Optical Method for the Determination of the Oxygen-Transfer Capacity of Small Bioreactors Based on Sulfite Oxidation

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Abstract: The growth of microorganisms may be limited by operating conditions which provide an inadequate supply of oxygen. To determine the oxygen-transfer capacities of small-scale bioreactors such as shaking flasks, test tubes, and microtiter plates, a noninvasive easy-to-use optical method based on sulfite oxidation has been developed. The model system of sodium sulfite was first optimized in shaking-flask experiments for this special application. The reaction conditions (pH, buffer, and catalyst concentration) were adjusted to obtain a constant oxygen transfer rate for the whole period of the sulfite oxidation reaction. The sharp decrease of the pH at the end of the oxidation, which is typical for this reaction, is visualized by adding a pH dye and used to measure the length of the reaction period. The oxygen-transfer capacity can then be calculated by the oxygen consumed during the complete stoichiometric transformation of sodium sulfite and the visually determined reaction time. The suitability of this optical measuring method for the determination of oxygen-transfer capacities in small-scale bioreactors was confirmed with an independent physical method applying an oxygen electrode. The correlation factor for the maximum oxygen-transfer capacity between the chemical model system and a culture of *Pseudomonas putida* CA-3 was determined in shaking flasks. The newly developed optical measuring method was finally used for the determination of oxygen-transfer capacities of different types of transparent small-scale bioreactors. *Biotechnol Bioeng* **74**: 355–363, 2001.
Keywords: oxygen transfer; sulfite oxidation; optical method; shaking flasks; test tubes; microtiter plates

INTRODUCTION

Small-scale bioreactors such as shaking flasks, test tubes, or microtiter plates are used in high numbers in biological screening projects. Experimental investigations in these small bioreactors are often the first step in developing a large-scale fermentation process. Both the characterization of the bioreactors and obtaining on-line information during the culture experiments becomes more difficult the smaller the bioreactors are. Microtiter plates, in particular, are critical in this respect. The question whether there is sufficient mixing, supply of oxygen and removal of carbon dioxide is difficult to quantify in this small scale. In the case of aerobic organisms the rate of oxygen supply is most frequently assumed to be determining for the microbial growth.

The oxygen transfer rate (OTR) is generally defined as

$$OTR = k_L a \cdot (C_{02}^{*} - C_L) = k_L a \cdot L_{O2} \cdot (p_G - p_L) \qquad (1)$$

Besides the oxygen solubility in the liquid phase (L_{O2}) and the driving partial pressure gradient between gas and liquid phase ($p_G - p_L$) the volumetric gas–liquid mass-transfer coefficient ($k_L a$) is the fundamental parameter. The mass-transfer coefficient (k_L) is predominantly influenced by the operating conditions as the agitation or the shaking intensity for small-scale bioreactors and the physicochemical properties of the medium (e.g., viscosity, ionic strength) under investigation. With increasing agitation or shaking intensity the liquid-side boundary layer thickness and therefore, the mass-transfer resistance decreases and the gas–liquid mass-transfer rate reaches higher values. The specific mass-transfer area (a) of bubble-aerated bioreactors is influenced by the agitation intensity and the aeration rate. Also, the composition of the liquid medium, i.e., the concentration of dissolved compounds influences the gas–liquid mass transfer. Higher concentrations or ionic strength lead to lower oxygen solubility (Schumpe, 1993; Weisenberger and Schumpe, 1996) and lower mass-transfer coefficients (Linek et al., 1970; Zieminski and Whittemore, 1971) due to smaller diffusion coefficients (Akita, 1981; Ho and Ju, 1988). The coalescence properties of the medium and, therefore, the mass-transfer area may drastically change. While, in nonbaffled shaken bioreactors the coalescence properties of the medium do not play a role because there is no sparged bubble aeration. Due to the specific regular rotating movement of the liquid in unbaffled shaking bioreactors a very well-defined gas–liquid mass-transfer area, in contrast to all other types of bioreactors, is obtained. The oxygen partial pressure in the gas phase (p_G) depends,

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on one hand, on the oxygen consumption in the liquid phase and on the other hand, on the oxygen supply from the outside of the bioreactor. In the case of small-scale bioreactors essentially the mass-transfer resistance of the sterile barrier determines this oxygen supply. For an oxygen-partial pressure in the gas phase of air (~ 0.21 bar), a bulk-liquid-oxygen partial pressure (p_L) near to zero and a defined concentration of the compounds in the liquid phase, the oxygen-transfer rate will be termed in what follows as maximum oxygen-transfer capacity (OTR_{max}) of a bioreactor. If the oxygen-partial pressure in the gas phase (p_G) is reduced, e.g., by a sterile barrier to a value smaller than in the ambient, the term oxygen-transfer capacity (OTR) will be used (in case $p_L \approx 0$).

Ideally, oxygen-transfer capacities should be measured in the biological culture under investigation. As this involves all the difficulties of sterile handling and the measurement of the dissolved-oxygen concentration, it is an inconvenient way to conduct systematic oxygen-transfer experiments. A common strategy is to simulate the conditions of a microbial culture by chemical model systems. The right choice of the model system and its physicochemical properties such as oxygen solubility, diffusion coefficients, or the presence of surface-active agents determines the degree of usefulness of the experiments. Aqueous sodium sulfite solutions are routinely employed as model systems to characterize the oxygen-transfer capacity of aerobic fermentors (Bailey and Ollis, 1986; Nielsen and Villadsen, 1994; Schügerl, 1991). Using the appropriate amount of catalyst, the sulfite oxidation can be adjusted in such a manner that under all operating conditions investigated the bulk-liquid-oxygen partial pressure (p_L) may be assumed to be zero. This avoids the need to measure the dissolved-oxygen concentration.

Several experimental set ups to determine the maximum oxygen-transfer capacity (or the volumetric gas–liquid mass-transfer coefficient) are described in literature for different sizes of bioreactors. A good review about the published methods for different chemical and microbial model systems is given by Sobotka et al. (1982). The described methods are often divided into two groups according to their applicability either to chemical model systems (Bartholomew et al., 1950; Cooper et al., 1944; Lara Marquez et al., 1994; Linek, 1966; Morimoto et al., 1979) or to microbial systems (Anderlei and Büchs, 2001; Bandyopadhyay et al., 1967; Duetz et al., 2000; Henzler and Schedel, 1991; Hirose et al., 1966; Hospodka, 1966; McDaniel and Bailey, 1969).

For some of these methods the dissolved-oxygen concentration has to be measured. Because the size of conventional oxygen electrodes is not negligible in comparison to the size of the considered small-scale bioreactor, the electrodes may act as baffles and even cause bubble entrainment. The hydrodynamic flow behavior of the chemical model system or the fermentation broth may strongly be influenced. This would lead to undefined changes in the oxygen transfer that would not reflect the conditions of the considered original bioreactor. Miniaturized electrodes applicable in small-scale bioreactors that do not influence the hydrodynamic flow behavior are not available yet. Optical methods using oxygen-sensitive fluorescence dyes in combination with optical fibers may be a possible solution.

In small-scale bioreactors all methods mentioned above are generally not applicable without accepting disadvantages with respect to sensor size and simplicity. Further, no feasible method is at present available to investigate the impact of the sterile barrier on the total oxygen transfer.

In this article a new method which allows an extremely easy, noninvasive determination of the maximum oxygen-transfer capacities in all kinds and scales of transparent small-scale bioreactors is presented. This method has been used for the preliminary characterization of different culture vessels (shaking flasks, test tubes, and microtiter plates) concerning the oxygen-transfer capacity including the oxygen transfer through the sterile barrier used and the oxygen transfer from the gas to the liquid phase inside the bioreactor.

THEORY OF THE SULFITE-OXIDATION METHOD

The sulfite method is based on the oxidation of sodium sulfite to sulfate by oxygen. The reaction is catalyzed by a number of metal ions such as Co^{2+}, Cu^{+}, Fe^{+}, and Mn^{+}, which may also be present as impurities in the educts (Linek and Vacek, 1981). Normally, a specific amount of copper or cobalt salt is added to the sulfite solution. The stoichiometric equation of the oxidation reaction is as follows:

$$SO_3^{2-} + 0.5\, O_2 \xrightarrow{Catalyst} SO_4^{2-} \tag{2}$$

The sulfite oxidation implies a nontrivial series of elementary steps as described by Linek and Vacek (1981). The oxidation of sulfite by the reaction of Eq. (2) is almost instantaneous.

The different regimes of the sulfite oxidation can be classified by the Hatta number (Ha) as

$$Ha = \frac{reaction\ rate}{mass\ transfer\ rate} = \frac{\sqrt{\frac{2}{n+1} \cdot k_n \cdot C_{O2}^{*\,n-1} \cdot D_{O2}}}{k_L} \tag{3}$$

with n as the reaction order for oxygen, k_n as the nth-order reaction constant, C_{O2}* as the equilibrium oxygen concentration at the gas–liquid interface, D_{O2} as the diffusion coefficient for oxygen in the solution and k_L as the mass-transfer coefficient. Measurements of the oxygen-transfer capacity should be conducted in the nonaccelerated reaction regime, when the reaction in the liquid side boundary layer is slow. Therefore, the following condition has to be fulfilled (Lara Marquez et al., 1994; Linek and Vacek, 1981):

$$\mathrm{Ha} < 0.3 \tag{4}$$

At Hatta numbers (Ha) higher than 0.3 the reaction acceleration has to be taken into account (Linek and Vacek,

1981; Robinson and Wilke, 1974; Sharma and Danckwerts, 1970).

The procedure to measure the maximum oxygen-transfer capacity is much easier, if the dissolved-oxygen concentration in the bulk liquid can be assumed to be negligible. This is fulfilled, if the reaction rate is significantly higher than the mass-transfer rate, which may be expressed by the reaction number (R) (Lara Marquez et al., 1994):

$$R = \frac{k_n \cdot C_{O2}^{*\,n-1}}{k_L a} > 10 \tag{5}$$

If this condition is not fulfilled ($R < 10$) but the reaction constant k_n is known, the dissolved-oxygen concentration (C_L) in the bulk liquid may be calculated by a mass balance:

$$C_L = \left(\frac{OTR}{k_n}\right)^{1/n} \tag{6}$$

Then, the maximum oxygen-transfer capacity of the considered small-scale bioreactor can be determined by:

$$OTR_{max} = OTR \cdot \left(\frac{C_{O2}^*}{C_{O2}^* - C_L}\right) \tag{7}$$

To work in the appropriate regime the reaction parameters have to be carefully adjusted. Influencing parameters on the reaction rate k_n are primarily the catalyst concentration, pH, the temperature, and even light irradiation (Gmelin, 1963; Linek and Vacek, 1981). From Equation (3) it becomes obvious that generally with decreasing oxygen-transfer capacities and concomitantly decreasing mass-transfer coefficients (k_L) the catalyst concentration and therefore, k_n has to be reduced to keep within the condition [Eq. (4)] of the nonaccelerated reaction regime (Ruchti et al., 1985).

Linek and Tvrdik (1971) and Reith and Beek (1973) derived kinetic equations accounting for the influences of catalyst concentration, pH, and temperature on the sulfite-oxidation reaction rate k_n. Specifically the effect of pH on the reaction rate k_n should be noted. Yasunishi (1977) observed a marked decrease of the oxygen absorption rate respectively the reaction rate with a decrease of pH from 10 to about 7.8 and an acceleration of the reaction at a further decrease of the pH. Fuller and Crist (1941), Gmelin (1963), Linek and Tvrdik (1971), and Reith and Beek (1973) confirm the described tendency of the absorption rate at pH values higher than 8. The increase of the oxygen-absorption rate and therefore, the reaction rate k_n at pH values smaller than 7 has also been observed by Veljkovic et al. (1995) and van Ede et al. (1995).

A freshly prepared sodium sulfite solution without pH adjustment has a pH higher than 9. Typically during the course of the oxidation the pH first decreases slowly and then at the end of the reaction drops sharply to a pH of about 5. Sulfite, which is a weak acid, also acts as a pH buffer ($pK_S = 7.2$). It is converted to the strong acid sulfate during the reaction which has no buffering properties. As a result the pH drops in the described way. Consequently, the following dissociation equilibrium

$$HSO_3^- \leftrightarrow SO_3^{2-} + H^+ \tag{8}$$

has to be taken into account. Quite often the initial pH is adjusted to a specific value between 8 and 9 prior to the oxidation (Reith and Beek, 1973; Ruchti et al., 1985).

MATERIALS AND METHODS

Sodium sulfite (98% purity, Roth, Karlsruhe, Germany), cobalt sulfate (Fluka, Neu-Ulm, Germany), Bromothymol blue (Fluka, Neu-Ulm, Germany) and Na_2HPO_4/NaH_2PO_4 phosphate buffer (Merck, Darmstadt, Germany) were used without further purification. All experiments were carried out with a 0.5*M* sulfite solution prepared with deionized water. Before and during the preparation of the sodium sulfite solution the liquid was gassed with technical-grade nitrogen to avoid a prior oxidation of the sulfite.

According to the methods of Ho et al. (1988) and Schumpe (1993) an oxygen-diffusion coefficient D_{O2} of $5{,}4 \cdot 10^{-6}$ m^2/h and an oxygen solubility L_{O2} of $9 \cdot 10^{-4}$ mol/L bar was calculated for the applied electrolyte solution at 22°C.

The reaction order n and the kinetic reaction constant k_n of the developed sulfite system was investigated in a 1.5-L bioreactor (VSF Fermentor, Bioengineering, Wald, Switzerland). For the determination of the oxygen-transfer rate in the bioreactor the exhaust gas was analyzed with an polarographic oxygen analyzer (model 26082, Orbisphere Laboratories, Geneva, Switzerland). To obtain different oxygen-transfer rates the stirring speed was varied at a constant aeration rate of 1.1 vvm. For each level of the oxygen-transfer rate the dissolved-oxygen concentration was determined with an polarographic oxygen electrode (Part. No. 341003050, Mettler Toledo, Steinbach, Germany).

The preculture of *Pseudomonas putida* CA-3 (kindly provided by W. Duetz, ETH Zürich, Switzerland) was grown in a 250-mL Erlenmeyer flask (without baffles) filled with 25 mL of complex medium (2 g/L glucose, 5 g/L yeast-extract, 5 g/L peptone; Merck, Darmstadt, Germany) at 30°C on an orbital shaker (250 min^{-1}, shaking diameter 12.5 mm). The main culture was grown in 250-mL flasks filled with 30 mL mineral medium (Evans et al., 1970) supplemented with nitrilo-triacetic-acid (4 m*M*) (Fluka, Neu-Ulm, Germany) as complexing agent, glucose (150 m*M*) as the sole carbon and energy source and a K_2HPO_4/KH_2PO_4 buffer (Fluka, Neu-Ulm, Germany) (200 m*M*) at pH 7.2. The flasks were shaken at 250 min^{-1} at a shaking diameter of 25 mm.

The pH was measured online with sterilizable pH electrodes (405 DPAS SC K8S/120, Mettler Toledo, Steinbach, Germany) installed in specially modified shaking flasks connected to a pH-meter.

A shaker modified for our purposes was kindly supplied by the Kühner AG, Basel, Switzerland. It allows five different shaking diameters from 3 to 50 mm and shaking

frequencies up to 1000 min^{-1}. 250-mL shaking flasks (Schott, Mainz, Germany), test tubes of 10-mL maximum filling volume (culture tubes Ø16 × 160 mm, Schott, Mainz, Germany) and deep-well plates with up to 1-mL filling volume (Megaplate, Polylabo, Geneva, Switzerland) were investigated.

Optical Determination of Oxygen-Transfer Rates

The investigated small-scale bioreactor was monitored during the oxidation reaction by a digital CCD camera (DCR-TRV890E, Sony, Germany). For measurements of longer duration the CCD camera is set to interval recording (int rec) with a recording time (rec) of 2 s and a standby interval (interval) of 5 min. The experimental set-up for the determination of the oxygen-transfer capacity in, e.g., microtiter plates is shown in Figure 1. A specially developed transparent plastic box was flushed with humidified air to diminish the evaporation during the measurement of the maximum oxygen-transfer capacity.

Reference System

As a reference for the present method the maximum oxygen-transfer capacities in shaking flasks were determined in sulfite solutions and in a microbial culture with a novel apparatus (Anderlei and Büchs, 2001; Büchs and Anderlei, 1999). With this apparatus an online determination of the oxygen-transfer rates was possible under sterile conditions. Essentially, the decrease of the oxygen concentration in the gas phase is repeatedly measured in a gas-tight closed shaking flask. An experimental set-up employing the same measuring method was built and used for test tubes.

This method was adapted for use in microtiter plates. In Figure 2 the experimental set-up for the determination of the maximum oxygen-transfer capacity in microtiter plates is shown. After flushing a microtiter plate with air, an oxygen electrode (Type: Fast Silver Bullet, Part. No. 6850930, Dräger, Lübeck, Germany) was fitted to a single well of the

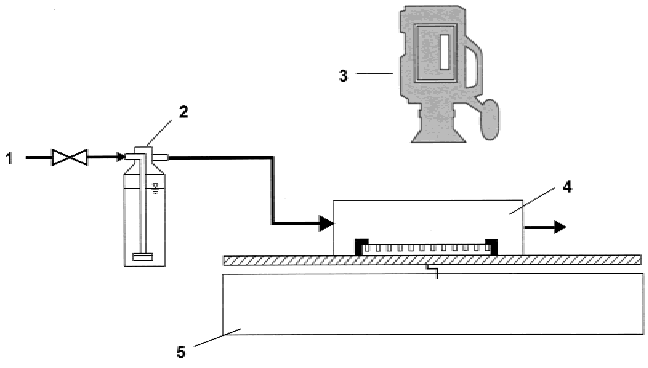

Figure 1. Optical measuring device for the determination of the maximum oxygen transfer capacity (OTR_{max}) in microtiter plates: (1) air supply; (2) gas humidifier; (3) CCD camera; (4) controlled environment box; (5) rotary table shaker.

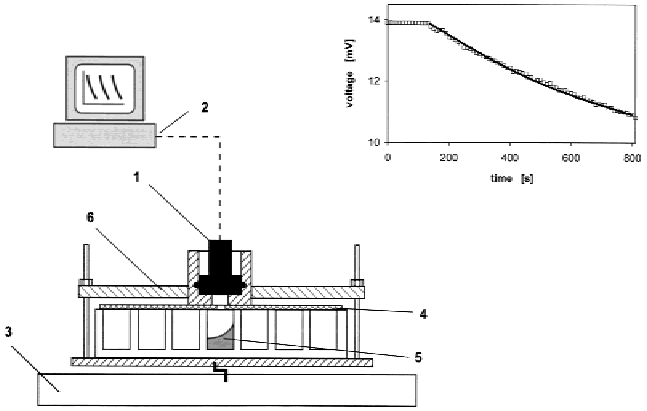

Figure 2. Experimental set-up for determination of the maximum oxygen-transfer capacity (OTR_{max}) in microtiter plate wells by electrochemical sensors and raw data of the sensor signal with mathematical approximation [Eq. (10)]: (1) oxygen sensor; (2) data acquisition; (3) rotary table shaker; (4) flexible silicon plate; (5) microtiter plate well; (6) electrode holder.

plate in a gas-tight manner using a flexible silicon rubber plate (Riplate, Ritter, Mönchengladbach, Germany) perforated with a hole of 3.5-mm diameter. The rate of oxygen consumption from the headspace of a well was recorded.

The change of the oxygen partial pressure in the head space (p_G) due to the oxygen consumption can be described by the following differential equation for ideal gases:

$$\frac{dp_G}{dt} = -R_M \cdot T \cdot OTR \cdot \frac{V_L}{V_G} \tag{9}$$

with the molar gas constant (R_M), the temperature (T), the volume of the gas phase (V_G), and the liquid phase (V_L) in the well. Insertion of Equation (1) in Equation (9) and integration over time leads to the logarithmic expression for p_G:

$$ln\frac{P_G}{P_{G0}} = -t \cdot R_M \cdot T \cdot k_L a \cdot L_{O2} \cdot \frac{V_L}{V_G} \tag{10}$$

The initial partial pressure of oxygen in the gas phase (p_{G0}) is 0.2095 bar at ambient conditions.

The slope of the signal of the electrode (U) shows a decreasing tendency, as the partial pressure gradient between the gas in the headspace p_G and the liquid phase ($p_L \approx 0$) decreases with time (see Fig. 2). The output voltage of the oxygen sensor starting from the initial value (U_0) at ambient conditions is approximated by Equation (11)

$$ln\frac{U}{U_0} = -t \cdot k_x \tag{11}$$

with the exponent k_x as fitting parameter. The direct proportionality between the output voltage [Eq. (11)] and the partial pressure of oxygen [Eq. (10)] is given by:

$$k_L a \cdot L_{O2} = \frac{k_x}{R_M \cdot T} \cdot \frac{V_G}{V_L} \tag{12}$$

The maximum oxygen-transfer capacity ($p_{G0} = 0.21$ bar; $p_L = 0$ bar) is finally defined by:

$$OTR_{max} = k_L a \cdot L_{O2} \cdot p_{G0} = \frac{k_x}{R_M \cdot T} \cdot \frac{V_G}{V_L} \cdot p_{G0} \qquad (13)$$

RESULTS AND DISCUSSION

For the presented method a constant oxygen-transfer rate in the nonaccelerated reaction regime is necessary to determine the maximum oxygen-transfer capacity. Thus, the influence of the cobalt concentration and of the pH has first been investigated to ensure the appropriate reaction regime and to avoid changes of the oxygen-transfer rate during the course of the reaction. These investigations have been carried out in shaking-flask experiments.

Optimization of the Cobalt Catalyst Concentration

Cobalt has been chosen as catalyst (Linek and Vacek, 1982). To find a suitable concentration an oxygen-transfer rate of 0.004 mol/L/h was assumed as the lowest value for the characterization of the small-scale bioreactors. For this lowest value the reaction rate k_n of the sulfite oxidation has to be adjusted to avoid working in the accelerated reaction regime. The following operating conditions for the 250-mL shaking-flask experiments have been chosen: a diameter for the orbital movement of the shaking machine (shaking diameter) of 25 mm, a filling volume of 50 mL, and a shaking frequency of 130 min^{-1}. The cobalt concentration was varied between 3.3 10^{-8} and $10^{-6}M$ (Fig. 3). The appropriate cobalt concentration for the nonaccelerated reaction regime is obtained when a constant oxygen-transfer capacity with variation of the cobalt concentration appears (Linek and Vacek, 1981). From Figure 3 a cobalt concentration of $10^{-7}M$ was chosen. This is in the range of cobalt concentrations used by other authors (van Ede et al., 1995; Linek and Vacek, 1982; Veljkovic et al., 1995).

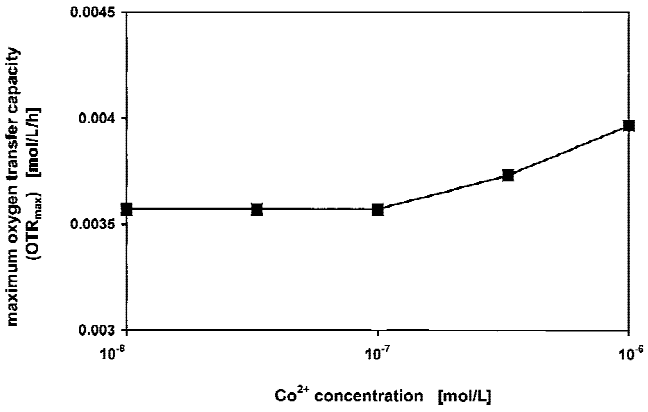

Figure 3. Maximum oxygen-transfer capacity (OTR_{max}) in 250-mL shaking flasks at different cobalt catalyst concentrations, 50-mL filling volume, 25-mm shaking diameter, 130 min^{-1} shaking frequency, 22°C, initial pH 8, 0.5M Na_2SO_3, 0.012M phosphate buffer, 2.4 $10^{-5}M$ bromothymol blue.

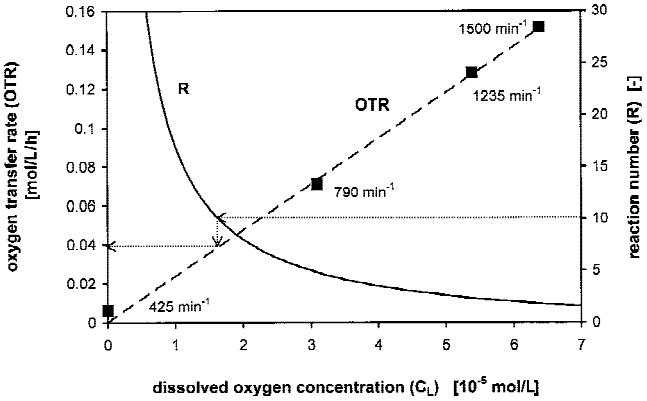

Figure 4. Oxygen-transfer rate (OTR), dissolved-oxygen concentration (C_L) and reaction number [R, Eq. (14)] in a 1.5-L bioreactor at different stirring speeds, constant aeration rate of 1.1 vvm, 22°C, initial pH 8, 0.5M Na_2SO_3, $10^{-7}M$ $CoSO_4$, 0.012M phosphate buffer.

To check if the conditions [Eqs. (4) and (5)] are fulfilled, the dissolved-oxygen concentration in the liquid phase of a 1.5-L bioreactor was measured at different oxygen transfer rates. As illustrated in Figure 4, a linear dependency between the oxygen-transfer rate and the dissolved-oxygen concentration is observed, which means that the sulfite oxidation is of first-order in oxygen ($n = 1$). This is consistent with the reaction order found in the literature for small cobalt concentrations (Linek and Vacek, 1981). At higher cobalt concentrations the reaction is of second-order in oxygen (Linek and Vacek, 1981). A first-order reaction constant k_1 of 2385 L/h results from the slope of Figure 4.

Rearranging of Equation (3) for a first-order reaction, the minimum mass-transfer coefficient (k_L) can be calculated as:

$$k_L > \frac{\sqrt{k_1 \cdot D_{O2}}}{0.3} = 0.38 \frac{m}{h} \qquad (14)$$

Using Equations (1), (5), and (6) for a first-order reaction the reaction number (R) as function of the dissolved-oxygen concentration can be derived as:

$$R = \frac{(C^*_{O2} - C_L)}{C_L} > 10 \qquad (15)$$

As illustrated in Figure 4, the condition of a negligible oxygen concentration in the bulk liquid (R > 10) is only fulfilled for an oxygen-transfer rate of less than 0.04 mol/L h. In this case the oxygen-transfer rate is equal to the maximum oxygen-transfer capacity ($OTR = OTR_{max}$).

Optimization of the pH

The drop of the pH from about 9.2 to 5 during the sodium sulfite oxidation without initially adjusted pH typically leads to a decrease in the oxygen-transfer rate during the

course of the reaction (Δ, Fig. 5). At the beginning of the reaction the measured oxygen-transfer rates are especially high, as the high pH values lead to elevated reaction rates k_n. To diminish this decrease of the oxygen-transfer rate during the oxidation reaction the pH should be kept at an almost constant value. To achieve this goal the buffering properties of the sulfite solution between a pH value of 8 and 6 can be taken advantage of. Thus, the pH was initially adjusted by sulfuric acid to a value of 8 avoiding the high reaction rates k_n at pH values above 8. As a result the oxygen-transfer rate remains at an almost constant value until a pH of 7 is reached and increases with a further decrease of the pH (●, Fig. 5) as consequence of the increasing reaction rate. At some operating conditions even higher increases of the measured oxygen-transfer rate than shown in Figure 5 appeared at the end of the reaction. To avoid the accelerated reaction regime at a pH smaller than 7, a 0.012*M* Na_2HPO_4/NaH_2PO_4 phosphate buffer was added to keep the pH at a constant level for as long as possible. The addition of higher phosphate buffer concentration led to a higher ionic strength resulting in a decrease of the oxygen solubility and the diffusion coefficient. Smaller values for both led to a reduction of the oxygen-transfer rate. The comparability between the obtained results from a typical fermentation medium and those of our chemical model system is then no longer given. Higher buffer concentration than 0.012 *M* would also interfere with our measuring strategy as a minimum pH drop is needed to monitor the end of the reaction as explained below.

As a result of the optimization of the catalyst concentration and the pH during the reaction, the oxygen-transfer capacity is constant over the reaction time and has a sharp decrease to zero when all the sulfite is oxidized (□, Fig. 5). In a shaking flask equipped with a pH-electrode it was shown (Fig. 6) that at the end of the reaction the pH sharply decreases from 7 to about 3.4 without disturbing the constancy of the oxygen-transfer rate.

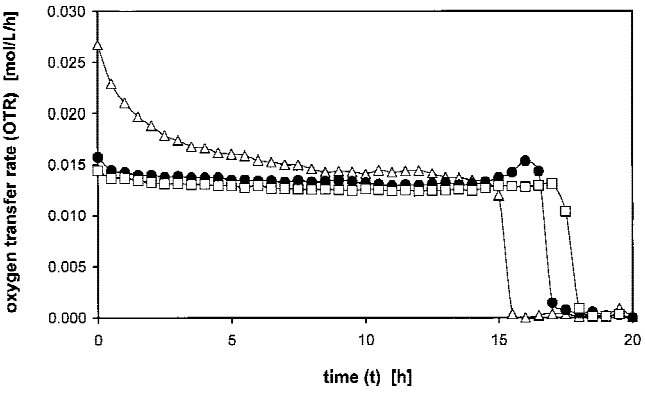

Figure 5. Oxygen-transfer rate (*OTR*) in 250 mL shaking flasks, 30-mL filling volume, 25-mm shaking diameter, 250 min^{-1} shaking frequency, 22°C, 0.5*M* Na_2SO_3, 10^{-7}*M* $CoSO_4$, (△) initial pH at 9.2, (●) initial pH at 8, (□) initial pH at 8 and 0.012*M* phosphate buffer.

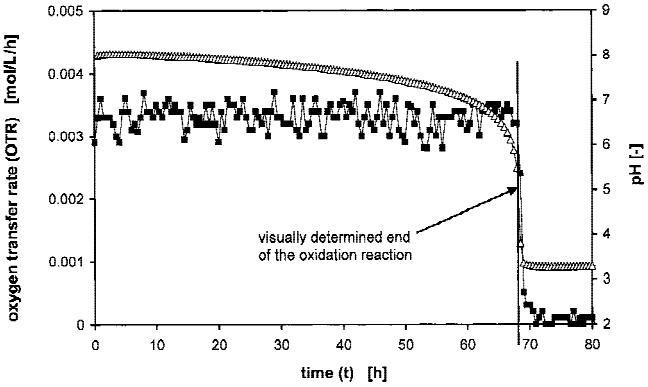

Figure 6. Development of (△) the pH and (■) the oxygen-transfer rate (OTR) during sulfite oxidation in 250-mL shaking flasks, 75-mL filling volume, 25 mm shaking diameter, 130 min^{-1} shaking frequency, 22°C, pH 8, 0.5*M* Na_2SO_3, 10^{-7}*M* $CoSO_4$, 0.012*M* phosphate buffer, 2.4 10^{-5}*M* bromothymol blue; the total consumption of sulfite was visually determined by evaluating the color change of the pH indicator on the recorded CCD camera images.

Determination of Oxygen-Transfer Rates by Optical Means

The integration of the oxygen-transfer rate over time yields the total oxygen consumption during the oxidation reaction. Smaller oxygen-transfer rates result in longer reaction times at the same sulfite concentration and total amount of oxygen consumed. This phenomenon of a time-dependant constant level of the oxygen-transfer rate in combination with a pH drop at the end of the reaction describes the principle of the novel method. At a fully stoichiometric transformation of a constant amount of sulfite to sulfate the consumed oxygen is determined by the reaction of Equation (2). If the length of the sulfite-oxidation reaction is determined the constant oxygen-transfer rate can be calculated by:

$$OTR = \frac{C_{Na_2SO_3} \cdot \nu_{O_2}}{t_{OX}} \tag{16}$$

with *OTR* as oxygen-transfer rate,

as molar sodium sulfite concentration, $\nu_{O2} = 0.5$ as stoichiometric coefficient for oxygen [see Eq. (2)] and t_{OX} as reaction time for the complete oxidation.

The length of the period for the sulfite oxidation may easily be determined by measuring the sharp decrease in pH at the end of the reaction. In small-scale bioreactors the effects of pH-electrodes with respect to the hydrodynamic flow behavior have to be considered. As described above for oxygen electrodes, pH-electrodes immersed in the liquid may act as baffles and cause a disturbance of the hydrodynamic flow, which may strongly affect the mass transfer. In the presented method electrodes are completely avoided. The pH drop from about 7, that occurs close to the end of the reaction to the final value of 3.4 is visualized by a pH indicator. The pH indicator bromothymol blue ($pK_S = 7.0$) with a color change from dark blue at pH 7.3 to yellow at pH 6.2 was chosen. An influence of bromothymol blue on

Table I. Maximum oxygen-transfer capacities of shaking flasks, test tubes, and microtiter plates for arbitrarily selected operating conditions obtained by (1) the optical determination of the duration of the sulfite oxidation as described here and (2) the measurements of the decrease of the oxygen concentration in the head space of the bioreactors by an oxygen electrode (reference method); the standard deviation has been determined from a number of experiments indicated in parenthesis.

	OTR_{max} [mol/L/h]	
	Novel optical method	Reference method
Shake flask (250 mL) with pH-electrode 130 min^{-1}, d_o = 25 mm, V_L = 75 mL	0.0036 ± 5.5% (6 exp.)	0.0033 ± 5.4% (6 exp.)
Test tube (O16 × 160 mm) 300 min^{-1}, d_o = 25 mm, V_L = 3 mL	0.0050 ± 4% (5 exp.)	0.0055 ± 5.4% (4 exp.)
Microtiter plate (deep-well) 300 min^{-1}, d_o = 50 mm, V_L = 1 mL	0.0163 ± 2% (10 exp.)	0.0161 ± 1.5% (5 exp.)

the maximum oxygen-transfer capacity has not been observed (data not shown).

For the determination of the oxygen-transfer capacity the considered transparent small-scale bioreactor is monitored during the sulfite oxidation by a CCD camera with an internal time recording for overnight operations (for example). The recorded video images are later evaluated to spot the color change from blue to yellow at the end of the reaction. The time from the beginning of the experiment until the color change indicates the length of the reaction. From this the maximum oxygen transfer capacity can be calculated by Equation (16). As shown in Figure 6, the visually determined end of the reaction shows good agreement with the true length of the reaction.

For the evaluation of the new optical method, oxygen-transfer capacities in shaking flasks, test tubes, and microtiter plates were each determined by two means: (1) the optical determination of the duration of the sulfite oxidation, and (2) the measurement of the decrease of the oxygen concentration in the head space of the considered small-scale bioreactor, as described above. The different shaking conditions were randomly chosen. Therefore, the level of the different oxygen-transfer capacities do not represent a classification of the small-scale bioreactors tested.

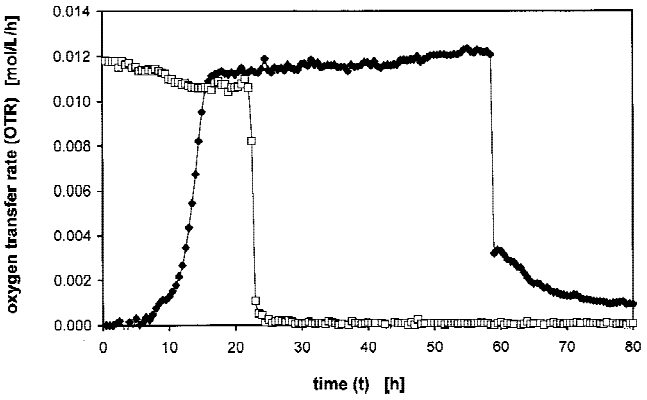

Figure 7. Oxygen-transfer rate (*OTR*) in 250-mL shaking flasks, 30-mL filling volume, 25 mm shaking diameter, 250 min^{-1} shaking frequency, 22°C: (□) 0.5*M* Na_2SO_3, 10^{-7}*M* $CoSO_4$ 0.012*M* phosphate buffer, initial pH 8, 2.4 10^{-5}*M* bromothymol blue; (♦) growth of *Pseudomonas putida* CA-3 in mineral medium (Evans et al., 1970) supplemented with nitrilotriacetic-acid (4 m*M*), glucose (150 m*M*) and K_2HPO_4/KH_2PO_4 (200 m*M*) buffer at pH 7.2.

In Table I the maximum oxygen-transfer capacities and their standard deviations at different operating conditions are shown. Good agreement is achieved in all cases between the two measuring methods. The value obtained in the shaking flask at the chosen shaking conditions (75-mL filling volume, 25-mm shaking diameter, 130 min^{-1} shaking frequency) determined by the optical method is 0.0033 mol/L/h compared to 0.0036 mol/L/h measured with the reference system. The results for the maximum oxygen-transfer capacity of test tubes and deep-well microtiter plates show a similar agreement between the two methods. The obtained differences between the mean values of the two methods are 10% and 2% for the test tubes and for the microtiter plate, respectively.

In each scale, several reactors have been monitored in parallel to obtain the standard deviation from the mean value of the maximum oxygen-transfer capacity. The accuracy of the novel method is in the same range as of the reference systems. At a standard deviation of less than 6% achieved in our experiments the optical method can be considered as sufficiently accurate.

The suitability of the chemical model system for the determination of the maximum oxygen-transfer capacity of a bioreactor was demonstrated by the oxygen-limited growth of *Pseudomonas putida* CA-3 in a shaking-flask experiment. The oxygen-transfer rate of an oxygen-limited growth of a microorganism typically is at a constant level during the period of oxygen limitation. This level of the oxygen-transfer rate determines the maximum oxygen transfer capacity of the reactor at the given culture conditions. The results of the new optical method should be in the same range of those obtained with a biological system. Figure 7 shows the oxygen-transfer rate of *P. putida* starting with a lag-phase and the exponential-growth phase. In the oxygen-limited growth phase the oxygen-transfer rate is nearly constant. At the end of the oxygen-limited growth phase no more carbon source is present in the medium. Thus, the oxygen-transfer rate sharply drops down. The oxygen-transfer rate measured with the chemical model system immediately reaches constant values when the experiment is

started. The maximum oxygen-transfer capacities obtained with the chemical model system and the growth of *P. putida* in shaking-flask experiments show only slight differences. As a result, the correlation factor for the maximum oxygen-transfer capacity between the chemical model system and the biological reference system is one.

CONCLUSION

A novel optical method to carry out mass transfer measurements in small-scale bioreactors based on the sulfite oxidation is presented. This method for the determination of maximum oxygen-transfer capacities is applicable for all kinds and shapes of transparent bioreactors. With a low-labor requirement and without the necessity of calibration this new optical method can also be used for miniaturized bioreactors (e.g., microtiter plates) with sufficient accuracy. The great potential of the noninvasive optical method is that no modifications of the investigated bioreactor are required. The bioreactor can be investigated as it is used for microbial growth experiments. As an additional feature the influence of the sterile barrier on the oxygen transfer of small-scale bioreactors can now be investigated as well. The only requirement is the optical accessibility of the liquid in the bioreactor. Depending on the optical characteristics of the lenses of the CCD camera several bioreactors may be studied in parallel, which may even be operated at different operating conditions such as, for example, filling volumes. Therefore, a very time efficient measurement is possible.

NOMENCLATURE

C_L	oxygen concentration in the bulk liquid	[mol/L]
C_{O2}*	maximum oxygen concentration at ambient conditions at the interface	[mol/L]
C_{Na2SO3}	molar sodium sulfite concentration	[mol/L]
d_o	orbital shaking diameter	[m]
D_{O2}	oxygen diffusion coefficient	[m^2/h]
k_1	first order reaction constant	[h^{-1}]
k_n	nth-order reaction constant	[−]
k_La	volumetric mass-transfer coefficient	[h^{-1}]
k_L	mass-transfer coefficient	[m/h]
k_x	mathematical constant	[h^{-1}] [Eq. (10)]
L_{O2}	oxygen solubility	[mol/L/bar]
n	reaction order	[−]
OTR	oxygen-transfer rate	[mol/L/h]
OTR_{max}	maximum oxygen-transfer capacity	[mol/L/h]
p_G	partial oxygen pressure in the gas phase	[bar]
p_{G0}	partial oxygen pressure in the gas phase at ambient conditions	[bar]
p_L	partial oxygen pressure in equilibrium with the bulk liquid	[bar]
R	reaction number	[−] [Eq. five]
R_M	molar gas constant	[J/mol/K]
t	time	[h]
t_{OX}	reaction time for the complete oxidation	[h]
T	temperature	[K]
U	voltage	[V]
U_0	initial voltage (at ambiance conditions)	[V]
V_L	liquid volume	[1]
V_G	gas volume	[L]
ν_{O2}	stoichiometric coefficient for oxygen	[−]

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