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Techniques for oxygen transfer measurement in bioreactors: a review

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Abstract

Oxygen is the most essential requirement for aerobic bioprocesses. The microbial growth in a bioreactor depends upon the oxygen transfer rate (OTR). The OTR is widely used to study the growth behavior of microbial and plant cell cultures. The mass transfer coefficient (k_La) determines the magnitude of the OTR. There are many techniques for measuring oxygen concentration and OTR in bioreactors. Zirconia, electrochemical, infrared, ultrasonic and laser cells are used to measure oxygen concentration in the liquid medium. Optical sensors are better alternatives to measure oxygen concentration in small bioreactors. Sulfite oxidation and gassing-out methods with a Clark-type electrode have been used for OTR measurements in bioreactors. Many new novel techniques have evolved recently for intermittent and continuous online measurement of OTR/k_La in various types of bioreactors. The present paper gives an overview of various measurement techniques and their limitations and/or suitability for measurement of OTR/k_La in various kinds of bioreactors, especially small bioreactors.

Keywords: oxygen measurement; sensor; oxygen electrode; shake flasks; oxygen transfer rate; OTR

INTRODUCTION

Availability of oxygen strongly affects the process performance of aerobic bioprocesses. These bioprocesses are mostly carried out in aqueous media where the solubility of oxygen is low owing to the presence of ionic salts and nutrients, and the rate of oxygen utilization by the microorganisms is high. Hence, oxygen transfer is an important and rate limiting step in bioprocesses.[1,2]

Changes in oxygen availability leads to drastic effects on fermentation kinetics.[3–8] An increase in the availability of dissolved oxygen in the culture medium often results in improved yield of secondary metabolites.[9] Oxygen limitation can be overcome by increasing the oxygen transfer rate (OTR). The introduction of pure oxygen into the fermenter to enhance the microbial cell harvest or product accumulation has attracted special attention.[10] Special aeration systems, e.g. aeration using oxygen enriched air and increased reactor pressure are also commonly applied techniques to increase oxygen availability.[11–14] During the last decade, the use of *in situ* production of oxygen[15] and use of oxygen vectors have gained significant interest.[16,17]

When oxygen is limited, the metabolic rate of the microorganisms decreases significantly and the culture may respond adversely to the resulting stress.[18] The addition of a non-aqueous organic phase (often called the oxygen vector) induced significant increase in oxygen transfer rate from air to microorganisms with no supplementary intensification of mixing.[19] These vectors have higher oxygen solubilization capacity than that of water.[17] Much of the oxygen transfer literature describes the effect of the presence of a second liquid phase on oxygen mass transfer for various organic compounds such as n-hexadecane,[20–25] n-dodecane,[17,8,20] perfluorochemicals,[26,27] oleic acid,[28] and vegetable oil.[23] The oxygen solubility in these compounds is about 15–20 times higher than that in water.[22,29–32] Generally, the oxygen-vectors have no toxicity against the cultivated microorganisms. In some cases, they could even be used as supplementary sources of carbon and energy.

Excellent reviews on the mechanistic aspects of the enhancement of the gas–liquid mass transfer because of the presence of a second dispersed liquid phase are available.[33–35] The addition of oxygen-vectors causes the appearance of four phases in the bioreactor: the gas phase (air), the aqueous phase, the liquid organic phase and the solid phase (biomass), with the formation of new interfacial areas between the gas and liquid phases. Among all the mechanisms of gas–liquid oxygen transfer, the most plausible mechanism assumes that the hydrocarbon is adsorbed onto the surface of oxygen bubbles, with or without the formation of a continuous film, the diffusion of oxygen from air to microorganisms occurring through oxygen vectors, and then, through the aqueous phase or directly to the cells adsorbed to hydrocarbon droplets or film surface.[32,36–38] The main resistance to oxygen transfer lies in the diffusion of oxygen through the aqueous boundary layer from the interface of the hydrocarbon-aqueous phase. This resistance is counteracted both by the increase in the interfacial area for oxygen transfer and the enhanced accumulation of oxygen in the organic phase, which acts as an oxygen reservoir.[32,36–38] Among the oxygen vectors, n-hexadecane[25] and n- dodecane[8] are found to have maximum potential for use in aerobic fermentations.

OTR could be rate limiting in high cell density culture (HCDC) techniques used for *E. coli*, which facilitated the production of recombinant proteins and non-protein biomolecular products such as amino acids and primary and secondary metabolites with

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Table 1. Top ten most cited articles in the ISI Web of Science database for 'All Years' (1983–2008) with 'Oxygen transfer' and *E. coli, C. glutamicum* and *Bacillus* in the topic (out of a total of 25 171 articles appearing: database searched 4.12.08). Note that early oxygen transfer articles only appeared late 1990s

1.	John GT, Klimant I, Wittmann C and Heinzle E, Integrated optical sensing of dissolved oxygen in microtiter plates: a novel tool for microbial cultivation. *Biotechnol Bioeng* **81**:829–836 (2003). Times cited: 70.	82
2.	Akesson M, Hagander P and Axelsson JP, Avoiding acetate accumulation in Escherichia coli cultures using feedback control of glucose feeding. *Biotechnol Bioeng* **73**:223–230 (2001). Times Cited: 48.	130
3.	Calik P, Calık G and Ozdamar TH, Oxygen transfer effects in serine alkaline protease fermentation by Bacillus licheniformis: Use of citric acid as the carbon source. *Enzyme Microbial Technol* **23**:451–461 (1998). Times cited: 46.	131
4.	Royce PN, Effect of changes in the pH and carbon–dioxide evolution rate on the measured respiratory quotient of fermentations. *Biotechnol Bioeng* **40**:1129–1138 (1992). Times Cited: 44.	132
5.	Calik P, Calik G, Takac S and Ozdamar TH, Metabolic flux analysis for serine alkaline protease fermentation by Bacillus licheniformis in a defined medium: Effects of the oxygen transfer rate. *Biotechnol Bioeng* **64**:151–167 (1999). Times Cited: 32.	133
6.	Schmid A, Kollmer A, Mathys RG and Witholt B, Developments toward large–scale bacterial bioprocesses in the presence of bulk amounts of organic solvents. *Extremophiles* **2**:249–256 (1998). Times Cited: 30.	134
7.	Calik P, Calik G and Ozdamar TH, Oxygen–transfer strategy and its regulation effects in serine alkaline protease production by Bacillus licheniformis. *Biotechnol Bioeng* **69**:301–311 (2000). Times Cited: 29.	135
8.	Anderlei T, Zang W, Papaspyrou M and Buchs J, Online respiration activity measurement (OTR, CTR, RQ) in shake flasks. *Biochem Eng J* **17**:187–194 (2004). Times cited: 28.	57
9.	Maier U, Losen M and Buchs J, Advances in understanding and modeling the gas–liquid mass transfer in shake flasks. *Biochem Eng J* **17**:155–167 (2004). Times cited: 24.	12
10.	Yang JD and Wang NS, Oxygen mass-transfer enhancement via fermenter headspace pressurization. *Biotechnol Prog* **8**:244–251 (1992). Times Cited: 22.	136

high productivities.[39,40] The scale-up of such aerobic fermentation processes demands the transfer of oxygen and meeting the oxygen requirement throughout the culture volume in the fermenter.[39,40]

OTR is widely used to study the growth behavior of microbial and plant cell cultures.[41] Almost every physiological activity is related to oxygen uptake in aerobic cultures. Physiological responses of aerobic microorganism to specific culture conditions like oxygen limitation, nutrient limitation and inhibiting factors are reflected by OTR.[41] The magnitude of OTR is greatly influenced by the liquid phase mass transfer coefficient (k_La). Therefore, it is essential to correctly determine the oxygen concentration in the bioreactor. This helps in determining accurate values of OTR/k_La.

The methods described for oxygen-transfer measurement are often divided into two groups according to their applicability either to chemical model systems,[10,42–45] or to microbial systems.[41,46–51] Table 2 compiles various techniques used in the literature for the measurement of oxygen.

Shaken systems are of diverse designs and varying volumes, ranging from shake flasks of hundreds of millilitres volume right down to microtitre plates (MTPs) of a few microlitres in volume. They are widely used in industry and academia as a tool for drug discovery, media, strain and product optimization, and process development. The measuring methods applied in small bioreactors like shaking flasks vary in terms of the flask shape and size, the type of flask closure and the measuring method used.

Sobotka *et al.*[52] provide a review of OTR measurement methods for different chemical and microbial model systems. In some of these methods the dissolved-oxygen concentration in the liquid phase has to be measured. For small-scale bioreactors, the size of the conventional oxygen electrodes is not negligible in comparison with the size of the bioreactor. Therefore, the electrodes act as baffles and even cause bubble entrainment and, thus the hydrodynamic flow behavior of the chemical model system or the fermentation broth is strongly influenced.[52] This leads to undefined changes in the oxygen transfer that do not reflect the conditions in the bioreactor. Miniaturized electrodes applicable to small scale bioreactors that do not influence the hydrodynamic flow behavior are not available yet.[52] Non-

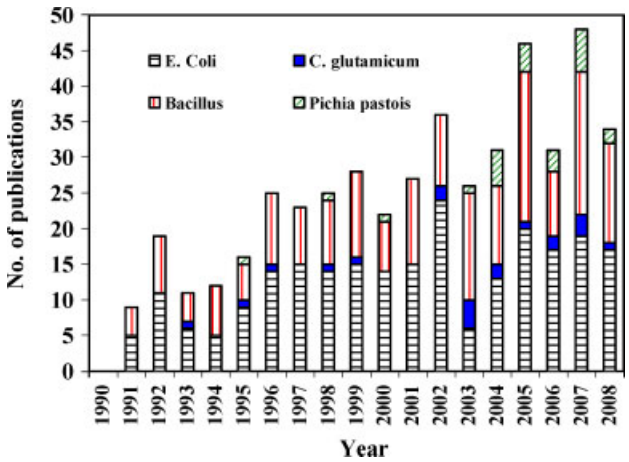


Figure 1. Number of research papers appearing with 'oxygen transfer' and *E. coli, C. glutamicum, Bacillus* and *Pichia pastories* in the topic as listed in the ISI Web of Science database for 'All Years' (1983–2008) (out of a total of 25 171 articles appearing: database searched 4.12. 08). Note that early oxygen transfer articles appeared in the late 1990s. The small number of papers in pre-1989 years means they cannot be properly shown in the figure.

invasive optical sensors are better alternatives to measure oxygen concentration in small bioreactors. Some researchers have used luminescence-based sensors for online measurement of oxygen in shake-flask systems.[1,53] The optical sensor system allows the robust and precise online measurement of dissolved oxygen in shake flasks.[53–57] Newly developed devices like RAMOS determine the optimal operating conditions, obviating the limitations in shaken bioreactors.[44,56,57]

A search of the ISI web of knowledge shows a number of research articles on oxygen transfer in culture medium with such microbial species as *E. coli, C. glutamicum, Bacillus and Pichia pastoris.* Oxygen transfer studies have been carried out using a variety of microorganisms and the most cited articles are shown in Table 1. Figures 1 and 2 show the yearly progress of publications

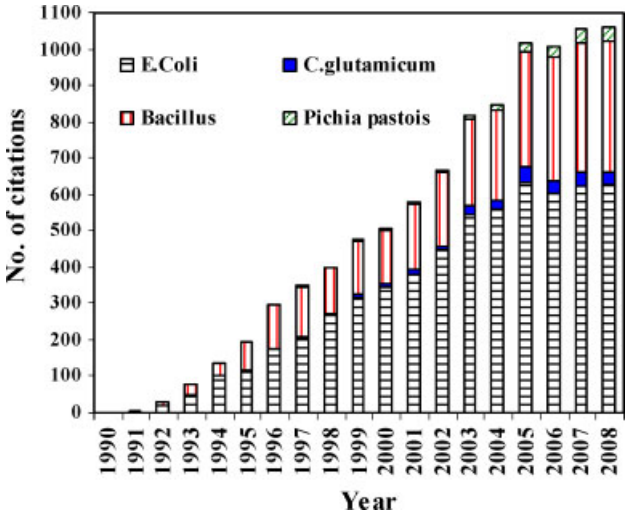


Figure 2. Number of citations of research papers appearing with 'oxygen transfer' and *E. coli, C. glutamicum, Bacillus* and *Pichia pastoris* in the topic as listed in the ISI Web of Science database 'All Years' (1983–2008) (out of a total of 25 171 articles appearing: database searched 4.12. 08; Total citations : for *E. coli, C. glutamicum, Bacillus* and *Pichia pastoris* were 6017, 257, 3066 and 173; average citations: for *E. coli, C. glutamicum, Bacillus* and *Pichia pastoris* were 25.18, 12.85, 16.48 and 7.21; h–index = 40, 7, 30 and 8).

and citations in the late twentieth century and during the present decade. These figures clearly indicate the increasing interest in the area of oxygen transfer in bioreactors.

Few authors have previously attempted to review the different methods of oxygen measurement in laboratory-scale bioreactors.[52] Van't Reit[58] and Rainer[59] described and discussed different methods for the determination of $k_L a$. However, none of the reviews were concerned with the oxygen measurement techniques employed in shake flasks, which are the most frequently used laboratory-scale bioreactors for screening and for bioprocess development. Recently, many new novel techniques have evolved for intermittent and continuous online measurement of oxygen transfer in various types of bioreactors. The present review aims to provide a critical assessment of various oxygen measurement techniques used in bioreactors.

MEASUREMENT OF OXYGEN CONCENTRATION

The oxygen measurement techniques include zirconia, electrochemical (also known as galvanic), infrared, ultrasonic and laser based methods.

A zirconium dioxide or zirconia lambda sensor is an electronic device based on a solid-state electrochemical fuel cell called the Nernst cell. It measures the proportion of oxygen (O_2) in the gas or liquid being analyzed. Its two electrodes provide an output voltage corresponding to the quantity of oxygen in the exhaust effluent relative to that in the atmosphere.

The majority of electrochemical systems are based on a Clark-type oxygen sensor. The Clark cell is a linear amperometric oxygen sensor based on the electrochemical cell with an external polarization voltage.[60] Its output is a current that is proportional to the oxygen content or more appropriately to the partial pressure of oxygen in the fluid.[61] The partial pressure can be expressed by the concentration level or percentage saturation. The output current is very small, ranging from several nA to several μA or even smaller for some models of oxygen sensors.[61] Commercially available dissolved oxygen measurement systems typically have an accuracy of 2.5–5%.[62] The theory of operation and practical usage of electrochemical oxygen sensors has been discussed extensively in the literature with several excellent reviews.[63]

Conventional galvanic oxygen sensor technology uses two dissimilar metal electrodes, typically silver and lead, which are consumed in the process of measuring oxygen. The sensors have a relatively short life-span of several months because the lead anode is consumed and the silver cathode is attacked by trace acid gases. Thus, the electrolyte becomes susceptible to contamination.[64] The galvanic sensors operate like a battery. As these sensors age, electrode sites become depleted and the sensor begins to read lower than the actual oxygen concentration. False low readings lead operators to believe that the process is proceeding normally when higher oxygen levels are causing poor product quality. The disadvantage of this type of electrode is that it consumes oxygen during the measuring process. The sample is required to be stirred during measurement, thus requiring long measurement times. Also, it is easily poisoned by the hydrogen sulfide.

The partial pressure sensor is very similar in construction to the electrochemical sensor in many ways. This sensor is mainly used for medical purposes, where the effect on the human body is the most important aspect of the measurement. It has the major advantage of not being sensitive to carbon dioxide. Since this sensor measures the partial pressure of oxygen directly, it is essential to compensate for ambient pressure.[65]

The paramagnetic oxygen sensor is a highly accurate measurement technique for oxygen concentration. All paramagnetic measuring instruments available in the market utilize the paramagnetic properties of oxygen. Oxygen is one of the very few gases with a strong magnetic susceptibility. The movement of the electrons within a molecule generates magnetic moments. However, such sensors are beset by high costs.

A paramagnetic measuring instrument is an obvious choice in those applications where the concentrations of contaminant gases are large. Such sensors are not damaged by CO_2 unlike electrochemical sensors. Typical areas of application for paramagnetic oxygen sensors include the measurement of oxygen in waste treatment gas, landfill gas and biogas and the monitoring of the oxygen concentration in putrefaction or fermentation processes.

Owing to the drawbacks of Clark-type oxygen sensors, optical sensors have gained importance in oxygen measurement.[66] The majority of the dissolved and gaseous oxygen optical sensors are based on luminescence techniques.[67–76] Optical sensors consist mainly of an oxygen- sensitive membrane and an optical system. The membrane contains an oxygen-sensitive dye and a supporting matrix in which the oxygen-sensitive dye is dispersed or dissolved. The optical system includes an excitation light source such as a light-emitting diode (LED) or a laser, a photon detector such as a photodiode or photomultiplier tube, and a bifurcated optical fibre acting as excitation and emission light waveguides.[77] Based on the use of oxygen-sensitive dyes, these optical methods can generally be divided into two major classes. The first type is polycyclic aromatic hydrocarbons and porphyrins, such as pyrenebutyric acid and tetraphenylporphyrin.[74,75] The second class is metallorganic complexes including ruthenium (II) and osmium (II) diamines, and platinum porphyrin complexes.[67–73,76] These sensors do not consume oxygen during the sensing process and also no reference electrode is required. They are immune to sample flow rate or stirring speed, and are immune to exterior electromagnetic field interference.

Recently, novel luminescence materials based on ruthenium complexes immobilized on silica gel particles have been developed. These luminescence materials are very stable, although extremely sensitive to molecular oxygen.[70,72] These materials have been employed in conjunction with some oxidases to develop optical biosensors such as glucose[78] and aspartame[77] biosensors. At present, optical dissolved oxygen sensors available in the market are expensive and relatively large, which makes them inconvenient for field use. In addition, software and personal computers are required for control and data acquisition using these sensors. These add to the overall cost.[79]

Measurement of dissolved oxygen in microtiter plates is of potential interest for the screening of oxygen-consuming enzymes (e.g. oxidases), aerobic cell activities, and biological degradation of pollutants, and for toxicity tests.

OXYGEN SENSORS FOR MINIATURE BIOREACTORS

The measurement of dissolved oxygen concentration using optical sensors is of particular interest in miniature bioreactors,[80,81] with a few commercial systems having been introduced in the recent past. One such commercial system uses an immobilized fluorescence sensor with a fluorescence signal intensity that is related to dissolved oxygen concentration.

Fluorescence methods[82] are considered to be superior to other optical and electrochemical methods because of their high sensitivity and their ability to measure fluorescence intensity as well as fluorescence decay time.[83,84] General problems encountered during the measurement of fluorescence intensity include fluctuations in light intensity and detector sensitivity, irregular sensor spots, light scattering, and intrinsic fluorescence of the sample. Therefore, the use of a second internal standard fluorophore with emission at a different wavelength has been proposed. The internal standard is insensitive to oxygen and serves as a reference; the fluorescence intensity of the actual sensing fluorophore is reduced by increasing oxygen concentration. Ideally, both fluorophores are excited at the same wavelength and emit light at two different wavelengths. John *et al*.[82] described the essential characteristics of microtiter plates with integrated online optical sensors of oxygen with respect to the monitoring of biocatalytic activities of enzymes and the whole cells. Oxygen transfer in two conventional fluorescence readers was determined using dynamic and steady-state calculations.

An alternative method is to fix a sensor inside the sample vessel for measurements in microtiter plates or directly in the reactor.[85] Although there are some reports on magnetic silica particles, the concept of combining optical sensor properties with magnetic properties is a new one.[86] Such magnetic sensor particles have good separability and high brightness of luminescence. Such sensors have many advantages: the sensor spot can be read out from the outside and isolated optically, the inhibition or toxicity of the dye towards sample components is minimized, and the sensor can be readily autoclaved. However, the fixing of the sensor spot inside the reactor can be cumbersome or even impossible sometimes. Chojnacki *et al*.[86] measured the oxygen transfer by magnetic sensor particles for the monitoring of oxygenation in bioreactors. Their preparation was based on the well-known silica-sol–gel technology, which yielded a sol mixture with highly versatile composition.

Alternatively, an oxygen indicator dye can be dissolved in the sample directly[87] or incorporated in dispersed micro-or-nano beads.[88,89] Such methods are very simple and readily adaptable. However, they suffer from such drawbacks as interference with the sample, sensitivity to turbidity, potential toxicity, and the necessity for large amounts of dye when measuring in volumes more than a few milliliters.

Optical sensors based on luminescence materials have been used widely for the measurement of oxygen in bioreactors, and this trend is likely to continue, as they do not have the drawbacks of the dye-based measurements.

OXYGEN TRANSFER RATE (OTR)

Earlier, oxygen absorption rate (OAR) used to be measured to see the effect on the growth of the microbial species.[90–92] The OAR was defined as milli-mole (mmol) of oxygen absorbed per liter (dm^3) of solution per minute. Currently, OTR is more widely used to discuss the transfer rate of oxygen and the various resistances that are encountered during the transfer of oxygen from gas to liquid phase in bioreactors.

OTR in an agitated gas–liquid system is obtained by oxygen balance across the gas–liquid interface. It is proportional to the driving force ($C_G/H - C_{O_2,L}$) and is given by the following equation:

$$OTR = k_L a\left(\frac{C_G}{H} - C_{O_2,L}\right) = k_L a(C^*_{O_2,GL} - C_{O_2,L}) = k_L a L_{O_2}(p_{O_2,G} - p_{O_2,L}) \quad (1)$$

where k_L is the individual mass transfer coefficient, which provides a measure of resistance for oxygen transfer across the gas–liquid interface, a is the specific surface area, C_G is the O_2 concentration in the gas phase, $C_{O_2,L}$ is the O_2 concentration in the liquid phase and H is Henry's constant.[93] The term $C_G/H = C^*_{O_2,GL}$ represents the oxygen concentration in the liquid phase at the gas–liquid interface.[94] L_{O_2} is the oxygen solubility and ($p_{O_2,G}$-$p_{O_2,L}$) is the driving pressure difference.[12] The magnitude of the OTR is greatly influenced by the $k_L a$. Equation (1) can be used to obtain the maximum oxygen transfer rate (OTR_{max}) under the conditions of negligible liquid phase resistance. In such a case,

$$OTR_{max} = k_L a C^*_{O_2,GL} = k_L a L_{O_2} p_{O_2,G} \quad (2)$$

Numerous techniques have been proposed in the literature (Table 2) for the determination of $k_L a$ based on measurement of the absorption rate of gas reacting with a dissolved chemical species in a reaction regime. A comparision of $k_L a$ values as obtained in shake flasks and microtitre plates is given in Table 3.

METHODS OF MEASURING OXYGEN TRANSFER RATE

Sulfite oxidation method

The sulfite oxidation 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^{+}.[95] The stoichiometric equation for the oxidation reaction in the presence of copper or cobalt salt is as follows:[96]

$$SO_3^{2-} + 0.5O_2 \xrightarrow{Catalyst} SO_4^{2-} \quad (3)$$

Measurement of the OTR is carried out in the non-accelerated reaction regime when the reaction in the liquid side boundary

layer is slow. This condition is satisfied when[95]

$$\text{Hatta number (Ha)} = \frac{\text{reaction rate}}{\text{mass transfer rate}} = \frac{\sqrt{\frac{2}{n+1}k_n(C^*_{O_2,GL})^{n-1}D_{O_2}}}{k_L} < 0.3 \quad (4)$$

where n is the reaction order for oxygen, k_n is the nth-order reaction constant, and D_{O_2} the diffusion coefficient for oxygen in the solution. At $Ha > 0.3$, the reaction acceleration has to be taken into account.[95,97,98] The application of the method requires knowledge of the kinetics of the homogeneous chemical reaction. Although the reaction of dissolved oxygen with sulfite ions has been studied by many authors,[45,99–101] there is poor agreement for (i) the reaction order in oxygen, (ii) the sulfite concentration at which the reaction becomes zero order in sulfite, and (iii) the region of the linear relation between reaction rate constant and cobalt catalyst concentration.[102]

Reaction parameters have to be adjusted for the reaction regime. Catalyst concentration, pH, temperature, and light irradiation are the main influencing parameters on the k_n.[95,103] Linek and Tvrdik[104] and Reith and Beek[105] proposed kinetic equations accounting for the influences of catalyst concentration, pH, and temperature on the k_n. A freshly prepared sodium sulfite solution, without pH adjustment, has pH >9. During the course of the oxidation, the pH first decreases slowly and then at the end of the reaction, it drops sharply to about 5. Sulfite is a weak acid and it 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 during the reaction as given above. Ruchti *et al*.[106] and Hermann *et al*.[96] have detailed the procedure for calculating k_La by the sulfite oxidation method in different regimes and conditions.

The sulfite oxidation method has been used for the determination of the interfacial area between gas and liquid in various absorption devices like mechanically agitated contactors,[101,102] packed columns,[107] and bubble columns.[108]

Dynamic method

Bandyopadhyay *et al*.[46] described a dynamic method for estimating k_La. This method is based on following the dissolved O_2 concentration during a brief interruption of the aeration (non-gassing) of the fermentation system. A fast-response, sterilizable, dissolved oxygen probe (like a Beckman laboratory model) is required to obtain the necessary data by this method. It is a direct method and is based on utilizing the fermentation system itself.

For a typical aerated fermentation system, the O_2 uptake rate under steady-state conditions is given by

$$O_2 uptake = r.X = OTR = k_La(C^*_{O_2,GL} - C_{O_2,L}) \quad (5)$$

where r is the specific O_2 uptake rate per unit mass of fermenting microorganisms; and X is the biomass concentration in the

Table 2. Measurement of oxygen by various methods as reported in literature

Type of bioreactor	Microorganism	Type of study	Measurement method	Reference
Shake flask	*S. cerevisae*	Culture and oxygen	Sulfite oxidation	Olson and Johnson[149]
Shake suction flask	*B. megaterium*	Growth and oxygen	Clark–type oxygen electrode	Schultz[137]
Shake flask	–	Theoretical aeration	Sulfite oxidation	Ecker and Lockhart[91–92]
Shake Flask	–	OAR	Sulfite oxidation	Auro *et al*.[44]; Corman *et al*.[90]
Shake flask	–	Oxygen	Sulfite oxidation	Auro *et al*.[44]; Rhodes and Gaden[138]
Shake flask	*S. marcescens*	Culture and oxygen	Chemical and biological	Smith *et al*.[139]
Shake flask and small laboratory fermenter	–	–	Reviewed all methods	Finn[140]
Fermenter	–	Mass transfer	Gassing-out and sulfite oxidation	Van't Riet[58]
Shake flasks having internal stainless–steel baffles	*S. marcescens*	Culture and oxygen	Membrane-type electrode	Heinemann *et al*.[141]
Shake flask	*E.coli* and *S. Viridoflavus*	Growth and candidin production	Sulfite oxidation	McDaniel and Bailey[51]
Shake flask (baffled and unbaffled)	*E. coil*	Growth and oxygen	Sulfite oxidation	McDaniel *et al*.[142]
Shake flask (baffled) and fermenter	*E. coil*	Growth and oxygen	Sulfite oxidation	McDaniel *et al*.[143]
Shake flasks	*S. tendae*	Culture and oxygen	Gas analyzer with mass spectrometer	Henzler and Schedel[48]
Shaking vessel type bioreactor	*A. belladonna*	Culture and oxygen	Polarographic dissolved oxygen electrodes	Kanokwaree and Doran[144]
Shaking vessel type bioreactor	–	–	Polarographic oxygen meter	Kato *et al*.[119]
Mechanical stirred and sparger–aerated vessels	*T. variabilis*	Culture and oxygen	Oxygen absorption, desorption, biological and electrode	Montes *et al*.[5]
Bioreactor	–	Culture and oxygen	Gassing out and oxygen electrode	Ozbek and Gayik[6]

Table 2. (*Continued*)

Type of bioreactor	Microorganism	Type of study	Measurement method	Reference
Shake flask	–	–	Sulfite oxidation and RAMOS	Anderlei and Buchs[41]; Maier and Buchs[11]
Shake flask	*Debaryomyces–hansenii*	Culture and xylitol production	Gassing out and oxygen electrode	Roseiro *et al.*[145]
Shake flask and fermenter	*E. coli*	Culture and oxygen	Optical sensor (luminescence)	Gupta and Rao[1]
Shake flask	*C. glutamicum*	Culture and oxygen	Optical sensor spot immobilized	Wittmann *et al.*[54]
Shake flask	*A. vinelandi* and *E. coli*	Culture and alginate production	Sulfite oxidation	Pena *et al.*[146]; Reyes *et al.*[147]
Shake flask	*P. putida*	Culture and oxygen	Sulfite oxidation	Hermann *et al.*[96]
Shake flask	*S. cerevisae and E. coli*	Culture and oxygen	Optical sensor(luminescence)	Tolosa *et al.*[53]
Bioreactor	*P. shermanii, S. cerevisia and P. chrysogenum*	Culture and oxygen	Gassing out and oxygen electrode	Cascaval *et al.*[8]
Bioreactor	–	Oxygen	Gassing out and oxygen electrode	Puthli *et al.*[117]
Shake flask	*C. glutamicum*	Effect on Carbon source (L–lactic acid)	Sulfite oxidation and RAMOS	Seletzky *et al.*[148]
Well plates	*B. subtilis*	Volumetric oxygen transfer coefficient	Optical dissolved oxygen probe	Duetz and Witholt[129]; Doig *et al.*[150]
Shake flask	*X. campestris*	Xanthan gum	Sulfite oxidation	Lotter and Buchs[55]
Shake flask	*S. cerevisiae* and *P. stipitis*	Culture and oxygen	Sulfite oxidation	Anderlei *et al.*[57]; Stockmann *et al.*[151]; Freyer *et al.*[152]
Shake flask	*C. glutamicum*	Effect on d–glucose and L–lactate	Dissolved oxygen probe	Baumchen *et al.*[153]
Shake flask	*A. vinelandii*	Culture and oxygen	Exhaust gas analyzer and respirometer	Pena *et al.*[154]
Shake flask	*P. putida*	Culture, oxygen and mass transfer	Oxygen meter with membrane probe	Nikakhtari and Hill[155]
Shake flask	*C. glutamicum*	Culture and oxygen	Exhaust gas analyzer and respirometer	Seletzky *et al.*[124]
Shake flask	*C. glutamicum, P. stipitis and S. cerevisiae*	Culture and oxygen	Sulfite oxidation	Akgun *et al.*[156]

Table 3. Comparison of k_La values as obtained in shake flasks and microtitre plates

Type of bioreactor	Medium/microorganism	N (rpm)	$k_La(s^{-1})$	Reference
Shaking vessel–type bioreactor	–	100 and 120	13 and 28	Honda *et al.*[157]
Miniature bioreactor	D-glucose and citric acid, and *E. coli*	1500 and 1850	0.0277–0.111	Lamping *et al.*[158]
Shake flask	PMB medium and *C. glutamicum*	200	150	Wittmann *et al.*[54]
Shake flask (baffled) without plug	Glucose and *E. coli*	250	59.2 ± 7.4 and 30.8 ± 6.7	Gupta and Rao[1]
Shake flask (baffled) having sponge plug	Glucose and *E. coli*	250	24 ± 3 and 28.4 ± 6	Gupta and Rao[1]
Shake flask (baffled) having cotton plug	Glucose and *E. coli*	250	30.4 ± 6 and 40.3 ± 5	Gupta and Rao[1]
Shake flask (baffled) having milk filter	Glucose and *E. coli*	250	53.9 ± 8 and 57.6 ± 7	Gupta and Rao[1]
Shake flask and stirred bioreactor	Glycerin and *P. pastoris*	50–500	0.7 and 0.12	Maier *et al.*[12]
Shake flask	–	50–300	0.006–0.026	Zhang *et al.*[159]
Microtitre plates	D-glucose and *E. coli*	500–1500	0.005–0.028 and 0.056–0.10	Zhang *et al.*[160]

fermenter. Since the transfer occurs over an integral volume, a mean driving force has to be used. However, there is considerable debate on the value of $(C^*_{O_2,GL} - C_{O_2,L})_{mean}$ to be used. For the non-gassing situation, the change in dissolved O_2 concentration is given by

$$dC/dt = -r.X \tag{6}$$

Equation (6) is not valid for the immediate period when the air is turned off as it takes some time for the hold-up air bubbles to escape from the fermenter. Also, at high agitation speeds measurable surface aeration occurs. This can be minimized by sparging N_2 over the surface or by lowering the agitation speed during the non-gassing period. However, sparging N_2 over the surface may affect the OTR.

For the aeration period following non-gassing, the change in dissolved O_2 concentration is given by

$$dC/dt = k_La(C^*_{O_2,GL} - C_{O_2,L})_{mean} - r.X \tag{7}$$

It is possible to estimate k_La by following the $C_{O_2,L}$ provided one has determined the proper value of $(C^*_{O_2,GL} - C_{O_2,L})_{mean}$ for use and has obtained $r.X$ from the non-gassing period. It may, however,

be noted that r is a constant only as long as $C_{O_2,L}$ is above the critical biological oxygen concentration and the organisms have not been unduly starved of oxygen.

If one assumes that the oxygen probe is measuring a bulk-average $C_{O_2,L}$ and that $C^*_{O_2,GL}$ can be represented by some mean value, then Equation (7) can be rewritten as follows:

$$C_{O_2,L} = -[1/(k_La)(dC/dt + r.X)] + C^*_{O_2,GL} \quad (8)$$

The value of k_La can be determined from a plot of $C_{O_2,L}$ versus dC/dt. This means that only the measurement of dissolved oxygen concentration at various times is necessary. Since only a single measuring device is used, the results will always be internally consistent, and they do not depend upon a zero or reference measurement.

The dynamic method of Bandyopadhyay *et al.*[46] for estimating k_La has been used by several researchers.[109,110] This method, however, does not give consistent results when compared with the steady-state method of measurement of oxygen uptake rate. The most probable cause of the discrepancy between measurements by the dynamic and steady-state methods is due to the effect of very small gas bubbles in viscous broths.[111] The existence of a fraction of very small bubbles in viscous gas–liquid systems affects the measurement of dissolved oxygen concentration and, therefore, the measurement of k_La by the dynamic method. This requires a correction in the measured value of k_La, depending on the small bubble hold-up.[112] The drawbacks notwithstanding, this method of estimating k_La is recommended more often than not, even though fast response, sterilizable oxygen probes are now available.

Ducros *et al.*[110] determined the k_La values from the transient data of dissolved oxygen obtained during degassing and aeration periods. The probe response was corrected by the first-order lag model.[113] The equation used for k_La calculation is given below:

$$C = Z_1 + \frac{Z_2}{1 - k_La\tau_E}e^{-k_Lat} + Z_3e^{-t/\tau_E} \quad (9)$$

where C is the oxygen concentration shown by the probe (g L^{-1}), $Z_1 = C^*_{O_2} - (Q_{O_2}X/k_La)$ is a parameter, Z_2 and Z_3 the integration constants, $C^*_{O_2}$ the saturation concentration of dissolved oxygen (g L^{-1}), Q_{O_2} the specific oxygen uptake rate (g g^{-1} h^{-1}) and τ_E is the probe time constant (h).

Optical method

In small-scale bioreactors, pH electrodes immersed in the liquid may act as baffles and affect the hydrodynamics. This may, therefore, affect the actual value of OTR/k_La as calculated by oxygen electrodes. Hermann *et al.*[96] used a method that completely avoided the use of *pH* electrode. This method is based on the sulfite oxidation reaction which makes a shift in *pH* when the total amount of sulfite is converted to sulfate. The shift in pH is observed by a pH sensitive dye (bromthymol blue). The pH drop from about 7 (which occurred close to the end of the reaction) to the final value of 3.4 was visualized by a pH indicator. Hermann *et al.*[96] recorded this color change by means of a camera. The time from the start of the experiment to the color shift indicated the time of the oxidation reaction (t_{ox}).[114]

OTR is then determined as:

$$OTR = \frac{C_{Na_2SO_3}\nu_{O_2}}{t_{ox}} \quad (10)$$

where $C_{Na_2SO_3}$ and ν_{O_2} are molar sodium sulfite concentration and the stoichiometric coefficient for oxygen, respectively. k_La can then be calculated from the relation:

$$k_La = \frac{OTR}{L_{o_2}p_{O_2,G}} = \frac{C_{Na_2SO_3}\nu_{O_2}}{t_{ox}L_{o_2}p_{O_2,G}} \quad (11)$$

Before and during the preparation of the sodium sulfite solution, the deionized water is gassed thoroughly with nitrogen gas to avoid any prior oxidation of the sulfite. The evaluation of the interfacial area from absorption rates measured by the optical sulfite method holds under moderate reaction rate (zero order in sulfite) regime only.

Gassing-out method

The dynamic gassing out method was first described by Suijdam *et al.*[94] The culture vessel is first filled to working volume with a reference medium. The medium is then purged of oxygen by bubbling nitrogen through it until the medium is oxygen-free.[1] The nitrogen gas is sparged in water kept at 50 °C before entering the shake flask in order to avoid temperature changes in the liquid phase.[115,116] In this way a constant temperature during the experimental gassing out run is ensured.

The oxygen dissolution rate is determined with the help of an oxygen probe. The dynamic time constant of the oxygen probe must be less than 1 s so as to ensure that there is no interference of the time lag in the measurements of dissolved oxygen concentration.[117] k_La is determined by direct measurement of the rate of increase in dissolved oxygen concentration, after it was lowered by passing dry nitrogen gas (oxygen free), through the system.

For shake flasks, the dynamic response of the process can be described by the following equation:

$$k_La = \frac{-\ln\left(\dfrac{C^*_{O_2,GL} - C_{O_2,L}}{C^*_{O_2,GL} - (C_{O_2,L})_o}\right)}{t} \quad (12)$$

where $(C_{O_2,L})_o$ is the oxygen concentration in the liquid phase initially (time (t) = 0). This method has the advantage that it can be applied to different media (for establishing the effect of media composition on oxygen mass transfer) and that it does not involve chemical reactions that could affect the measurement precision and the liquid film resistance.[5,118] The limitation of the gassing-out method lies in the fact that it involves a non-respiring system which is not the case in the actual fermentation conditions.[5,6]

k_La can be measured by the method proposed by Kato *et al.*[119] Initially, dissolved oxygen is eliminated by adding sodium sulfite with small amounts of cobalt sulfate as a catalyst, which does not affect the physical properties of the liquid. The dissolved oxygen in the liquid is purged with nitrogen gas, so that $(C_{O_2,L})_o$ becomes zero. Therefore, the oxygen concentration changes due to the oxygen transfer from the liquid surface are measured with a polarographic digital dissolved oxygen meter. The k_La is then obtained from Equation (12). The change in oxygen concentration in the liquid with time is so slow that the response delays of the cell can be neglected.

The gassing-out method of k_La measurement is associated with a limitation. This method necessitates the use of membrane-type electrodes, the response time of which may be inadequate to reflect the true change in the rate of oxygenation over a short period of time.[120] The probe response time, which is the time needed to record 63% of a stepwise change, should be much smaller than the mass transfer response time of the system ($1/k_La$).[121] Various researchers have used the static gassing out method for determining k_La values.[6,7]

RAMOS (intermittent on-line) method

A major disadvantage of shake flasks as an experimental bioreactor is the lack of online monitoring and control.[122,123] A new OTR measuring system (termed respiration activity monitoring system (RAMOS)) combined online oxygen and carbon dioxide measurement in shaking bioreactors.[41] The OTR is measured by periodically repeating an automated measuring cycle composed of a measuring phase (flask closed air-tight, 10 min) and a rinsing phase (continuous air flow, 20 min). During the rinsing phase, air is flushed through the measuring flask at a specific flow rate, thus, recreating the average oxygen supply of a standard flask with cotton plug.[57]

At the beginning of the measuring phase, inlet and outlet valves of the measuring flask are closed. Respiration of the microorganisms leads to a decrease in the partial pressure of oxygen, and to an increase in the carbon dioxide partial pressure in the headspace of the measuring flask. The partial pressures are monitored by an electro-chemical oxygen sensor and a differential pressure sensor. Assuming linear changes in the measuring phase, OTR is calculated from the decrease of the partial oxygen pressure (Δp_{o_2}) in the headspace of the shake flask having gas volume (V_G) by using the following equation:

$$OTR = \frac{\Delta p_{o_2}}{\Delta t}\frac{V_G}{RTV_L} \tag{13}$$

where V_L is the liquid volume, R the gas constant, T the temperature and Δt the time interval of measurement.

The carbon dioxide transfer rate (CTR), and thus, the respiratory quotient (RQ) are calculated from the following equations:

$$CTR = \frac{\Delta p_{co_2}}{\Delta t}\frac{V_G}{RTV_L} \tag{14}$$

$$RQ = \frac{CTR}{OTR} \tag{15}$$

After the measuring phase, the valves are opened again, and the next measuring cycle starts. To allow the monitoring of weakly respiring cell cultures, the accuracy and the precision of the measurement is increased by recalibrating the oxygen sensors before each measuring phase using the steady state gas composition at the end of the rinsing phase. This also compensates for the signal drift.[41,57,124]

The gas concentration in the headspace of a normal flask equipped with a sterile barrier, e.g. a cotton plug, is calculated using the method of Mrotzek *et al.*[125] The air flow rate through the measuring flask during the rinsing phase is adjusted such that the gas concentration in the headspace of the measuring flask is equivalent to that in the normal shake flask. The establishment of equivalent fermentation conditions in the measuring flask and the normal shake flask with reference to the hydrodynamics and the gas concentration in the headspace is required for comparison of the systems. Thus, results determined with RAMOS can be transferred to the normal shake flasks running in parallel with the measuring flasks. Anderlei *et al.*[57] used RAMOS to measure OTR in the growth of two types of yeast, a bacterial culture and an animal cell culture.

The RAMOS method can be used simultaneously for measuring OTR in 6–12 parallel fermentation vessels, with a measuring interval of 10–30 min. Amount of sample required for measuring OTR is in the range 5–100 mL.

Exhaust gas analyzer (continuous on-line) method

In this method, the OTR is calculated by specifying the oxygen concentration difference between the inlet gas stream ($O_{2,in}$) and the outlet gas stream ($O_{2,out}$). The OTR is measured by a magneto-mechanical exhaust gas analyzer (EGA). To avoid the influence of variations of the aeration rate on the EGA, the outlet gas stream is first dried by a cooler, and then the volume flow through the EGA is kept constant with a thermal mass flow controller. EGA is calibrated prior to each experiment with nitrogen and a test gas (e.g. 25% O_2, 5% CO_2 and 70% N_2).[124] In a fermentor, OTR by this method is calculated by the following equation:

$$OTR = \frac{q}{V_{mo}}[(O_{2,in}) - (O_{2,out})] \tag{16}$$

where q is the specific aeration rate and V_{mo} is the molar gas volume under standard conditions. This continuous method can be used to measure OTR in one to five parallel fermentation vessels, however, the amount of sample required for OTR measurement is high (~250 mL). This limits the use of this method to high volume bioreactors.

Respirometer (off-line) method

This method depends on the measurement of decreasing dissolved oxygen concentration with time after the culture vessel has been aerated and then the aeration was stopped. The OTR can be calculated, using L_{O_2} from the following equation:

$$OTR = \frac{DO_2}{\Delta t}L_{O_2} \tag{17}$$

where DO_2 is the dissolved oxygen concentration in the liquid phase. A respirometer with an electro-chemical oxygen electrode can also be used to measure the OTR of a complex medium sample drawn from standard shake flasks with cotton plug, and also of medium samples drawn from a fermenter.[124] Respirometer can be used for bioreactors of any shape, however, its use is limited because of its requirement for manual handling.[124]

ESTIMATION OF K_LA IN MINIATURE BIOREACTORS

Many methods are available for determining k_La in small-scale bioreactors. These include conventional methods (e.g. cobalt-catalyzed oxidation of sulfite and the dynamic gassing out methods), and fast enzymatic methods such as bio-oxidation of glucose oxidase or catechol (C230).

Conventional methods

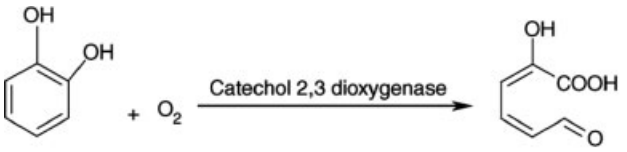
The dynamic gassing-out method is often preferable to the sulphite oxidation method for the determination of k_La values

in miniature bioreactors.[126] However, the dynamic gassing out method is difficult to use in mini-titreplates (MTPs) as shaking has to be stopped long before dissolved oxygen tension (DOT) measurement in order to get accurate readings. This alters the mass transfer environment at a critical moment.

The cobalt-catalyzed oxidation of sulphite method is suitable for mini-volumes of less than 1 mL.[96] However, the high salt concentration (usually 0.5 mol L^{-1}), reduces the maximum solubility of oxygen to a level well below that in low-salt concentration (0.1–0.2 mol L^{-1}) culture media. As the method consistently underestimates the maximal OTR, a correction factor of (approximately 30%) is necessary when translating the results to microbial cultures. A second drawback of this method is the high surface tension in comparison with microbial cultures, leading to an underestimation of potentially achievable OTRs in small-scale cultures that have a well diameter of less than 8 mm.[127]

C230 method

Ortiz-Ochoa *et al*.[128] estimated OTR based on the bio-oxidation of catechol forming 2-hydroxymuconic semialdehyde (HS), catalyzed by the enzyme catechol-2, 3-dioxygenase (XylE):



1D

A XhoI restriction fragment, containing the XylE gene (which forms part of the toluene oxidation pathway expressed from the Tol plasmid in *P. putida*), was inserted into the multicopy plasmid pUC8 to yield pQR109. The gene expression was controlled by the lac promoter in *E. coli JM107*. This enzyme uses 1 mol of molecular oxygen to convert 1 mol of catechol into 1 mol of HS. HS is a bright yellow compound that can be quantified at 425 nm. If the enzyme concentration is in excess and the bio-oxidation rate is zero order, then the rate at which the product is formed, *d(HS)/dt*, will equal the oxygen transfer rate (OTR). Since

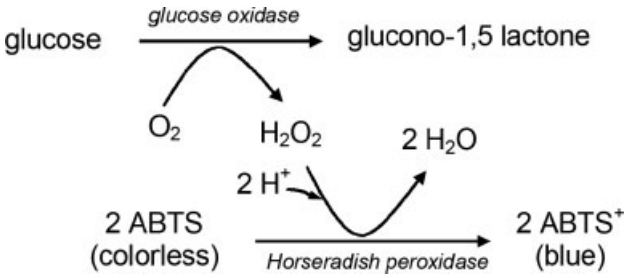
$$\mathrm{OTR} = k_L a(C^*_{O_2,GL} - C_{O_2,L}), \text{ and } C_{O_2,L} = 0, \qquad (18)$$

$k_L a$ is then determined by measuring the linear rate of formation of the brightly colored HS. Here t is time, $C^*_{O_2,GL}$ is the saturation concentration of oxygen, and C is the actual concentration of oxygen in the liquid. It is a rapid method and makes no assumptions about the kinetics, and is well suited to $k_L a$ evaluation in MTPs and other small-scale devices. The C23O method yields similar $k_L a$ values to those from the dynamic gassing-out method.

Glucose oxidase method

Duetz and Witholt[129] estimated OTR using an enzymatic method involving glucose oxidase, horseradish peroxidase (HRP), and 2,2-azino-bis 3-ethybenz-thiazoline-6-sulfonic acid (ABTS). In the glucose oxidase method, a 120 mL bottle is filled with 100 mL of an aqueous solution (pH 7.0) containing 100 mmol L^{-1} K_2HPO_4/KH_2PO_4 buffer, 1.5 U mL^{-1} (measured at 25 °C) glucose oxidase, 5 U mL^{-1} HRP, and 1 mmol L^{-1} ABTS. The bottle is closed and is flushed with nitrogen (100 mL min^{-1}) for 30 min. Subsequently, 1 mL of a nitrogen-flushed glucose solution (1 mol L^{-1}) is added. The (empty) vessels to be tested are mounted on an orbital shaker, shaken at the desired frequency. Subsequently, appropriate amounts of the anoxic mixture (pre-warmed at 25 °C) is added rapidly (within 1 s) to the (shaking) vessel. After a defined time period (between 15 and 60 s), the reaction is stopped by the rapid addition of an equal volume of HCl (1.0 mol L^{-1}) to the (still shaking) vessel. The time period, after which the reaction is stopped is chosen so as to result in an absorbance between 1 and 3 at 725 nm (A_{725}). The A_{725} is measured using a spectrophotometer.

A scheme of the coupled reaction in the glucose oxidase method is shown below:



2D

It is assumed that 1 mmol L^{-1} of oxygen consumed correlates with an increase of 19.1 in A_{725} (value before correction for the dilution due to the addition of HCl). This value been determined by supplying the ABTS mixture described above with a limited concentration of glucose (100 µmol L^{-1}), and the subsequent measurement of A_{725} (after all glucose was consumed, and after acidification).[129]

Both glucose oxidase and C23O methods are suitable for cultures in the 10–200 mL range. The C23O method is more elegant because the colored compound (a semi-aldehyde) is formed in a single-step reaction in a 1:1 proportion to the amount of oxygen consumed. The glucose-oxidase method shows a hazy stoichiometry, and therefore, requires calibration with another method (such as the cobalt-catalyzed oxidation of sulphite) but has the advantage that all reagents being used are commercially available.

CONCLUSIONS

This paper provides a review of various types of oxygen sensing elements and techniques that have been employed for the measurement of oxygen transfer in bioreactors. The majority of electrochemical systems are based on a Clark-type oxygen sensor which has been widely used for oxygen measurement. Conventional galvanic oxygen sensor technology uses two dissimilar metal electrodes, typically silver and lead, which are consumed in the process of measuring oxygen. The paramagnetic oxygen sensor is an accurate measurement technique for oxygen concentration. Non-invasive optical sensors are better suited to measure oxygen concentration in small bioreactors, as they circumvent a variety of problems associated with conventional probes. In recent years, optical sensors based on luminescence materials have been used widely for the measurement of oxygen in bioreactors, and this trend is likely to continue.

Sulfite oxidation and gassing out methods have been widely used for the determination of $k_L a$ values, although this method

has come under severe criticism by several researchers because the reaction rate constant varies in a peculiar way. The main disadvantage of using the gassing out method for the calculation of k_La is the slow response time of the electrode to changes of oxygen concentration in the liquid. Chemical methods are now generally not used for the determination of k_La in bioreactors because of the enhancement of k_La values obtained due to chemical reaction. These methods are beset with many uncertainties and may even physically alter the system's physicochemical properties. Recently, novel techniques such as respirometry and solubility methods have been developed. The new measuring systems like the respiration activity monitoring system (RAMOS) and the exhaust gas analyzer (continuous on-line) method combine online oxygen and carbon dioxide measurements in bioreactors. A survey of the oxygen measurement in shaken and other type of minibioreactors is also provided. The future trend includes the integration of optical oxygen sensing technology with integrated online measurement of oxygen parameters.

NOTATION

a	Specific mass transfer area
C_G	Oxygen concentration in the gas phase
$C_{Na_2SO_3}$	Molar sodium sulfite concentration
$C^*_{O_2,GL}$	Oxygen concentration at the gas–liquid interface
$C_{O_2,L}$	Oxygen concentration in the liquid phase
$(C_{O_2,L})_o$	Oxygen concentration in the liquid phase at time (t) = 0
H	Henry constant
Ha	Hatta number
k_L	Mass transfer coefficient
k_La	Overall gas–liquid mass transfer coefficient
k_n	nth-order reaction constant
L_{O_2}	Oxygen solubility
n	Reaction order for oxygen in sulfite oxidation method
$p_{O_2,G}$	Oxygen partial pressure in gas phase
$p_{O_2,L}$	Oxygen partial pressure in liquid phase
Δp_{o_2}	Difference in oxygen partial pressure in the head space of shake flask
Δp_{co_2}	Difference in carbon dioxide partial pressure in the head space of shake flask
R	Gas constant
t	Time
t_{ox}	Time of the oxidation reaction
T	Temperature
ν_{O_2}	Stoichiometric coefficient for oxygen
V_G	Gas volume of head space of shake flask
V_L	Liquid filling volume

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