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A system for accurate on-line measurement of total gas consumption or production rates in microbioreactors

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ABSTRACT

A system has been developed, based on pressure controlled gas pumping, for accurate measurement of total gas consumption or production rates in the nmol/min range, applicable for on-line monitoring of bioconversions in microbioreactors. The system was validated by carrying out a bioconversion with known stoichiometric relation between gas consumption and substrate conversion, that is, the enzymatic oxidation of glucose to gluconic acid. The reaction was carried out in a stirred microreactor with a working volume of 100 μL, whereby the oxygen consumption was monitored on-line. Subsequently the system was applied to determine the oxygen transfer capacity of the microbioreactor. The dissolved oxygen concentration was measured with an optical dissolved oxygen sensor, which was integrated near the bottom of the reactor. Different stirrer sizes and geometries were investigated for their effect on the mass transfer of oxygen. A maximal k_La of $156 \pm 10\,h^{-1}$, allowing a maximal O_2-transfer rate up to 50 mmol O_2/L/h, was reached which is sufficient to grow cells aerobically in (fed-)batch mode at relatively high biomass concentrations.

1. Introduction

The development of miniaturized bioreactor systems for high throughput experimentation is an emerging and rapidly progressing area of applied research. The development of such systems aims to combine a small working volume with good monitoring and control features in order to speed up experimentation in the field of biotechnology.

Gaseous compounds are important substrates or products in many biological processes, including fermentations. Sufficient oxygen supply and carbon dioxide removal is one of the most frequently occurring problems associated with the use of high-throughput cell cultivation systems (Minas et al., 2000; Duetz and Witholt, 2001; Hermann et al., 2001; Duetz and Witholt, 2004; Hermann et al., 2003).

As far as we know no attempts have been undertaken to directly measure the gas consumption or production in 100 μL-scale bioreactors for high throughput cultivation. However, on-line gas consumption or production measurements can provide reliable and quantitative on-line information on bioconversion rates, such as biomass growth and product formation (Cooney et al., 1977), and is state of the art in bench- or pilot-plant-scale bioreactors.

Pressure sensing is a reliable and straightforward technique for the on-line quantification of gas consumption or production in bioprocesses were only a single gas is involved (Wick et al., 2001; Anderlei et al., 2004) and can easily be scaled down. This methodology could be very useful for studying anaerobic or (de)nitrifying cultivations as well as enzymatic conversions like oxygenases. Widely employed techniques to gain information on cellular or enzymatic performance in small bioreactors are turbidity and fluorescence, respectively. However both techniques have severe limitations, i.e. the turbidity measurement suffers from a decrease in sensitivity when it is miniaturized and fails at high biomass concentrations, whereas fluorescent detection requires an appropriate assay. Measurement of gas consumption or production trough pressure based sensing could then be a viable alternative to monitor the performance of bioprocesses in microreactors.

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We developed a pressure based system capable of the quantification of gas consumption or production in the nmol/min range. This system was validated by carrying out the enzymatic oxidation of glucose to gluconic acid in a stirred microbioreactor with a working volume of 100 μL, as oxygen consuming system. The microbioreactor was equipped with a magnetically driven stirrer bar and an optical dissolved oxygen sensor. Subsequently this technique was applied to measure the oxygen transfer capacity of the microbioreactor using the enzymatic oxidation of glucose to gluconic acid.

2. Materials and methods

2.1. Construction of the pressure based gas pumping and measurement system

A highly sensitive pressure sensor (Sursense® DC001NDC4, Honeywell Sensing and Control, Freeport, USA) with a measurement range of ± 2.5 mbar was connected to the leak-tight headspace of the microbioreactor. To make sure that the measurement was not influenced by changes in atmospheric pressure, which could occur during the experiment, the second port of the differential pressure sensor was connected to a reference vessel. The reference vessel was made out of PMMA and had a volume of 577 μL, similar to the reactor headspace.

A custom made LabVIEW™ (National Instruments, Austin, USA) software routine, running on a standard PC was applied to log the signal from the pressure sensor via a DAQ-card (Advantech-PCI-1710HGL, Milpital, USA). The pressure in the headspace was controlled within 0.5 mbar with the used of a syringe pump (KD Scientific 210, Antec Leyden BV, Zoeterwoude, The Netherlands) that could either supply gas to or remove from the headspace of the microbioreactor. The syringe pump was controlled via the RS232-port of the PC. The cumulative amount of gas addition/removal was logged via the LabVIEW™ routine.

2.2. Design and fabrication of the microbioreactor

The microbioreactor consisted of three PMMA layers (see Fig. 1). The middle layer contained the liquid phase of the reactor. In this 3 mm thick middle layer a hole was drilled with a diameter of 7 mm, resulting in a reactor volume of 115 μL. Four fluidic ports consisting of stainless steel capillaries (OD: 500 μm ID: 150 μm) were mounted perpendicular to the reactor wall to connect the reactor chamber with the outer world (see Fig. 2). Through these ports the reactor could be filled e.g. with glucose and/or enzyme solutions or emptied.

An optical dissolved oxygen (DO) sensor (IMP-PSt1-900/2.5-600/0.35-140/1-TF-YOP PreSens Precision Sensing GmbH, Regensburg, Germany) was mounted in the 1 cm thick bottom layer, 0.5 mm below the bottom surface of the reactor. A vertical channel with a diameter of 1 mm connected the 140 μm sensor tip to the reactor.

The 2 cm thick top plate contained a headspace above the liquid level with a diameter of 7 mm and height of 15 mm, resulting in a gas volume of 577 μL. For this configuration the specific surface area for gas transfer was 335 m^2/m^3.

Two stainless steel tubes (OD: 1/16″, ID: 1 mm) were mounted in the head plate to serve as gas inlet/outlet connections to/from the headspace of the reactor.

In order to obtain a liquid and gas tight system, gaskets made out of a 200 μm thick PDMS sheet were mounted in between the different PMMA layers. Six bolts were used to hold the three PMMA layers together (see Fig. 1).

The reactor was placed on a magnetic stirring motor (KMO 2, IKA-Werk Janke & Kunkel KG, Germany). An *u*-shaped holder that was screwed on the stirring motor ensured an exact positioning of the reactor. During all experiments the motor was set at a stirring

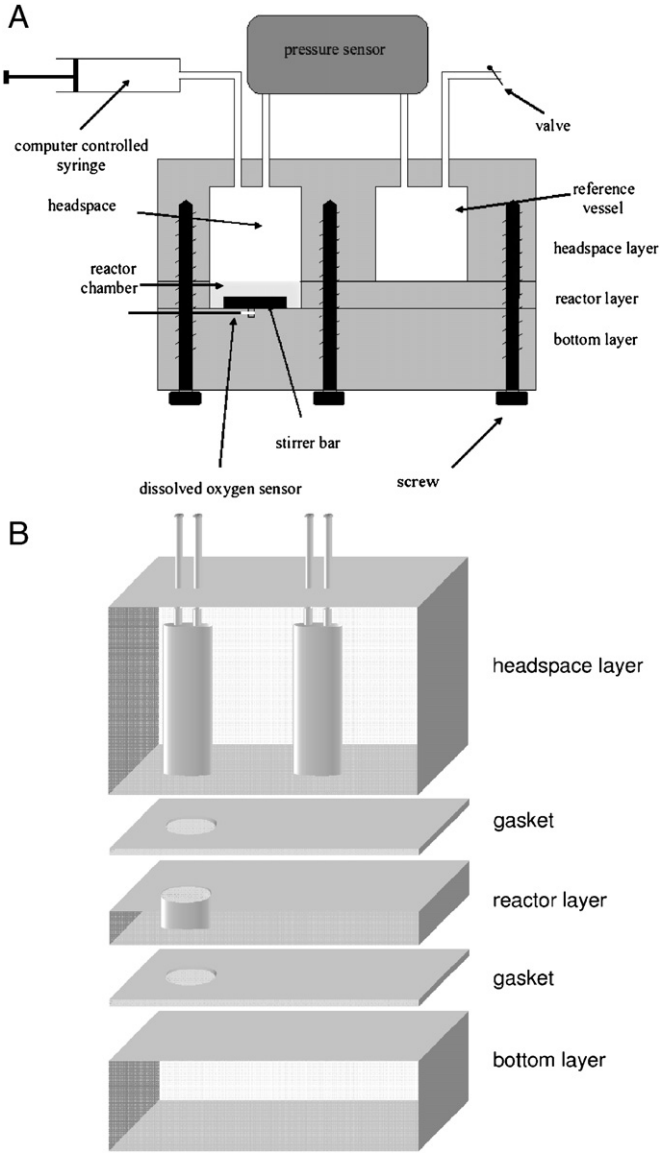

Fig. 1. (A) Schematic representation of the 100 μL bioreactor, equipped with pressure based gas measurement system and (B) exploded view of the reactor.

speed of 1100 rpm. The reactor was equipped with a small stainless steel stirrer bar, of which four different types were used, namely (1) large dowel 1.93∗4.75 mm, (14 μL), (2) large bar 1.57∗1.91∗4.75 mm (14 μL) and (3) small dowel 1.93∗2.46 mm (7 μL) and (4) small bar 1.57∗1.91∗2.90 mm (9 μL) (kindly provided by V&P Scientific Inc, San Diego, USA). The complete reactor-setup was placed in a temperature controlled cabinet maintained at 30 ± 0.1 °C.

2.3. Enzymatic conversion of glucose

The oxidation reaction of glucose towards *D*-glucono-1,5-lactone catalyzed by the enzyme glucose oxidase (GOx) (Sigma-Aldrich, cat. nr. g2133) was applied as oxygen consuming system in the liquid phase of the reactor. Catalase (CAT) (Sigma-Aldrich, cat. nr.c1345) was added to remove the hydrogen peroxide formed during the oxidation of glucose. The overall stoichiometry of these two reactions is:

$$0.5\ O_2 + \text{glucose} \rightarrow \text{glucono-1.5-lactone} + H_2O \quad (1)$$

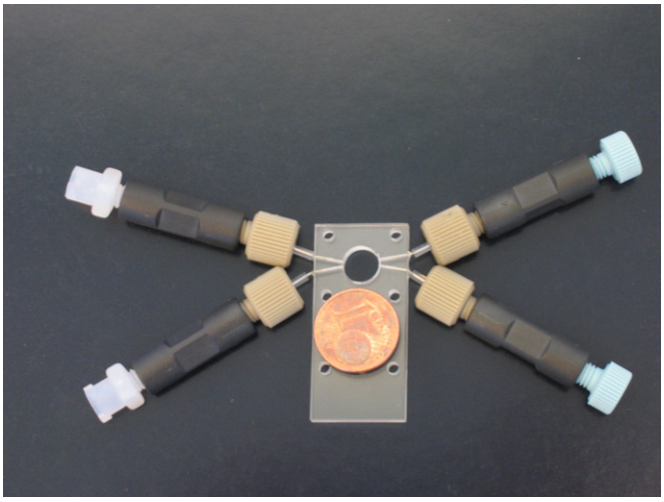

Fig. 2. Picture of the reaction chamber of the 100 μL bioreactor with fluidic connections.

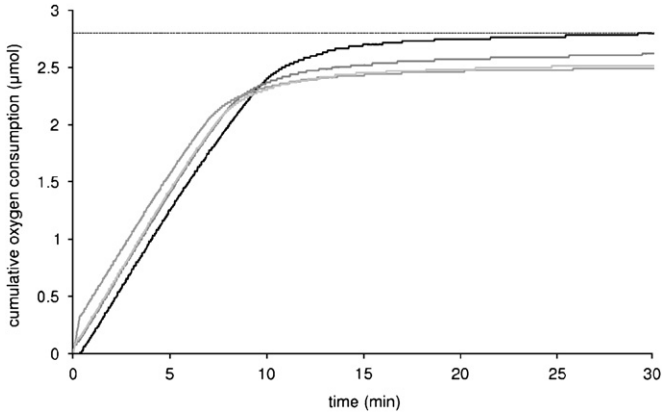

Fig. 3. Quadruplicate measurement of the cumulative oxygen consumption during the oxidation of glucose for the determination of the k_La. The large bar was used as stirrer. The dashed line indicates the maximal theoretical cumulative oxygen consumption based on the stoichiometry of the reaction.

A mixture of GOx and CAT was dissolved in 200 mM K_2HPO_4/KH_2PO_4 buffer at a pH of 6.9 in a concentration of 157 and 3 kU/L, respectively. Glucose-1-hydrate (106 mM) was dissolved separately in a 200 mM K_2HPO_4/KH_2PO_4 buffer at a pH of 6.9. Before each experiment the headspace was flushed with pure oxygen for at least 20 min at a flow rate of 1.5 mL/min to assure an oxygen concentration of 100% in the gas phase of the reactor. Furthermore a syringe (500 μL Hamilton GASTIGHT® syringe 1750 LTN) was filled with pure oxygen and was placed in the computer controlled syringe pump.

Directly after the reactor was filled with the glucose and enzyme solutions, the control software was started and the oxygen supply to the batch reaction was measured.

2.4. Measurement of the oxygen transfer capacity

The oxygen uptake rate (OUR) [mol/m^3/s] was calculated from the amount of oxygen supplied to the system per unit of time. The k_La was calculated from Eq. (2), assuming pseudo steady state conditions whereby the rates of oxygen consumption and transfer can be considered equal:

$$\mathrm{OUR} = k_La \cdot (C^*_{O_2} - C_{O_2}) \quad (2)$$

In this equation C_{O_2} and $C^*_{O_2}$ depict the oxygen concentration in the liquid and the saturation oxygen concentration at the oxygen/liquid interface [mol/m^3], respectively, a is the specific surface area for aeration [m^2/m^3] and k_L depicts the overall mass transfer coefficient for oxygen [m/s].

3. Results and discussion

3.1. Measurement of the oxygen consumption rate during enzymatic oxidation of glucose in the 100 μL bioreactor

Fig. 3 shows the results of four replicate experiments wherein the cumulative oxygen consumption during enzymatic oxidation of glucose in the microreactor was measured by means of the pressure controlled oxygen supply system. As can be seen the oxygen consumption rate, e.g. the slope of the measured cumulative oxygen consumption, was very reproducible and appeared constant during the first 8–10 min of the experiment. This corresponds with the oxygen limited region where excess amounts of enzyme and substrate are present and the reaction rate is entirely determined by the maximum oxygen transfer rate, from the gas to the liquid phase, of the reactor. As long as the reaction runs oxygen limited, the oxygen transfer rate should remain constant, i.e. at its maximum value. Only when the concentration of the substrate glucose starts to become limiting, the oxygen consumption rate, and thus the oxygen transfer rate will decrease. This was indeed observed from the recorded cumulative oxygen consumption which started to level off approx. 8–10 min after the start of the experiment.

The average total amount of oxygen consumed in the four experiments was 2.6 ± 0.14 μmol. This matches very well with the 2.8 μmol that is expected based on the total amount of glucose added and the stoichiometry of the reaction. In theory the cumulative amounts should be identical, being equal to half of the molar amount of the added glucose. It is known from the reaction stoichiometry that the oxidation of glucose consumes one oxygen molecule per molecule glucose with formation of one molecule of hydrogen peroxide. To remove the hydrogen peroxide produced in this reaction the enzyme catalase was added to convert the produced hydrogen peroxide into water thereby producing half a mol of oxygen per mol peroxide converted. The net stoichiometry of the reaction is therefore half a mol of oxygen consumed per mol of glucose oxidized. The amount of oxygen which was expected to be consumed if all glucose had been consumed under the chosen experimental conditions and according to the reaction stoichiometry is therefore 2.8 μmol. This is indicated in Fig. 3 with a dashed horizontal line. The observed variations could be attributed to variations in the amounts of glucose supplied to the reactor and/or oxygen leakage.

However, Fig. 3 shows that after the cumulative oxygen consumption levels off, no real horizontal plateau is reached but still a minor amount of oxygen is supplied to the reactor. This might indicate that some oxygen disappears from the system by leakage. Assuming that the reaction is finished after 18 min and the oxygen supplied after that point is caused by compensation of leakage alone, it can be calculated that the average oxygen leakage was approximately 5 nmol O_2/min. However, as this is only 2–4% of the oxygen consumption rate during the linear part of the experiment, oxygen leakage can be considered negligible.

3.2. Determination of the oxygen transfer rate

The mass balance for oxygen in the liquid phase is written as:

$$\frac{dC_{O_2}}{dt} = k_La \cdot (C^*_{O_2} - C_{O_2}) - \frac{1}{2} q_s \cdot C_{GOx} \quad (3)$$

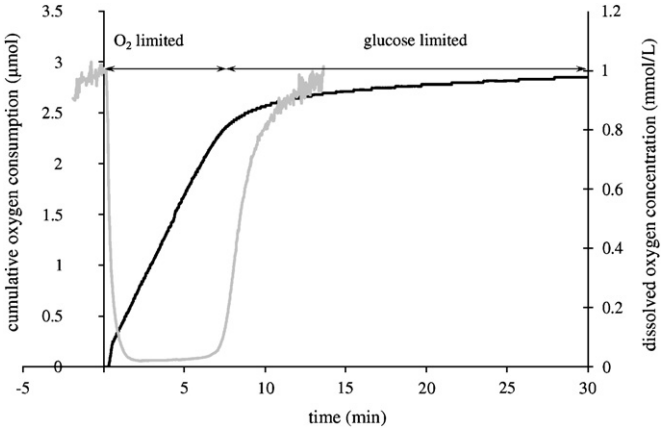

Fig. 4. Measurement of both the dissolved oxygen concentration (−) and cumulative oxygen consumption (−) during the oxidation reaction of glucose under oxygen limiting conditions. The large dowel was used for stirring.

Table 1
Measured k_La values using different types of stirrer bars at a rotation speed of 1100 rpm

Type	Large dowel	Large bar	Small dowel	Small bar
Dimensions (mm)	1.93∗4.75	1.54∗1.91∗4.75	1.93∗2.46	1.54∗1.91∗2.90
Occupied volume (µL)	14	14	7	9
k_La (h^{-1})	156 ± 10	139 ± 3	70.1 ± 5.4	83.1 ± 2.6

For measuring the maximum oxygen transfer capacity, the driving force ($C^*_{O_2} - C_{O_2}$) should be maximal. To obtain this the glucose oxidase reaction should be carried out under oxygen limiting conditions, whereby the dissolved oxygen concentration, C_{O_2}, is close to zero. This was achieved by using sufficiently large amounts of both enzymes (glucose oxidase and catalase) and the substrate glucose. The maximum oxygen transfer capacity rate (OTR^{max}) is then equal to:

$$OTR^{max} = k_La \cdot C^*_{O_2} \quad (4)$$

To confirm this we measured the dissolved oxygen concentration on-line during the enzymatic reaction. Fig. 4 shows the measured cumulative oxygen consumption and the dissolved oxygen concentration profiles. It can be seen that the dissolved oxygen concentration decreases rapidly after the reaction starts and remains at a low value of approximately 20 µmol/L until almost all the glucose is consumed. This is a very low value compared to the saturation concentration, $C^*_{O_2}$, which is 1200 µmol/L. This result clearly and convincingly shows that the reaction proceeds under oxygen limiting conditions during the first 6 min.

To be sure to be in the oxygen limited region, the k_La values were determined from the slope of the cumulative oxygen consumption curve between 3 and 5 min of the reaction.

3.3. k_La measurements using different stirrer geometries

Three different magnetizable stirrers were tested. Table 1 shows the calculated k_La values for the different types of stirrers. It can be inferred from these results that the choice of the size and shape of the stirrer has a significant effect on the mass transfer, as could be expected. The maximum k_La we could achieve was $156 \pm 10\,h^{-1}$ in the stirred microbioreactor, which is significantly larger then the k_La reported for current microbioreactor designs (e.g. Zhang et al., 2006) and should be sufficient to grow cells in fed-batch mode at relatively high biomass densities. With a specific surface area for aeration of $335\,m^2/m^3$ it follows that the $k_L = 0.47$ m/h, which agrees with the range of known k_L-values (van 't Riet and Tramper, 1991). It was found that the calculation of the k_La yielded very reproducible results with a standard deviation of less than 10%.

4. Conclusions

We developed a system, based on pressure controlled pumping of gas, for measurement of total gas consumption or production in the nmol/min range, applicable for on-line monitoring of bioconversions in microreactors. Validation of the system by carrying out a bioconversion with known stoichiometric relation between gas consumption and substrate conversion, that is the enzymatic oxidation of glucose to gluconic acid, proved that the measurements were accurate and reproducible. This measurement system can be applied directly to quantify the gas production rate in anaerobic fermentation processes carried out in microbioreactors, such as alcoholic fermentation. Hereby the rate of CO_2 production can e.g. be used to quantify product formation, as it is directly related to the ethanol production rate (Hatzinikolaou et al., 1996). However, in aerobic fermentation processes both oxygen consumption and carbon dioxide production occur. In this case oxygen consumption can still be quantified if it is accompanied by an efficient technique for the removal of the produced CO_2, e.g. a scrubber containing an alkaline solution.

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