

Application of the sodium sulfite method for monitoring the oxygen transfer in shake flasks with the wireless sensor system SENBIT®

Eva-Maria Materne, Julia Glazyrina, Stefan Junne, Peter Neubauer

*Laboratory of Bioprocess Engineering, Institute of Biotechnology, Technische Universität Berlin.
Ackerstrasse 71-76, ACK24, D-13355 Berlin, Germany.*

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Introduction

Background

The oxygen transfer rate (OTR) of a bacterial culture system is of great importance for all aerobically growing microorganisms. As the solubility of oxygen in water is low, the oxygen availability is very critical during microbial growth in submerged cultivations. Hence, the actual OTR is a commonly employed parameter for quantifying the physiological state of an aerobic culture. 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 cultivation (Anderlei and Büchs, 2001). If the oxygen demand of a culture exceeds the oxygen transfer capacity through the gas-liquid interface, the growth rate is only a function of the mass transfer rate and provides no information about the true growth rate of the microorganism (Van Suijdam et al. 1978). Hence, the avoidance of limitations is crucial, especially within investigations of basic process conditions, the evaluation of kinetics, and in screening. If a screening is performed under unknown oxygen limitation, an unwanted selection pressure may be caused. This leads to a high degree of variability in an early stage of bioprocess development. In order to harmonize screening experiments, suitable operating conditions ensuring sufficient oxygen supply for cultures must be determined.

Many research groups work with small and miniature size bioreactors, as they are easy to handle, are characterized by low expenditures, and have low requirements of laboratory space. Especially in the fields of starter culture preparation, media optimization, as well as the cultivation of clone libraries, protein expression studies and high throughput screening for biocatalysts, small scale cultivation vessels are well applied. However, controlling the reactions proceeding in the media of these small-scale reactors to a degree similar to that of a lab or pilot-scale reactor, is hardly possible. Oxygen limitations are encountered frequently in these bioreactors. Hence, it is of great importance to use cultivation systems, which are characterized by a high OTR.

The maximum OTR in Ultra Yield™ flasks from Thomson Instrument Company (San Diego, CA) was studied during this project. These flasks have a baffled base, which increases the agitation in the liquid as well as the available surface area for oxygen transfer at the gas-liquid interface. Previous studies have shown that they yield high cell densities (Brodsky and Cronin, 2006b). It was assumed, that these high cell densities are due to a high OTR compared to other shake flasks. This had to be verified in the presented study.

This study describes a new method, based on a wireless monitoring system, for the determination of the OTR in shaken culture systems, based on the sodium sulfite method but independent on titration or camera use. It can be performed even if there are no possibilities for inserting electrodes into the liquid, or for optically monitored reactions proceeding in these flasks.

The oxygen transfer rate

As the solubility of oxygen in water is low, the oxygen consumption of a culture has to be compensated by continuous aeration.

The oxygen transfer can be described by the following equation:

$$N_{O_2}^* = k_L \cdot A \cdot (c_{O_2}^* - c_L) \quad (1)$$

$N_{O_2}^*$ represents the stream of amount of oxygen, $c_{O_2}^*$ is the oxygen concentration at the phase boundary surface, c_L the oxygen concentration in the liquid phase, A is the phase boundary surface and k_L the mass transfer coefficient.

If one correlates equation (1) to the liquid volume V_L , the oxygen transfer rate (OTR) is obtained:

$$OTR = \frac{N_{O_2}^*}{V_L} = k_L \cdot \frac{A}{V_L} \cdot (c_{O_2}^* - c_L) = k_L \cdot a \cdot (c_{O_2}^* - c_L) \quad (2)$$

a is called the specific gas-liquid surface area. As k_L and a are difficult to measure separately, they are often combined as one parameter and called volumetric oxygen transfer coefficient ($k_L a$). This coefficient is hard to determine exactly and depends on the following factors:

- The operating conditions (here: shaking velocity and amplitude, aeration rate)
- The design of the cultivation vessel (here: flask shape and size, surface properties of the flask material, aeration type and type of stirrers)
- The physical properties (surface tension, viscosity of the medium, diffusion coefficient and concentration of dissolved materials like salts and glucose)

Methods for determining the $k_L a$

There are a number of different physical and chemical methods for determining the $k_L a$. These can be subdivided into unsteady state and steady state measuring methods, depending on whether the concentration of the gas dissolved in the liquid changes with the time or whether it remains constant. A good overview of the most important methods is given by (Garcia-Ochoa and Gomez, 2009; Suresh et al. 2009). A summarization is given in Tab.1.

TABLE 1
Methods for determining the $k_L a$

Method	References
Unsteady state	
Dynamic method in biological system	(Van Suijdam et al. 1978; McDaniel and Bailey, 1969)
Measurement of gas phase	(Henzler and Schedel, 1991; Anderlei et al. 2004)
Dynamic gassing out method	(Veglio et al. 1998; Hirose et al. 1966; SCHULTZ, 1964a)
Steady state	
Sulfite method	(Linek and Vacek, 1981; Hermann et al. 2001)
Hydrazine Method	(Zlokarnik, 1999)

Unsteady state methods

One of the currently most often used strategies for determining the $k_L a$ belongs to the unsteady state measuring methods. Hereby the oxygen is removed from the liquid by degassing with nitrogen or by binding chemically to sodium sulfite. Then the aeration is switched on again. The increase in oxygen is detected by a polarographic oxygen electrode. The change in dissolved oxygen concentration over time can be used to calculate $k_L a$.

Another common technique applied is the dynamic response method, which is often used during the course of cultivation. But as both methods rely on the utilization of oxygen electrodes inserted into the liquid, they cannot be used in most small-scale reactors.

Steady state methods

The sulfite method belongs to the steady state measuring methods. It was mainly used before the invention of the polarographic oxygen electrodes. An oxygen sink is provided by the addition of sodium sulfite to the system, which binds oxygen chemically. The reaction is catalyzed by Co^{2+} . The concentration of oxygen in the liquid is close to zero. The oxygen absorption rate could be determined from the change in the normality of the sulfite solution with respect to the time. In former days, the remaining amount of sodium sulfite was continuously monitored by iodometric titration. Samples were taken several times during the course of the reaction. Hence, many titrations had to be performed. Then, the oxygen adsorption rate could be obtained based on the decrease of the sulfite concentration over time. This method was very laborious and time consuming, especially if all the titrations were performed by hand.

The sodium sulfite method

A chemical model system is used as an artificial oxygen consumer. In aqueous sodium sulfite solutions, anions are oxidized almost immediately by the incoming dissolved oxygen. This reaction is catalyzed by metal ions like Co^{2+} , Fe^{2+} and Cu^{2+} .



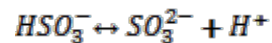
This simplified reaction was described in more detail by Linek and Vacek (1981).

Several different chain reaction mechanisms have been published for this reaction, as well as different reaction orders of sulfite, oxygen and catalysts, determined under homogeneous or heterogeneous conditions. Furthermore, there are many different variations of the sulfite-oxidation method. Sobotka and Prokop (1982) presented a good overview of some chemical methods that are in use.

Generally, the catalyst concentration is very important for the sulfite-oxidation method: if it is adjusted correctly, the bulk-liquid oxygen partial pressure (p_l) should be close to zero. Hence, the reaction rate is larger than the physical mass-transfer rate. If the reaction rate was too high, the reaction will no longer be first order with respect to oxygen. In this case, the mass transfer represents no longer the limiting step for oxygen transfer from the gas to the liquid phase. The resulting reaction is located exclusively at the interfacial surface area. A cobalt concentration of 10^{-7}M insures that the reaction rate is larger than the mass-transfer rate, while the reaction at the liquid surface is still not enhanced. This necessity was revealed previously by several authors (Linek and Vacek, 1981).

Apart from the catalyst concentration the reaction rate is influenced by the temperature, the pH, and even by light irradiation. These parameters have to be controlled to stay in a range suitable for the

experiments. The pH is of major importance within this context, as it has a big impact on the reaction rate. This is due to the fact that the dissociation equilibrium



depends strongly on the pH. However, in the range of $7.5 < \text{pH} < 9.0$, where the experiments are generally performed, the $\frac{c_{HSO_3^-}}{c_{SO_3^{2-}}}$ ratio varies from 6×10^{-3} to 2×10^{-4} . $c_{HSO_3^-}$ remains very small compared to $c_{SO_3^{2-}}$ (Linek and Vacek, 1981).

A freshly prepared sodium sulfite solution usually has a pH of 9 to 10. The OTR is very high at a pH of 10. It starts to decrease in parallel with the pH, which drop from 10 to 8. Hence, the sulfite solution has to be adjusted to pH 8 in the beginning to avoid fluctuating reaction rates.

The oxygen absorption rate remains almost constant until the pH of 7 is reached. Then it rises as a consequence of the increased reaction rate (Yasunishi, 1977). To avoid this increase, a buffer is added to keep the pH stable for as long as possible.

It can be concluded that for the case the reaction rate (R) is much higher than the mass transfer rate, the dissolved oxygen concentration in the liquid can be assumed to be negligible. This assumption makes it much easier to estimate the maximum oxygen transfer capacity.

The reaction rate is defined as follows (Hermann et al. 2001):

$$R = \frac{k_n \cdot C_{O_2}^{n-1}}{k_L a} > 10 \quad (4)$$

If this condition is not fulfilled ($R < 10$), but the reaction constant k_n is known, the dissolved oxygen concentration in the liquid (c_L) can be calculated as follows:

$$c_L = \left(\frac{OTR}{k_n} \right)^{1/n} \quad (5)$$

Then, the maximum oxygen transfer capacity can be calculated with:

$$OTR_{max} = OTR \cdot \left(\frac{c_{O_2}^*}{c_{O_2}^* - c_L} \right) \quad (6)$$

Further development of the sodium sulfite method (the method of Hermann et al., 2001)

Hermann et al. (2001) proposed to analyze the course of the reaction by the help of the pH. During the reaction, sodium sulfite's buffering capacities are lost and the production of the more acidic sodium sulfate results in a lower pH. This drop of pH is recorded and used for the calculation of the OTR.

As Hermann et al. (2001) worked with very small culture vessels like deep well plates and shake flasks, they could not insert pH or oxygen sensors into their reaction vessels. Instead, they used a pH indicator to determine the termination of the reaction. The color change was observed by a charge-coupled device camera. This end point determination method has the advantage that no sensor is introduced into the liquid and that the change of the hydrodynamic flow described below is omitted.

The length of the complete sulfite oxidation reaction (t_R) is used to calculate the average specific OTR (Linek et al. 2006):

$$N_{ave} = \frac{C_{Na_2SO_3}}{v \cdot t_R} \quad (7)$$

With $C_{Na_2SO_3}$ as the initial sulfite concentration and $v=2$ as a stoichiometric coefficient for sulfite (see Formula 3), N_{ave} represents the OTR over the whole measuring period. If it is assumed that the oxygen flux is constant during the reaction while the catalyst concentration and pH remains stable, the following relation can be assumed:

$$N_{ave} = k_L a \cdot c_{O_2}^* = OTR_{max} \quad (8)$$

When combining eqs. 7 and 8, it can be concluded, that a smaller OTR lead to a longer reaction time t_R .

Material and Methods

Basic considerations

The above described sodium sulfite method is best suited for the determination of k_La values in the Ultra Yield™ flasks. Still, some adjustments to the method of Herman et al. (2001) had to be performed.

The experiments of this study were performed at a sodium sulfite concentration of 0.25 M. This is in contrast to the experiments of Herman et al., who performed his experiments at a sodium sulfite concentration of 0.5 M. This reduction of the sodium sulfite concentration was done to shorten the reaction time. In order to prove that the experiments are still performed under conditions where the reaction regime is not accelerated, a control experiment had to be conducted. Therefore, a 0.25 M sodium sulfite solution, containing 10^{-7} M cobalt catalyst and 0.07 M buffer was aerated in a fermenter with 2 L working volume at different stirrer speeds. The dependency between the dissolved oxygen concentration, the OTR, and as result the reaction order with respect to oxygen was obtained.

Further, the end point of the reaction was not determined by the drop of pH and the corresponding color change of the pH indicator, but with an amperometric oxygen electrode. The Ultra Yield™ flasks for which the k_La values were determined, are turbid. Hence, it was not possible to spot a color change in the medium. Furthermore, it was not possible to insert an electrode in the flask, which also would have changed the fluid characteristics. Therefore, the reaction was started in an Ultra Yield™ flask. After a predetermined time, the solution was poured from the Ultra Yield™ flask to a SENBIT® flask. The SENBIT® flasks were equipped with a pO_2 electrode and consequently the dissolved oxygen profile could be observed.

The oxygen concentration in the liquid remained close to zero during the whole reaction time until the conversion from sulfite to sulfate stopped. The time recorded from the beginning of the reaction in the SENBIT® flask to the time point, where the dissolved oxygen concentration in the liquid increased rapidly after all of the sulfite had reacted to sulfate, was termed $time_{SENBIT\ flask}$ (Fig. 1). This duration was used to calculate the average specific OTR of the Ultra Yield™ flask.

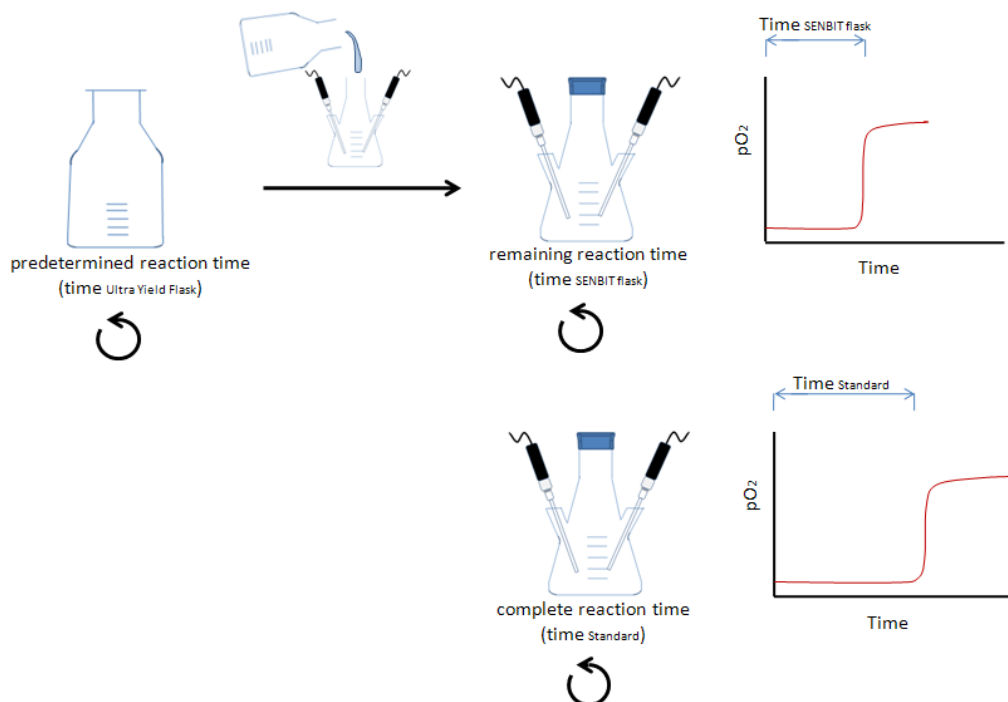


Fig. 1: Experimental procedure of the k_La measurement

To convert the results obtained in the SENBIT® flasks to the k_La values of the Ultra Yield™ flasks, standard experiments were performed. In those experiments, the reaction was exclusively observed in the SENBIT® flasks. The parameters of these standard experiments were adjusted to fit those of the Ultra Yield™ flasks. Hence, different filling volumes were used, as the reactions in the Ultra Yield™ flasks were performed with different filling volumes, too. The oxygen transfer rates ($OTR_{standard}$) were calculated for those standard experiments based on the reaction time ($time_{standard}$) as described above (eq. 7).

Inserting the $time_{SENBIT\ Flask}$ obtained from the reaction in the SENBIT® flasks, and the knowledge of the reaction time in the Ultra Yield™ flasks ($time_{Ultra\ Yield\ Flasks}$), the OTR in the Ultra Yield™ flasks ($OTR_{Ultra\ Yield\ Flask}$) could be calculated:

$$\frac{(time_{standard} - time_{SENBIT\ flask})}{time_{Ultra\ Yield\ Flask}} \times OTR_{standard} = OTR_{Ultra\ Yield\ Flask} \quad (9)$$

The OTR value obtained from eq. 9 can be used to calculate the oxygen transfer coefficient. Therefore, eq. 2 can be reduced to

$$OTR = k_La \cdot c_{O_2}^*$$

as the dissolved oxygen concentration in the liquid is assumed to be zero. The incoming oxygen reacts immediately with sodium sulfite. The oxygen concentration at the phase boundary surface was calculated to be $0.000215 \text{ mol L}^{-1}$ (Linek and Vacek, 1981).

Ultra Yield™ flasks

The primary goal of the work was to determine the k_La values of the Ultra Yield™ flasks (UYF) as received from Thomson Instrument Company (San Diego, CA) (Fig. 2). The sizes and recommended filling volumes of these flasks are listed in Tab. 2.

The Ultra Yield™ flasks are disposables, have a baffled base and a more vertical wall construction compared to other shake flasks, and they also have a wide neck, i.e. opening. The Baffles increase the agitation in the liquid as well as the available surface area for oxygen transfer at the air-liquid interface. Studies have shown that these flasks yield high cell densities and are especially well-suited for protein expression studies (Brodsky and Cronin, 2006a).

Those flasks are available in four sizes as can be seen in Table 2. It is recommended to use the flasks with a filling volume of up to 40%. The experiments in this work were done with filling volumes of 20% and 40%.

TABLE 2
Specification of the Ultra Yield Flasks

UYF used, ml	filling volume	
	20%, ml	40%, ml
125	25	50
250	50	100
500	100	200
2500	500	1000



Fig. 2: UltraYield flasks with total volumes of 2.5L, 500ml, 250ml, and 125ml

Previous studies have shown that the utilization of plugs in shake flasks can limit the mass transfer significantly, so that the oxygen level in the headspace can decrease up to 6 % v/v. Carbon dioxide can accumulate up to as high as 15 % v/v (SCHULTZ, 1964b). Oxygen transfer in shake flasks depends on the flow of air through the plug. As a consequence, the length of the neck of the flask or the type of closure greatly affects the oxygen transfer to the surface of the liquid. In this work, the AirOTop Seal from Thomson Instrument Company (San Diego, CA) was used.

SENBIT® System

The SENBIT® system used during this study consists of shake flasks, standard electrodes and a data transmission and acquisition system (Fig. 3). The shake flask possesses four baffles and three positions to introduce electrodes into the liquid phase (1 L Schott DURAN glass ware, retrace code 01268185).

The single-rod pH glass electrode and the amperometric DO sensor are both connected to transmitters which read, amplify and convert the signals of the sensors. The ISM band of 433MHz is used to send the data from the sensors to a computer (Vasala et al., 2006). This band is strong enough to penetrate solid barriers. In usual laboratory conditions (including closed incubation chambers) a distance of up to 100m can be covered.



Fig. 3: SENBIT Flask equipped with a pO₂ electrode

Hence, measurements from various locations are possible, while the data is collected by a central-fed workstation. In the workstation, the data is further processed and visualized.

The dissolved oxygen concentration was observed continuously and as soon as all of the sulfite had reacted to sulfate, the dissolved oxygen concentration increased immediately. The time correlating to this end of reaction is t_R .

Experimental Design with MODDE

All experimental design studies were performed with the software tool MODDE 8 (Umetrics Inc., Umeå, Sweden).

The aim of experimental design is to find the optimal number of experiments that have to be performed to yield certain information. Furthermore, the points, where to perform those experiments, are indicated, so that interactions between the different parameters can be observed.

The primary experimental objective of this work was to identify the most influential parameters and to get an estimation of the range of $k_L a$ at different operating conditions. This screening objective requires only a few experiments in relation to the number of parameters. It was best suited for our needs.

The experiments with the Ultra Yield Flasks were performed only on one shaker. Here the influence of the shaking velocity and the filling volume were observed. The experiments were performed at 25 °C. Due to technical reasons a narrow range of shaking velocities was studied.

TABLE 3

Experimental design with MODDE; the parameters shaking velocity and filling volume were defined in MODDE. The experiments were calculated with the screening objective. A full factorial design with 3 center points was chosen. The experiments were performed at 25°C.

Exp No	Exp Name	Run Order	Incl/Excl	Shaking velocity (rpm)	Filling volume
1	N1	3	Incl	150	20%
2	N2	1	Incl	190	20%
3	N3	2	Incl	150	40%
4	N4	6	Incl	190	40%
5	N5	5	Incl	170	20%
6	N6	4	Incl	170	20%
7	N7	7	Incl	170	20%

Firstly, the input parameters, their range and the responses were defined in the program MODDE. "Screening" was chosen as experimental objective. The experimental design was created as shown in Tab. 3.

After the experiments had been performed, the results were computed into the program. Then the regression analyses yielded a model reflecting the changes in the parameters to the changes in the results. This model indicated which parameters influence the response and which do not. The results of the regression analyses are attached in the appendix.

Experimental Procedure

A sodium sulfite solution of 0.25 M (98 % purity, Karl Roth Chemikalien GmbH, Karlsruhe, Germany), 10^{-7} M cobalt sulfate (Fluka Chemie AG, Buchs, Switzerland), and 0.007 M K_2HPO_4/NaH_2PO_4 Sørensen buffer (98 % purity, Karl Roth Chemikalien GmbH, Germany) was used, to work in the non enhanced reaction regime (Tab. 4). The initial pH was adjusted to 8 with 1 M sulfuric acid solution.

TABLE 4
Composition of the reaction media

Na ₂ SO ₃ , M	CoO ₄ S, M	Sørensen buffer, M
0.25	10^{-7}	0.007

- The solution was prepared with deionized water (already containing the buffer). It was gassed with nitrogen prior to and during dissolving the sodium sulfite in it in order to avoid prior reaction of sulfite to sulfate.
- After adjusting the pH, it was stored at 4°C and shielded of light irradiation.
- The solution was decanted carefully into a graduated cylinder and from there into the reaction flask. Prior shaking of the solution has to be omitted.
- The programmed and calibrated electrodes were inserted into the flasks and the reading of the values was switched on before the flask was shaken.

- The cobalt catalyst was only added shortly before the reaction started.
- During the experiments using the Ultra Yield™ flasks, the shaker was stopped after a predetermined time. Then the solution was poured from the Ultra Yield™ flask to the SENBIT® flask. Care had to be taken that no drops remained in the Ultra Yield™ flask and that the liquid did not ingest air bubbles during the pouring.
- The remaining reaction time was determined in the SENBIT® flasks.
- When the reaction finished, no more sulfite was present to react to sulfate. Hence, a sharp increase in the dissolved oxygen concentration was observed.

The experiments were performed on an orbital shaker with 2.5 cm shaking diameter (VKS75, Edmund Bühler GmbH, Tübingen, Germany) in an air-conditioned chamber at 25°C.

Results

To establish the new sodium sulfite method, some primary experiments were performed. The method of Hermann et al. (2001) was modified and the validity of the new method was proven.

Aim 1: Reproduction of the experiments performed by (Hermann et al. 2001).

A 0.5 M sodium sulfite solution containing 0.013 M buffer and 10^{-7} M cobalt sulfate catalyst was poured into a beaker and stirred with a magnetic stirrer bar. The course of the reaction was observed by a pH electrode connected to the SENBIT® data acquisition system and inserted into the liquid (data not shown). After a certain time, the reaction came to an end and a decrease of pH was observed.

Aim 2: Reduction of the duration of k_t determination

Application of the SENBIT® culture flask

Since the reaction was very slow, SENBIT® flasks were used as reaction vessels instead of a beaker to fasten the procedure for the following experiments. These flasks have a bigger OTR and it is easier to keep the standard conditions constant, while being able to use them in a temperature controlled room on an orbital shaker and at constant light intensity. The experiments in the beaker, on the contrary, had to be performed under the extractor hood without temperature control.

The reaction was performed in a SENBIT® flask equipped with a pO_2 and a pH electrode. 0.5M sodium sulfite solution containing buffer and catalyst were used (Fig. 4). The shaking intensity was set to 190 rpm at an orbital deflection of 25 mm at 25 °C.

At the end of the reaction, a first slow and then sharp drop of pH concomitant to a very steep increase in the dissolved oxygen concentration was recorded. The experiment demonstrated that the increase in dissolved oxygen concentration was even faster and more significant than the drop of pH. As a result, the pO_2 signal was used to determine the end of the reaction in the following experiments. The time period from the onset of the experiment to the time point when 80 % of saturation of dissolved oxygen concentration was reached was regarded as reaction time.

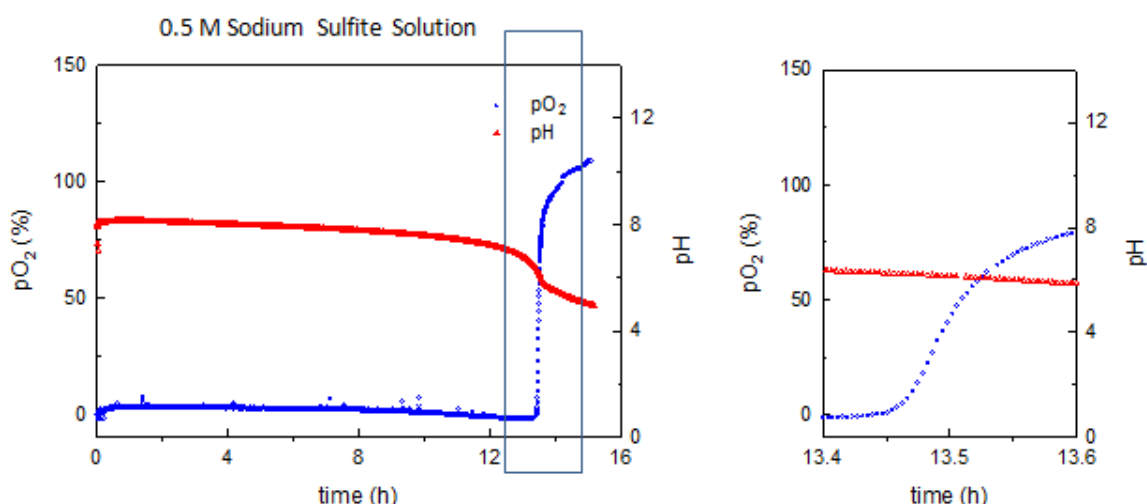


Fig. 4 : pO_2 and pH curve of the reaction of 0.5 M sodium sulfite solution with oxygen; 100 ml of 0.5 M sodium sulfite solution containing 0.013 M phosphate buffer with 10^{-7} M cobalt catalyst were filled into a SENBIT® flask and shaken on a horizontal shaker with 25 mm shaking diameter at 190 rpm.

Optimization of sodium sulfite concentration

In the next step, the sodium sulfite concentration was lowered from 0.5 M to 0.25 M. The cobalt catalyst concentration was kept at 10^{-7} M, all other reaction parameters were kept constant as well. Now, a reaction time of 6h was achieved (Fig. 5). This experiment showed that a buffer concentration of 0.013 M is too high for a 0.25 M sodium sulfite solution in order to recognize a profound pH decrease.

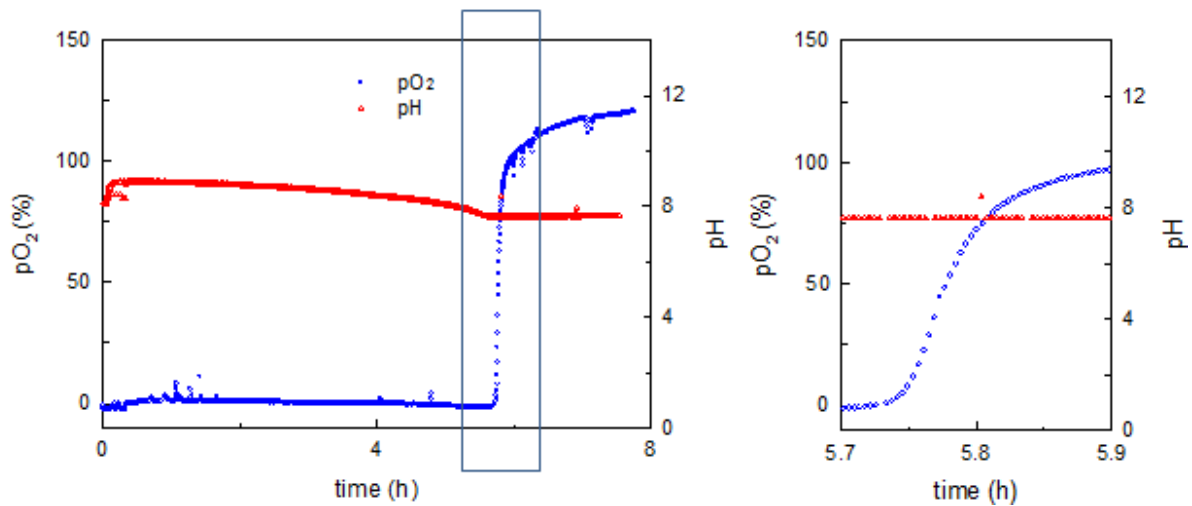


Fig. 5: pO₂ and pH curve of the reaction of 0.25 M sodium sulfite solution with oxygen; 100ml of 0.25 M sodium sulfite solution containing 0.013 M phosphate buffer and 10^{-7} M cobalt catalyst in SENBIT® flasks, shaken at 190 rpm on a horizontal shaker with 25 mm shaking diameter.

Aim 3: Verification of the dependency between dissolved oxygen concentration and transfer rate

In order to determine the $k_L a$ value with the sodium sulfite method, a constant OTR in the non accelerated reaction regime is required. If this is fulfilled, the reaction rate in the liquid side boundary layer is low and no reaction acceleration has to be taken into account. Therefore, the dependency between the OTR and the dissolved oxygen concentration was studied in a 2 L fermenter. The 0.25 M sodium sulfite solution was prepared in the reactor and supplemented with 10^{-7} M cobalt catalyst. The solution was then gassed with air at a constant rate. By changing the stirrer speed, the OTR was changed. A linear dependency between the OTR and the dissolved oxygen concentration was observed, as it can be seen in Fig. 6. That is, the sulfite oxidation is of first order with respect to oxygen. A first order reaction constant k_1 of 8000 L/h was obtained. The condition of a negligible oxygen concentration in the liquid ($R > 10$) is only fulfilled for an OTR of less than 0.17 mol/L/h as shown in Fig. 6. In this case, the OTR is equal to the maximum oxygen transfer capacity (OTR = OTR_{max}). For an OTR of more than 0.17 mol/L/h, the maximum oxygen transfer rate, and as a result the $k_L a$ value, can be calculated applying formulas (5) and (6).

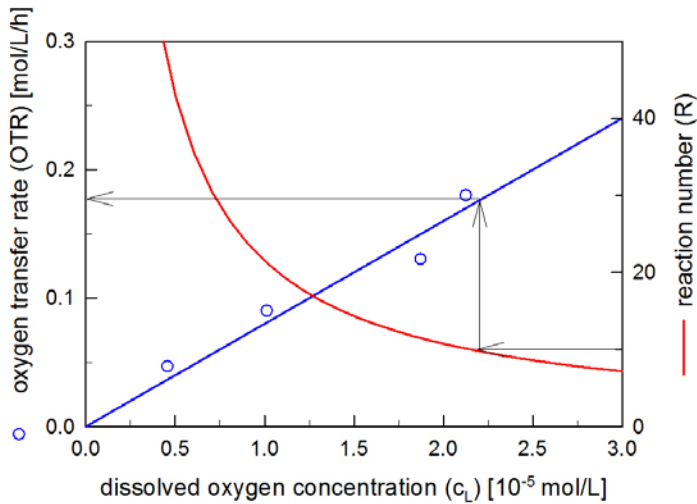


Fig. 6: Fermentation in reactor showing the dependency between dissolved oxygen concentration, oxygen transfer rate and reaction number; a 2 L reactor was operated at different stirrer speeds, with an aeration rate of 1 vvm at 25 °C, 0.25 M Na₂SO₃, 0.07 M buffer and 10⁻⁷ M CoSO₄

Aim 4: Impact of solution transfer between different reaction vessels

In order to get an estimation of the order of the error produced by pouring the liquid from the Ultra Yield™ flask to the SENBIT® flask, five pouring experiments were performed. 100 ml reaction medium was shaken at 190 rpm in a SENBIT® flask on an orbital shaker. Three flasks were stopped in between and the liquid was poured from the SENBIT® flask into an Ultra Yield™ flasks and back into the SENBIT® flask.

The determined values of the OTR are compared in Fig. 7. It could be shown, that there is no significant difference between the two groups of experiments. The standard deviation is in the range of 6 %.

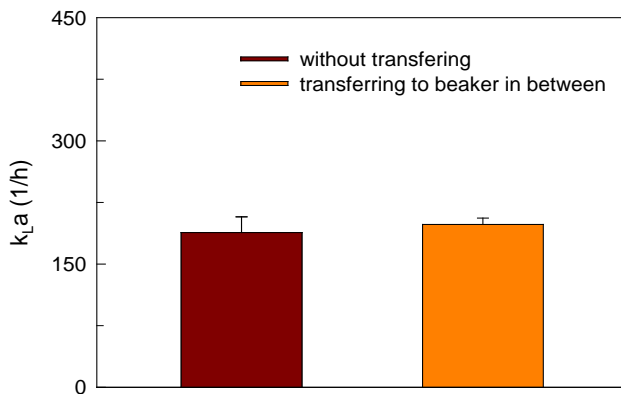


Fig. 7: Results of the transferring experiment; 100 ml reaction media was shaken at 190 rpm in a SENBIT® flask; the experiments were performed at 25 °C on an orbital shaker with 25 mm shaking diameter, 0.25 M Na₂SO₃, 0.07 M buffer, 10⁻⁷ M CoSO₄, initial pH8; In the transferring experiments the reaction was stopped and the liquid transferred to a beaker and back into the SENBIT® flask. These results are compared to those where no transfer had been performed.

The modified sodium sulfite method was used to determine the values of the OTR of the Ultra Yield™ flasks in the following experiments.

Aim 5: k_{La} values determined in SENBIT® cultivation flasks

The experimental setup required that six standard experiments had to be performed. These experiments were performed in triplicate in the SENBIT® flasks, without prior reaction of the sulfite solution in the Ultra Yield™ flasks. The obtained OTR values of the standard experiments were used to calculate the OTR values of the Ultra Yield™ flasks of the same filling volume.

TABLE 5

Experimental setup and results of the standard experiments performed in the SENBIT® flasks; the experiments were performed at 25 °C on an orbital shaker with 25 mm shaking diameter, 0.25 M Na₂SO₃, 0.07 M buffer, 10⁻⁷ M CoSO₄, initial pH8

Volume of Na ₂ SO ₃ solution, ml	Velocity, rpm	OTR, mol L ⁻¹ h ⁻¹	k _L a, h ⁻¹	Standard deviation of the k _L a
25	190	0.0516	239.82	14.27
50	190	0.0534	248.46	1.50
100	190	0.0478	222.54	16.82
200	100	0.0172	79.84	13.97
500	120	0.0077	35.86	1.27
1000	stirrer	0.0039	17.97	-

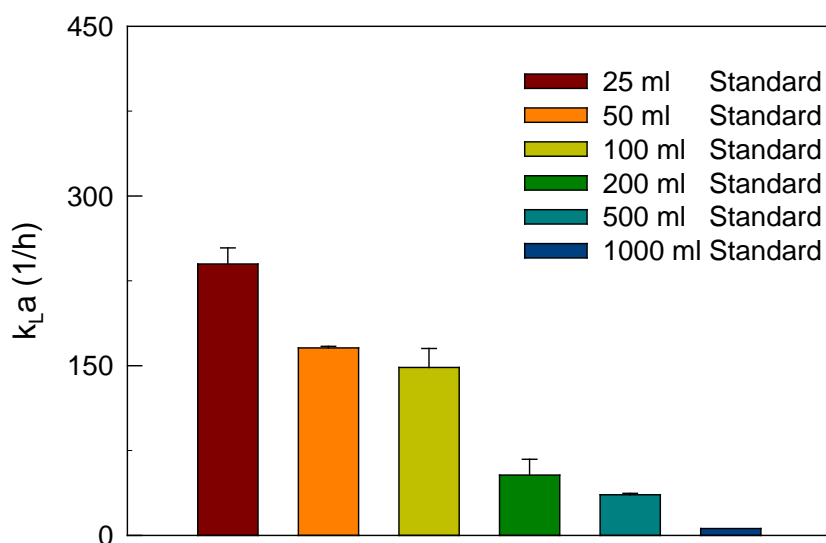


Fig. 8: k_La values determined only in the SENBIT® flasks; the experiments were performed at 25 °C on an orbital shaker with 25 mm shaking diameter at different shaking velocities, 0.25 M Na₂SO₃, 0.07 M buffer, 10⁻⁷ M CoSO₄, initial pH=8; different filling volumes of the sodium sulfite solution reacted with oxygen at predetermined shaking velocities in the SENBIT® flasks.

The smallest filling volume investigated was 25 ml. 25 ml of filling volume is not insuring that the electrodes are covered continuously with liquid during the experiment in the SENBIT® flasks. For this reason, the experiments containing 25 ml filling volume were performed in a 100 ml Erlenmeyer flask. The pO₂ electrode was fixed at the shake flask with adhesive tape. However, the largest filling volume applied exceeded the volume of the SENBIT® flask. In this case, the reaction proceeded in a stirred beaker. As the hydrodynamic flow behavior of the media changed with the filling volume, different shaker speeds were applied to prevent loss of media due to spill over.

The detailed reaction parameters are listed in Tab. 5. These standard experiments resulted in a reasonable distribution of the k_La values at the different conditions applied (Fig. 8).

Aim 6: k_La values determined in Ultra Yield™ cultivation flasks

The suggested experiments of the experimental design with MODDE for a variable velocity between 150 rpm and 190 rpm and a filling volume of 20 % and 40 % are shown in table 3. Those seven experiments were performed for each flask. The results of the experiments are summarized in table 6 and Fig. 9. For each flask, 5 different pairs of parameters were investigated. The center points were estimated in triplicate to obtain the experimental error. All experiments were performed at 25 °C on the same orbital shaker.

The lowest k_La values (4.79 mol L⁻¹ h⁻¹ and 6.66 mol L⁻¹ h⁻¹) recorded were those of the 125 ml Ultra Yield™ flask with 20 % filling volume at 150 rpm and of 250 ml Ultra Yield™ flask at 40 % filling

volume at 150 rpm. The largest $k_L a$ values ($422 \text{ mol L}^{-1} \text{ h}^{-1}$ and $399 \text{ mol L}^{-1} \text{ h}^{-1}$) were recorded in the 250 ml Ultra Yield™ flask for a filling volume of 20% at a shaking frequency of 190 rpm and in the 500 ml Ultra Yield Flask at 20 % filling volume and 190 rpm. The $k_L a$ increases with a decrease in filling volume from 40 % to 20 % and an increase in shaking speed. However, this was not observed for the 125 ml Ultra Yield™ flask.

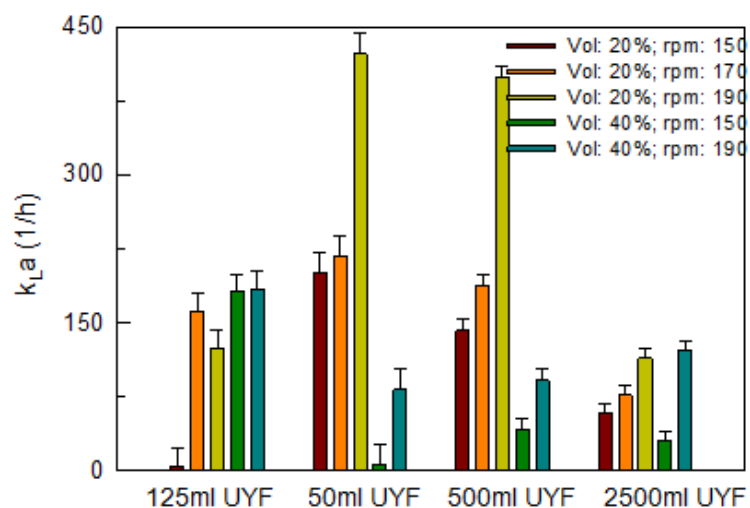


Fig. 9: $k_L a$ of the Ultra Yield™ flasks at different filling volumes and shaking velocities; the experiments were performed at 25 °C on an orbital shaker with 25mm shaking diameter, 0.25 M Na_2SO_3 , 0.07 M buffer, 10^{-7} M CoSO_4 , initial pH 8; the reaction media was allowed to react in the Ultra Yield™ flasks for a predetermined time, after which the liquid was transferred to the SENBIT® flasks. The $k_L a$ was determined by the time difference of the reaction in the SENBIT® flask and a standard reaction.

TABLE 6

$k_L a$ values determined for the different Ultra Yield™ flasks at different shaking velocities and filling volumes; all experiments were performed at 25°C on an orbital shaker with an orbit of 25mm

UYF, ml	Filling volume, ml	Shaking velocity, rpm	Reaction time in UYF, h	Time in SENBIT flask, h	OTR UYF, $\text{mol L}^{-1} \text{ h}^{-1}$	$k_L a$, h^{-1}
125	25	150	1	2.41	0.0010	4.79
125	25	170	1	1.67	0.0391	181.84
125	25	170	1	1.80	0.0324	150.73
125	25	170	1	1.80	0.0327	151.93
125	25	190	1	1.91	0.0267	124.41
125	50	150	1	1.61	0.0390	181.38
125	50	190	1	1.60	0.0395	183.86
250	50	150	1	1.53	0.0433	201.25
250	50	170	1	1.53	0.0433	201.30
250	50	170	1	1.49	0.0454	211.19
250	50	170	1	1.37	0.0518	241.01
250	50	190	1	0.64	0.0908	422.38
250	100	150	1	2.59	0.0014	6.66
250	100	190	1	2.25	0.0177	82.11
500	100	150	1	1.98	0.0305	142.02
500	100	170	1	1.81	0.0386	179.74
500	100	170	1	1.72	0.0432	200.83
500	100	170	1	1.79	0.0395	183.63
500	100	190	1	0.82	0.0859	399.43
500	200	150	4	5.31	0.0088	40.98
500	200	190	4	2.75	0.0196	91.30
2500	500	150	4	3.31	0.0127	58.97
2500	500	170	4	8.57	0.0147	68.57

2500	500	170	4	6.55	0.0186	86.66
2500	500	170	4	7.86	0.0161	74.93
2500	500	190	4	1.21	0.0245	113.77
2500	1000	150	4	25.65	0.0065	30.07
2500	1000	190	4	5.32	0.0261	121.44

Comparing the k_{La} values obtained in the Ultra Yield™ and the SENBIT® flasks, it was shown, that the Ultra Yield™ flasks from Thomson Instrument Company are characterized by significantly higher values of the OTR. The SENBIT® flask yielded at a normal working volume of 10 % an oxygen transfer coefficient of around 220 (h), whereas the 250 ml and 500 ml Ultra Yield™ flasks with a recommended working volume of 20 % reached an oxygen transfer coefficient, which is nearly doubled. For the 125 ml and 2500 ml Ultra Yield™ flasks, k_{La} values of a comparable magnitude as those of the SENBIT® flasks were obtained.

Discussion

The determination of the oxygen transfer coefficients of the Ultra Yield™ flasks verified the assumption, that these flasks are characterized by high k_{La} values. The oxygen transfer capacities were shown to be higher than those of the SENBIT® flasks. Comparing the Ultra Yield™ flasks to standard Erlenmeyer flasks, the difference becomes even more pronounced. Gupta and Rao (2003) proposed an empirical correlation for calculating the k_{La} value, as it would result from the sodium sulfite method, in shake flasks. By applying our operational parameters in the equation, the oxygen transfer coefficient of standard Erlenmeyer flasks was calculated (Tab. 7).

TABLE 7

Comparison of oxygen transfer coefficients of standard Erlenmeyer flasks and the four different Ultra Yield™ flasks; values of the Ultra Yield™ flasks have been determined experimentally, the values of the Erlenmeyer flask has been calculated (Atul Gupta and Govind Rao, 2003), a maximum inner shake flask diameter of 13 cm of the Erlenmeyer flask was assumed

Shaking velocity, rpm	k_{La} , /h				
	Erlenmeyer flask, 10 % filling volume	Ultra Yield™ flasks, 20 % filling volume			
		125 ml	250 ml	500 ml	2500 ml
150	34	5	201	142	59
170	40	161	218	188	77
190	45	184	422	399	114

The k_{La} values of the Ultra Yield™ flasks are up to 10 times higher than those calculated for the Erlenmeyer flasks.

Furthermore, the sodium sulfite method was established as a method for determining k_{La} values in small scale culture vessels. The new method was compared to methods described in the literature and the applicability was further proven by a reference experiment in a 2L fermenter. The results of the experiments concerning the Ultra Yield™ flasks were computed in MODDE and the model validity proven for three out of four flasks.

Hence, the sodium sulfite method is well-suited for the determination of the OTR in smaller culture vessels where it is hard to introduce oxygen sensors. In those small scale bioreactors, the size of the oxygen electrode is considerably big in comparison to the size of the culture vessel. The electrodes

may act as baffles. Hence, they influence the hydrodynamic flow behavior of the model system. This would lead to unpredictable changes in the oxygen transfer. These influences can be omitted by using the sodium sulfite method of this study.

Contrarywise, a very high concentrated salt solution is used to determine the OTR by the sodium sulfite method. The high salt concentration reduces the maximal solubility of oxygen to a level below that in low-salt culture media, which generally contains 0.1 – 0.2M salts (Duetz, 2007). This leads to lower oxygen solubility in the media. Hence, the OTR is underestimated. A correcting factor of approximately 1.3 was proposed by Duetz et al. (2007) when translating the results to microbial cultures.

Acknowledgements

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Nomenclature

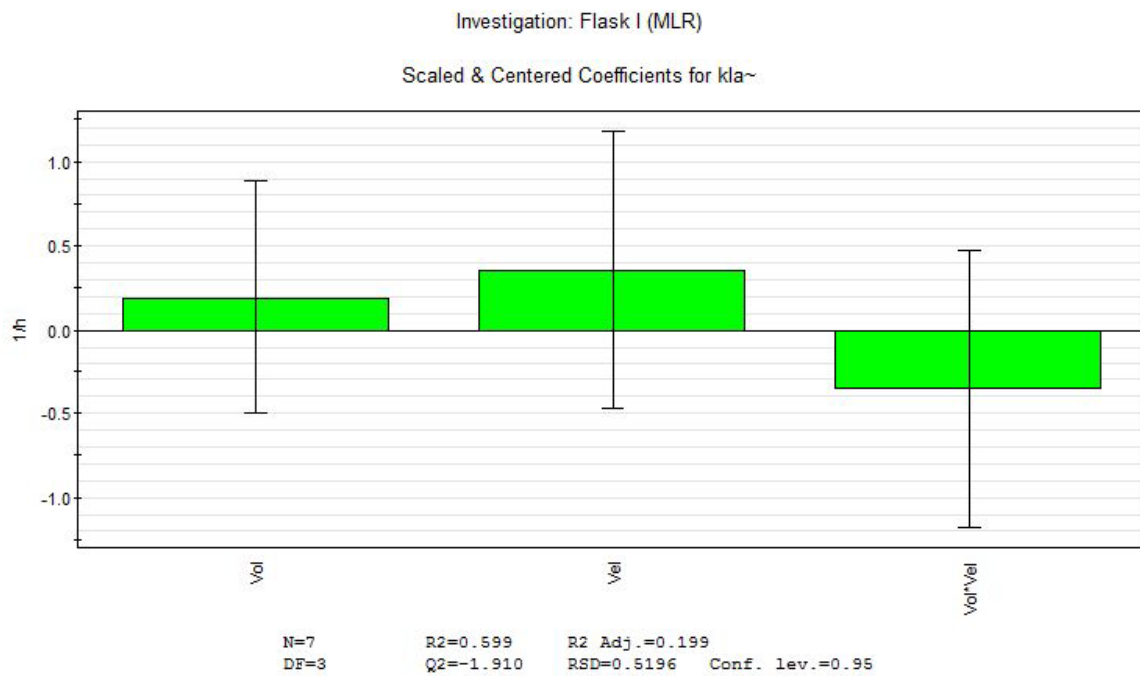
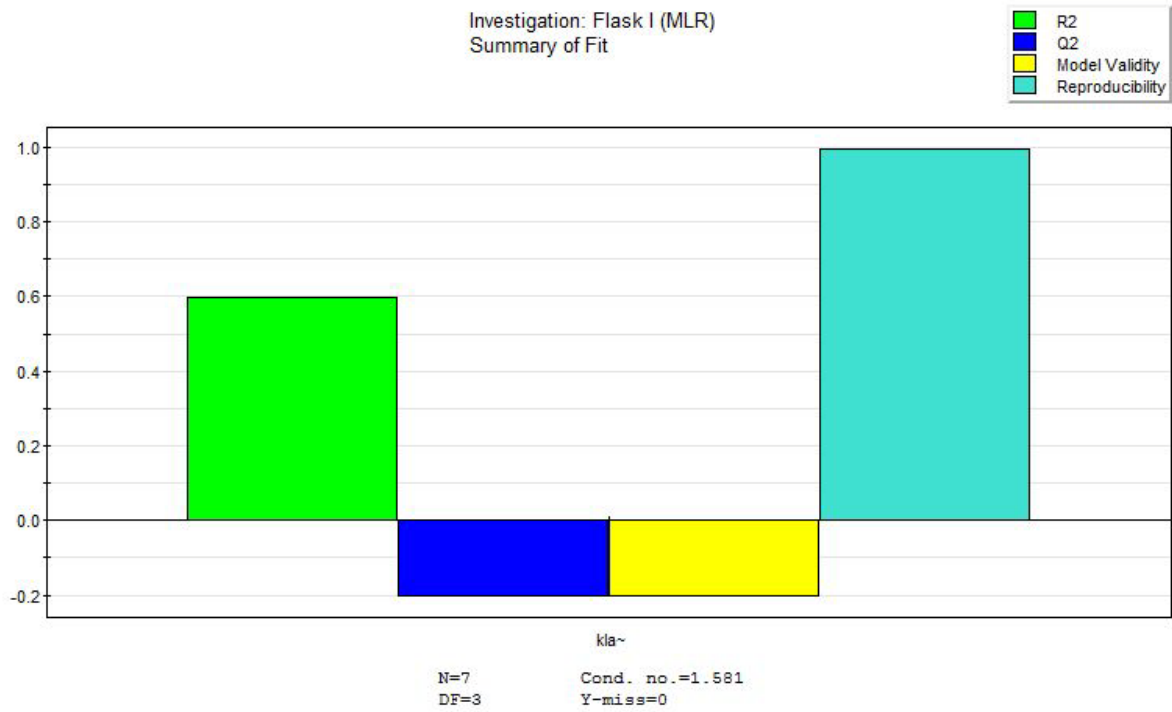
A	phase boundary surface
c_L	oxygen concentration in the liquid phase
$c_{O_2}^*$	oxygen concentration at the phase boundary surface
$c_{Na_2SO_3}$	initial sodium sulfite concentration
k_L	mass transfer coefficient
$k_L a$	volumetric mass transfer coefficient
k_n	reaction constant
N_{ave}	oxygen transfer rates over the whole measuring period ($N_{ave} = k_L a c^* = OTR_{max}$)
$N_{O_2}^*$	stream of amount of oxygen
OTR	oxygen transfer rate
OTR_{max}	maximum oxygen transfer capacity
R	reaction rate
$\frac{t_1}{t_2}$	time (start fitting and end fitting)
t_R	overall reaction time
v	stoichiometric coefficient
V_L	liquid volume

Reference List

- Brodsky O, Cronin CN. 2006a. Economical parallel protein expression screening and scale-up in *Escherichia coli*. *J Struct Funct Genomics* 7:101-108.
- Anderlei T, Büchs J. 2001. Device for the sterile online measurement of oxygen transfer rate in shaking flasks. *Biochem Engin J* 7:157-162.
- Van Suijdam JC, Kossen NWF, Joha AC. 1978. Model for Oxygen Transfer in a Shake Flask. *Biotechnol Bioengin* 20:1695-1709.
- Brodsky O, Cronin CN. 2006b. Economical parallel protein expression screening and scale-up in *Escherichia coli*. *J Struct Funct Genomics*.
- Garcia-Ochoa F, Gomez F. 2009. Bioreactor scale-up and oxygen transfer rate in microbial processes: An overview. *Biotechnol Adv* 27:153-176.
- Suresh S, Srivastava VC, Mishra IM. 2009. Techniques for oxygen transfer measurement in bioreactors: a review. Wiley InterScience.
- McDaniel LE, Bailey EG. 1969. Effect of shaking speed and type of closure on shake flask cultures. *Appl Microbiol* 17:286-290.
- Henzler H-J, Schedel M. 1991. Suitability of the shaking flask for oxygen supply to microbiological cultures. *Bioprocess Engin* 7:123-131.
- Anderlei T, Zang W, Papaspyrou M, Büchs J. 2004. Online respiration activity measurement (OTR, CTR, RQ) in shake flasks. *Biochem Engin J* 17:187-194.
- Veglio F, Beolchini F, Ubaldini S. 1998. Empirical models for oxygen mass transfer. *Process Biochem* 33:367-376.
- Hirose Y, Sonoda H, Kinoshita K, Okada H. 1966. Studies on oxygen transfer in submerged fermentations Part IV. Determination of oxygen transfer rate and respiration rate in shaken cultures using oxygen analyzers. *Agric Biol Chem* 30:49-58.
- SCHULTZ JS. 1964a. Cotton closure as an aeration barrier in shaken flask fermentations. *Appl Microbiol* 12:305-310.
- Linek V, Vacek V. 1981. Chemical-Engineering Use of Catalyzed Sulfite Oxidation-Kinetics for the Determination of Mass-Transfer Characteristics of Gas-Liquid Contactors. *Chem Engin Sc* 36:1747-1768.
- Hermann R, Walther N, Maier U, Büchs J. 2001. Optical method for the determination of the oxygen-transfer capacity of small bioreactors based on sulfite oxidation. *Biotechnol Bioengin* 74:355-363.
- Zlokarnik M. 1999. *Rührtechnik* Springer-Verlag GmbH. p 136-137.
- Sobotka M, Prokop A. 1982. Review of methods for the measurement of oxygen transfer in microbial systems. *Ann Rep Ferm Proc* 5:127-211.
- Yasunishi A. 1977. Effect of pH on oxidation rate of aqueous sodium sulfite solution. *J Chem Engin Japan* 3:154-159.
- Linek V, Kordac M, Moucha T. 2006. Evaluation of the optical sulfite oxidation method for the determination of the interfacial mass transfer area in small-scale bioreactors. *Biochem Engin* 27:264-268.
- Atul Gupta, Govind Rao. 2003. *A Study of Oxygen Transfer in Shake Flasks Using a Non-Invasive Oxygen Sensor*. Wiley InterScience.
- Duetz WA. 2007. Microtiter plates as mini-bioreactors: miniaturization of fermentation methods. *Trends Microbiol* 15:469-475.

Appendix

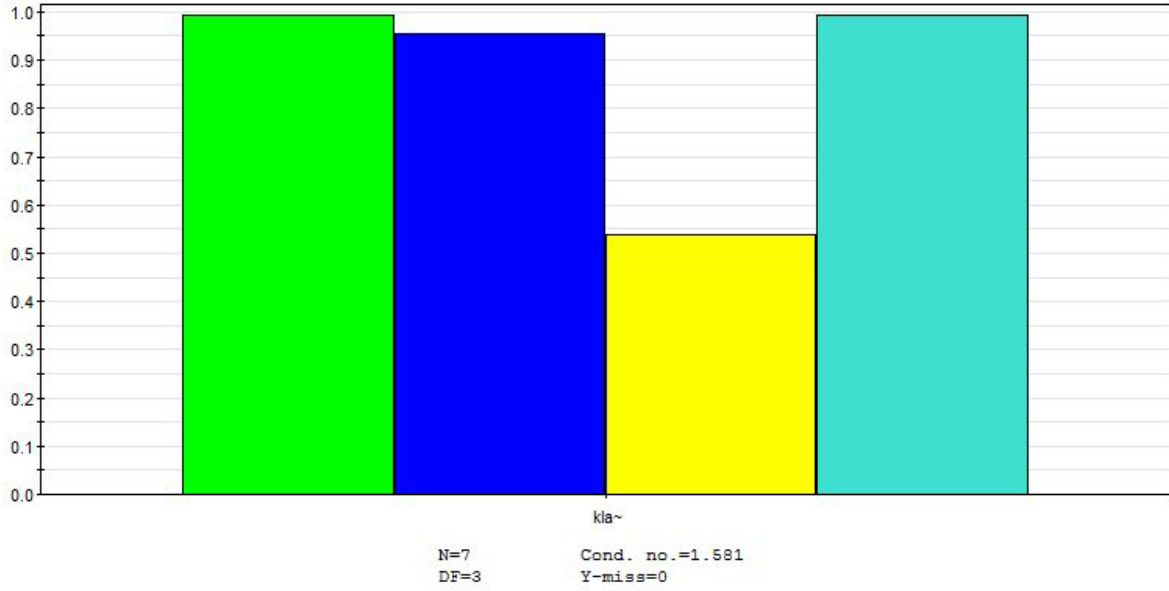
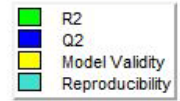
125ml Ultra Yield Flask



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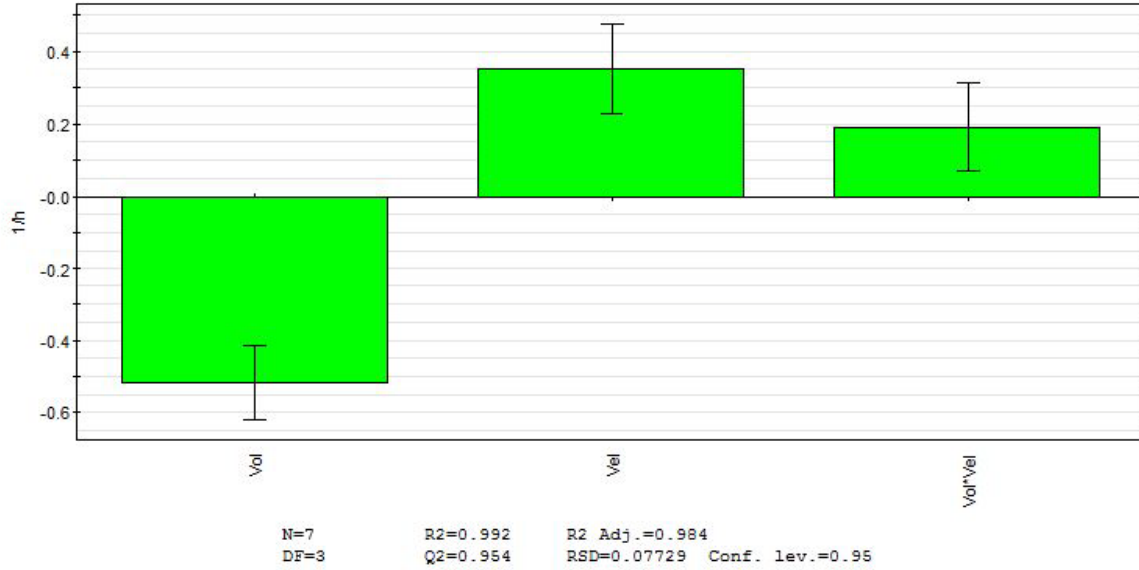
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Summary of Fit



Investigation: Flask II (MLR)

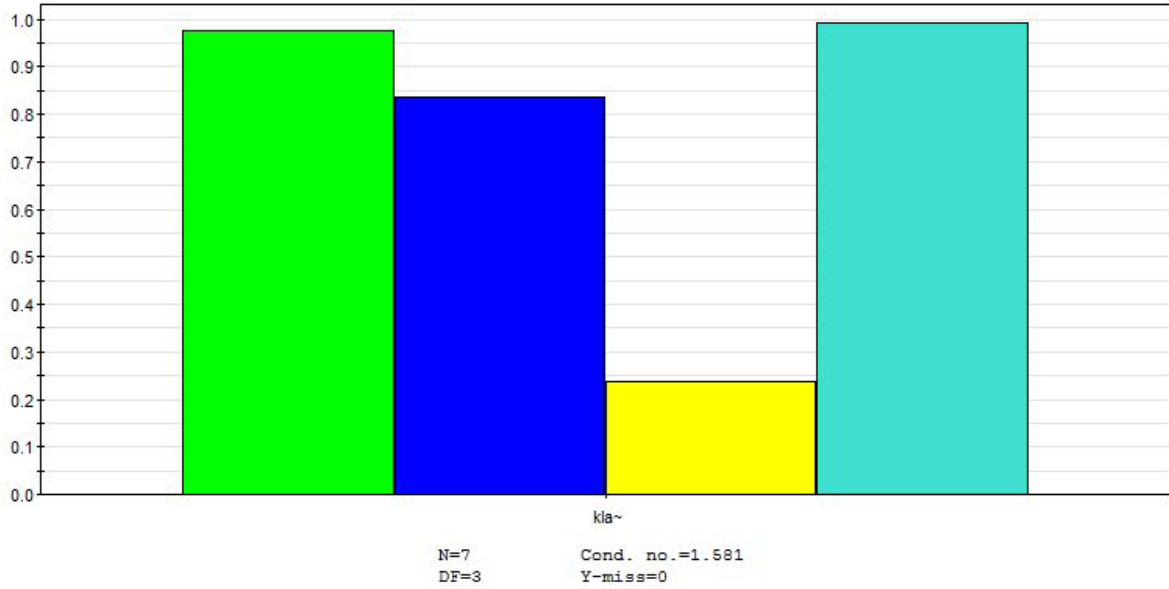
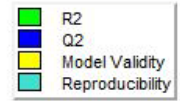
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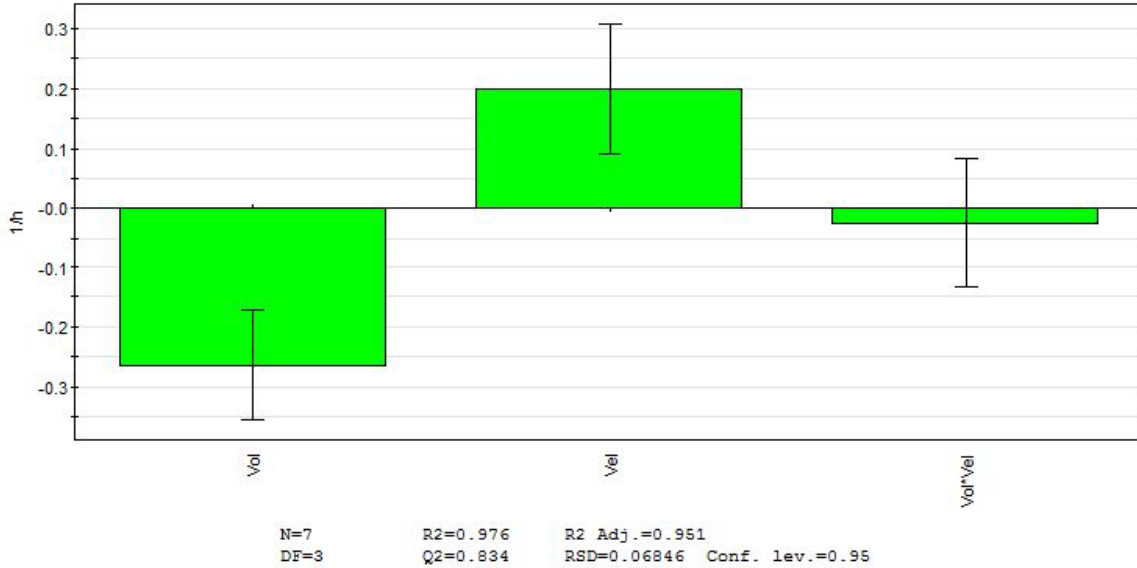
500ml Ultra Yield Flask

Investigation: Flask III (MLR)
Summary of Fit



Investigation: Flask III (MLR)

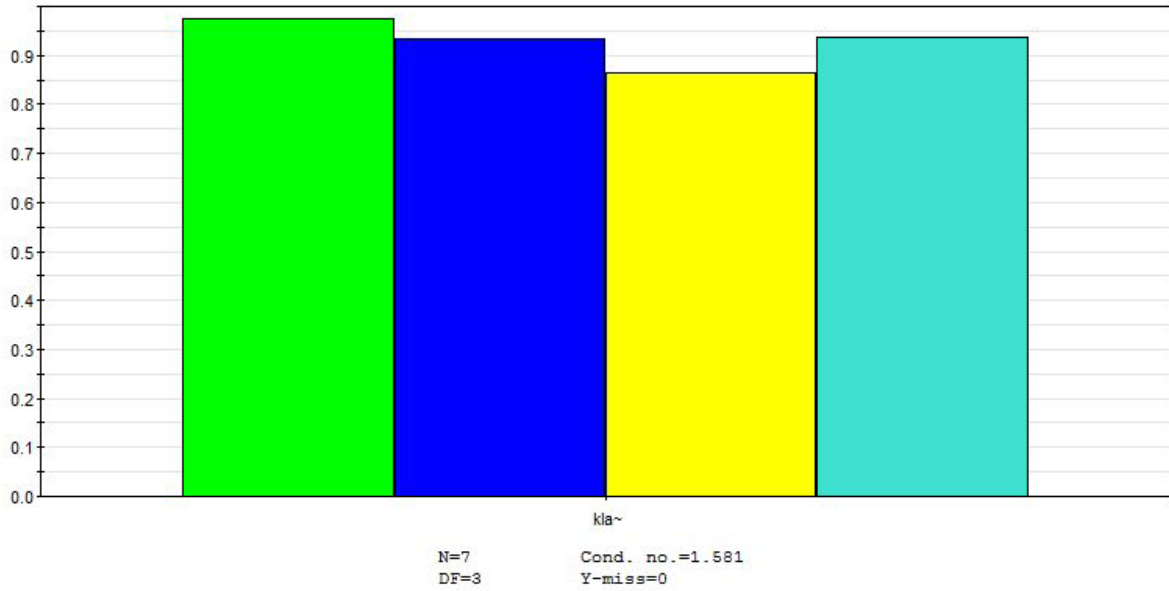
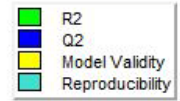
Scaled & Centered Coefficients for kla~



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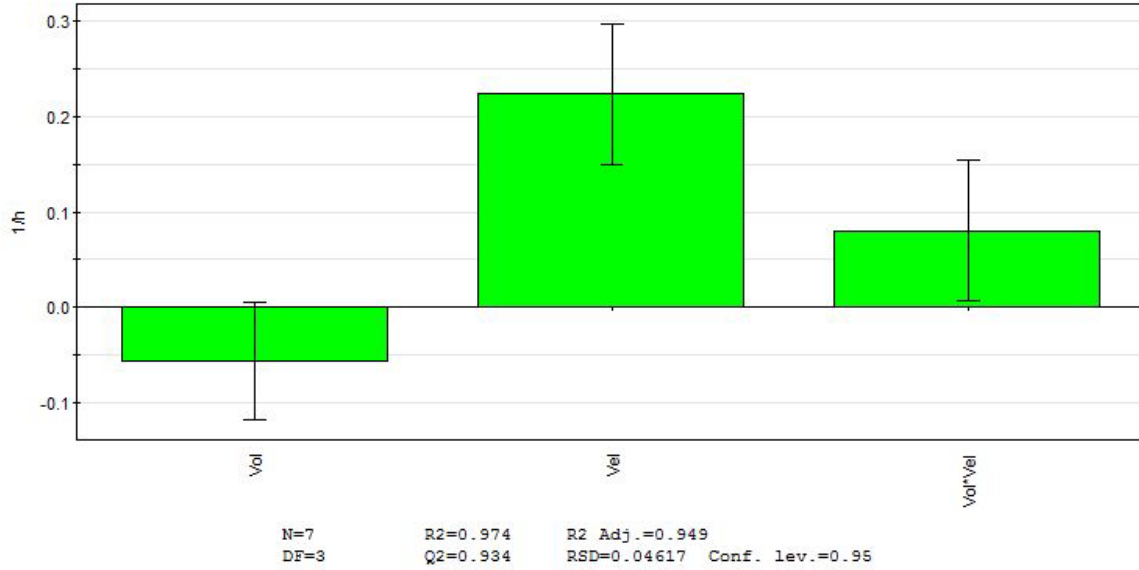
2500ml Ultra Yield Flask

Investigation: Flask IV (MLR)
Summary of Fit



Investigation: Flask IV (MLR)

Scaled & Centered Coefficients for kla~



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