipitis* is now consuming ethanol. The

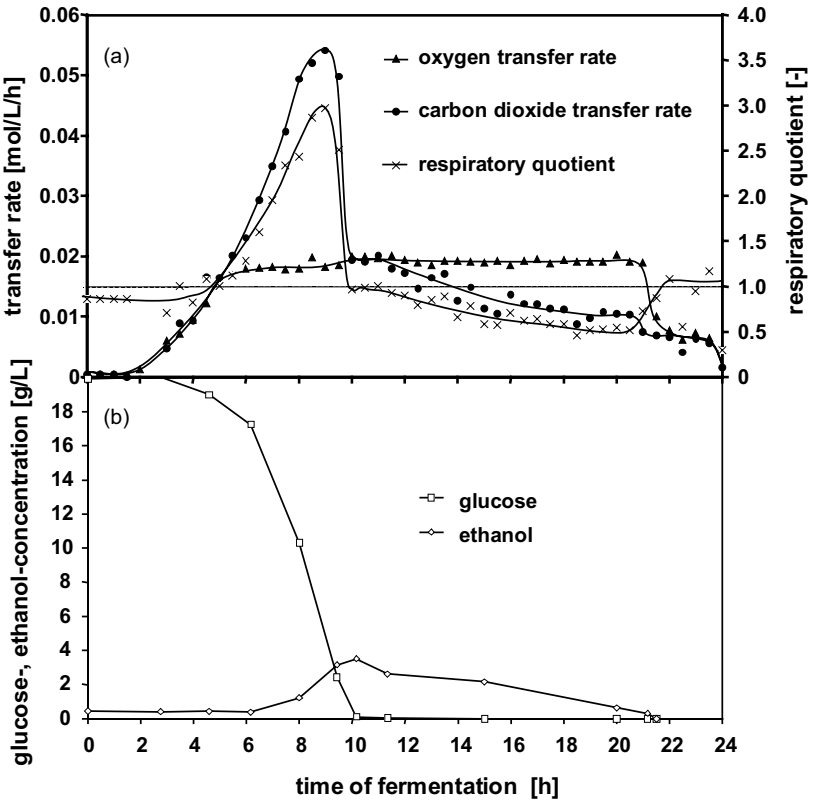

Fig. 5. (a) Oxygen and carbon dioxide transfer rates and the respiratory quotient of the fermentation of the yeast *P. stipitis*. (b) Glucose and ethanol concentration from the off-line analysis. Shaking conditions of the rotary shaker: 100 rpm shaking frequency, 50 mm shaking diameter, 30 °C fermentation temperature, 10 ml filling volume.

declining ethanol concentration in this time period as shown in Fig. 5b supports this hypothesis. The steep decreases in the oxygen and carbon dioxide transfer rates at hour 21 signal the depletion of the carbon source ethanol and thus the end of the fermentation. The off-line analysed samples shown in Fig. 5b verify the exhaustion of ethanol at this time.

3.3. *Corynebacterium glutamicum*

Fig. 6 shows two fermentations of the bacterium *C. glutamicum*, which were carried out with RAMOS in parallel. The conditions of the two fermentations differ only in the liquid filling volume (10 and 30 ml). In case of 10 ml liquid volume (Fig. 6a), the oxygen transfer rate exponentially increases after a short lag-phase. After 20 h, the oxygen transfer rate levels off and plateaus at approximately 0.039 mol/l h until hour 30, indicating an oxygen limitation. The subsequent drop down of the oxygen transfer rate indicates the depletion of the carbon source. The off-line analysis verifies the exhaustion of the initial carbon source glucose and the carbon source lactate which was produced in a small amount (<2 g/l) during the oxygen-limited growth phase. The second fermentation was carried out with 30 ml liquid filling volume in the measuring flask. The oxygen and the carbon dioxide transfer rate of the culture increase in parallel and level off after hour 15 (approximately 0.016 mol/l h). The maximum oxygen transfer capacity is much lower than at the fermentation with 10 ml liquid volume. The transfer rates plateaus until hour 40, indicating an oxygen limitation of the bacterial culture. The off-line measured lactate concentration verifies the oxygen-limited growth of the culture, as lactate increases up to 6 g/l in hour 40. Due to stoichiometric reasons (lactate has the same degree of reduction as glucose), this lactate formation can not be detected by the respiratory quotient (not shown). After hour 40, the carbon dioxide transfer rate drops, whereas the OTR still remains more or less on the same level. One reason for the drop of the OTR is the depletion of glucose, as indicated by the off-line data. Another reason is the termination of lactic acid production and of the concomitant release of CO_2 from the $CaCO_3$ buffer. The drop in oxygen and carbon dioxide transfer rates at hour 47 shows the end of the fermentation due to the depletion of the carbon source lactate.

3.4. *Hybridoma cells*

Fig. 7 shows four fermentations of a hybridoma cell line. In this experiment, two different media were compared using two parallel flasks each. All four cultures start with the same very low oxygen transfer rate level of 0.00005 mol/l h

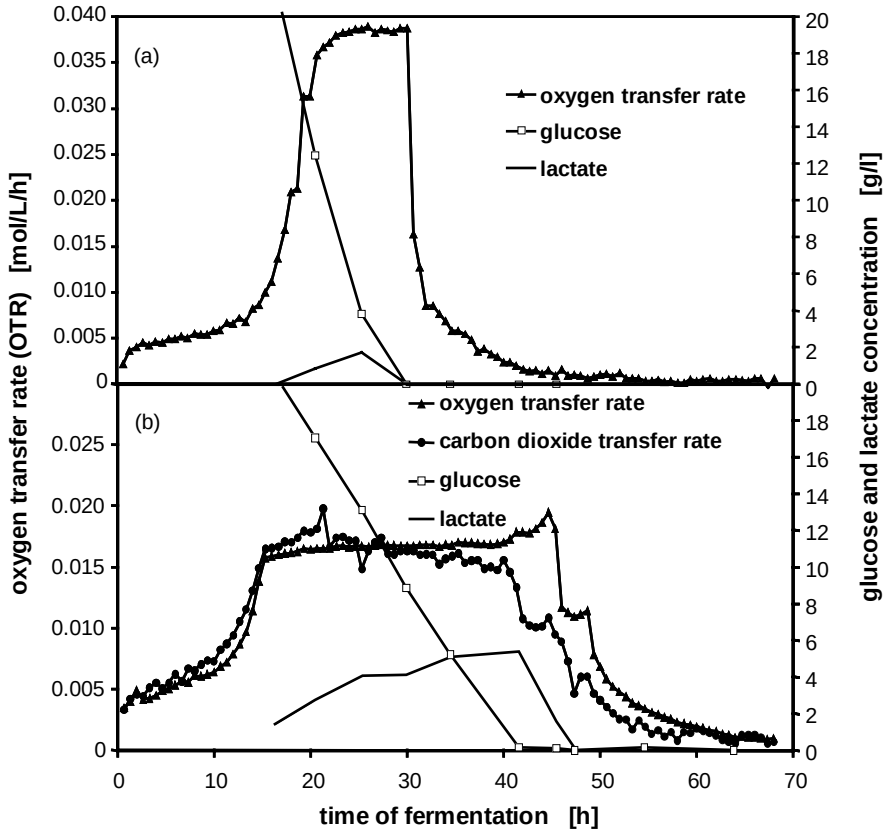

Fig. 6. Oxygen and carbon dioxide transfer rates, glucose and lactate concentration of the fermentation of the bacterium *Corynebacterium glutamicum*. Shaking conditions of the rotary shaker: 200 rpm shaking frequency, 50 mm shaking diameter, 30 °C fermentation temperature, and (a) 10 ml and (b) 30 ml filling volume.

and rise linearly until hour 35. The oxygen transfer rates of the cultures with the medium 1 keep on increasing linearly, while the oxygen transfer rates of the cultures with the medium 2 start to rise exponentially. At hour 55, the cell cultures reach their maximum oxygen transfer rates (medium 2: 0.0035 mol/l h and medium 1: 0.0029 mol/l h). Afterwards, the oxygen transfer rates of all cultures decrease very slowly until hour 138. Between hours 70 and 75, an oscillation of the oxygen transfer rate can be observed. This phenomenon is the result of sampling of the measuring flasks. To take this sample, the measuring flasks were removed from the incubator (37 °C) and placed in a laminar flow bench (20 °C)

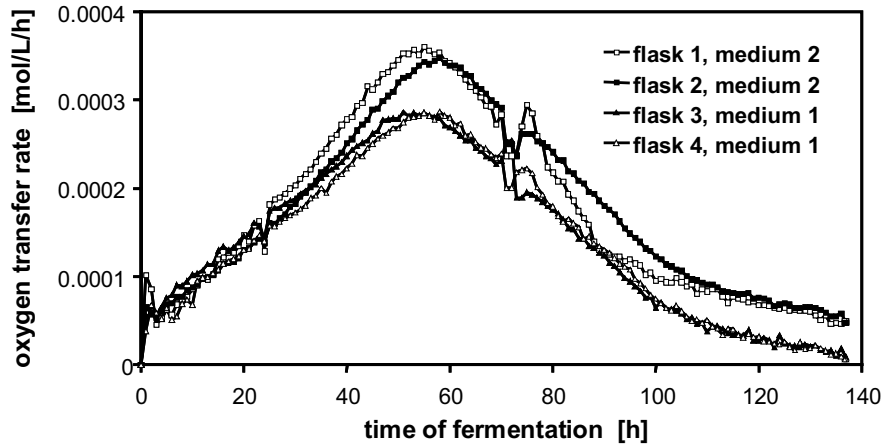

Fig. 7. Oxygen transfer rates of a fermentation of hybridoma cells. Two different media were used described in Section 2. Shaking conditions of the rotary shaker: 100 rpm shaking frequency, 50 mm shaking diameter, 37 °C fermentation temperature, 20 ml filling volume.

for approximately 10 min. This temperature shift is the reason for the observed oscillation of the oxygen transfer rate curve. Therefore, we prefer the method usually applied in our laboratory, taking samples from harvested normal shake flasks, which are run in parallel to the respective measuring flask and which are not replaced on the shaking machine.

This experiment shows that the oxygen transfer rate measurement of cell cultures in shake flasks is applicable, even though the respiration activity is 100 times lower than the respiration activities of bacteria or yeast cultures. Furthermore, respiration activity differences are detected for the slightly different media (Fig. 7). As these signal differences are significant and reproducible, the RAMOS device has proven to be suitable for cell culture process development in small scale. In future research work, it has to be determined which effect (viability of the cells, total cell number, metabolic activity, etc.) is responsible for the observed differences in respiration activity.

4. Conclusions

It was shown that the carbon dioxide transfer rate and of the respiratory quotient of shake flask cultures can provide important additional information about a fermentation process. This technique (RAMOS) enables the user to exactly determine the fermentation phases (respirative, fermentative, respiro-fermentative) without taking samples. Furthermore, the type of carbon source metabolised by the microbial culture can be identified with the respiratory quotient. It is noteworthy that results of the samples taken out of the normal shake flasks running in parallel to the measuring flask exactly reflect the information gained by RAMOS, e. g. depletion of a carbon source. In particular, the simultaneous drop of the respiration activity at the end of the fermentation and the depletion of the volatile component ethanol (analytical samples were taken form the normal shake flasks running in parallel to the measuring shake flasks) show that the ventilation of the measuring flask and the normal shake flask are comparable. Furthermore, RAMOS is applicable to the cultivation of cell cultures, even though the respiration activity is 100 times lower than that of a bacterial or yeast culture.

The newly developed measuring device (RAMOS) generates useful information about cultivating conditions and the physiological state of microorganisms in early stages of research and bioprocess development from many parallel reactors. Unsuitable screening conditions can be avoided based on these measurements and scale-up is facilitated. RAMOS combines advantages of the shake flask (easy to handle, enables several parallel fermentations) with the advantages of a stirred bioreactor, in particular, online measuring capabilities.

Acknowledgements

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