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Keywords:	activity assay, laccase, ascorbate oxidase, oxygen consumption rate, O2 optode sensor

Point by point response to reviewers

Reviewer 1:

There are no modifications asked by Reviewer 1.

Reviewer 2:

1. We have reduced the length of introduction (see text in red) as much as possible without decreasing its quality and comprehensiveness.
2. Fig. 5 presents oxygen concentration (DO [μM]) versus time, i.e. monitoring DO after the repetitive addition of substrate. Reaction volume indeed changed due to addition of substrate; but activity calculations were amended accordingly. Fig. 5 is therefore volume-independent.
3. The authors thank the reviewer for his/her valid suggestion, though they preferred to keep Fig. 7 and related result descriptions in the section 3.2.4 as all this material refers to the impact of k_La on activity (OCR) determination.
4. Disadvantages/limitations of proposed method are now added to concluding remarks (see text in red).

Research article

Dynamic measurement of oxidase activity based on oxygen consumption in open systems

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Keywords: activity assay, ascorbate oxidase, laccase, O_2 optode sensor, oxygen consumption rate

Abbreviations: AAO, L-ascorbate oxidase; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); BPA, bisphenol A; CP, *Coriolopsis polyzona* laccase; DMP, 2,6-dimethoxyphenol; DO, dissolved oxygen, EDTA: ethylenediaminetetraacetic acid; HPLC, high performance liquid chromatography; LNB, laccase-nanoparticle conjugates; M-buffer, citrate-phosphate buffer; OCR, oxygen consumption rate; TV, *Trametes versicolor* laccase

Abstract

Oxidases catalyze the oxidation of a variety of substrates with the concomitant reduction of molecular oxygen as a final electron acceptor. UV-visible spectrophotometry is a simple and high-throughput method commonly used to measure oxidase activities. However, drawbacks such as light scattering exist especially concerning the activity assessment of enzymes immobilized on supports. Monitoring of the universal cosubstrate O_2 circumvents these drawbacks. This study aimed at developing a methodology which allows activity measurement of many types of oxidases based on O_2 consumption applicable to various open systems. Dissolved oxygen in the reaction medium was monitored by an O_2 sensor and the reaction rate was deduced from the O_2 mass balance equation correcting for atmospheric diffusion. Common activity units ($\mu M_{product}$ min^{-1} or U L^{-1}) could be subsequently derived using calibration curves. The sensitivity of the method towards temperature, atmospheric pressure and ionic strength variations was evaluated and makes it possible to define operating windows for the simplification of the proposed methodology.

Nomenclature

Symbol	Unit	Description
OCR	μM_{O2} min^{-1}	Maximum oxygen consumption rate (= oxidase activity)
OCR_t	μM_{O2} min^{-1}	Oxygen consumption rate at any time *t*
ε	mM^{-1} cm^{-1}	Molar extinction coefficient
$C^*_{O_2}$	µM	Dissolved O_2 (DO) at saturation
$C_{O_2,t}$	µM	Dissolved O_2 (DO) at any time t
$C^m_{O_2,t}$	µM	Diffusion-free modeled DO
$\Delta c^{atm}_{O_2,t}$	µM	DO intake due to atmospheric diffusion
τ_x	s	Response time constant: time required for the probe to reach *x* % of the signal after a step change in DO concentration
k_La	s^{-1} or min^{-1}	Volumetric mass transfer coefficient
Km_{O2}	µM	Laccase Michaelis-Menten constant for O_2
Φ	%	Percent of error on the estimation of OCR
P_N	mbar	Normal atmospheric pressure (1013 mbar)
P_{atm}	mbar	Atmospheric pressure
V_M	L mol^{-1}	Molar volume of oxygen
P_w	mbar	Vapor pressure of water
α	dimensionless	Bunsen solubility coefficient
t_s	s or min	Sampling time *i.e.* time interval between DO measurements
T	Kelvin	Temperature
$[Cl^-]$	‰	Chloride concentration

1. Introduction

Oxidoreductases are very advantageous enzymes that catalyze the electron transfer from one substrate (e^- donor) to another (e^- acceptor), their recommended name being "donor:acceptor oxidoreductases". When molecular oxygen is the final electron acceptor, they are called "donor oxidases". For decades, extensive research has been conducted on laccase (EC1.10.3.2, benzenediol:oxygen oxidoreductase or phenoloxidase) which has a great potential in industrial and environmental biotechnology [1, 2]. Laccases are Cu-containing enzymes that catalyze one-electron oxidations by transferring four electrons from four substrate molecules to one molecule of molecular oxygen which is reduced to water (Figure 1). Ascorbate oxidase (*AAO*, EC1.10.3.3, L-ascorbate:oxygen oxidoreductase) is an oxidase that also belongs to the group of Cu-containing oxidases like laccases [3].

The most common method to determine enzyme activity is monitoring the consumption of a UV-Vis absorbing substrate or the formation of UV-Vis absorbing product by spectrophotometry ($\mu M_{substrate\ or\ product}$ min^{-1} or U L^{-1}). These methods are simple, sensitive and easy to implement. However, they suffer from various limitations like a narrow range of substrate concentrations that can be used not to exceed the detection limit of the spectrophotometer, the need for UV-Vis absorbing enzyme substrates or products, the assumption that only the monitored compound contributes to the absorbance at the given wavelength in the reaction medium and the possible occurrence of side reactions with the monitored compounds. Additionally, when this methodology is used to measure the activity of immobilized enzymes, light scattering, substrate/product sorption to supports, extensive activity dilution to prevent absorbance overflow, and an increased number of handling steps (e.g. centrifugation between absorbance readings in a kinetic study) constitute severe limitations of photometric assays.

In the case of O_2-consuming enzymes or oxidases, there are alternative methods to determine the enzyme activity that are able to circumvent the limitations of the spectrophotometric tests. Voltammetric methods measure the electron transfer (current during linear voltage sweep) between the enzyme-catalyzed reduction of the substrate and an electrode placed in close vicinity with the enzyme

molecule. This method has been reported for the determination of laccase activity and kinetic parameters [4, 5]. However, it requires enzyme immobilization on electrodes which makes it unsuitable for sensitive oxidases or carrier-bound enzymes and renders it laborious for routine assays. An alternative method is the measurement of the electron acceptor concentration i.e. the oxygen consumption rate (OCR [μM_{O2} min^{-1}]). The method typically relies on amperometric O_2 sensors that are also used in the determination of cell respiration (respirometry tests) and in microbial fermentations. A singular case is the so-called "Clark-type electrode" – an amperometric dissolved oxygen (DO) sensor – which is frequently applied to determine oxidase activity based on OCR in a hermetically closed system [5]. The experimental setup avoids having any air-containing headspace, so that only the oxygen dissolved in the medium is used in the biocatalytic process and, in absence of significant external influx of oxygen, very accurate measurements of biocatalytic OCRs are possible.

OCR determination methods in closed systems have distinct drawbacks that limit their applicability in oxidase activity assays. Often a small influx of oxygen is present and limits the resolution of OCR measurements [6]. Eliminating the diffusion of external oxygen into the reaction medium is not trivial [7]. Most plastics such as polyethylene, polypropylene, polystyrene, etc. used in research are permeable to oxygen to varying extents [8]. Oxygen diffusion in the reaction medium through plastic walls is well documented [9] and software of respirometry equipment usually integrate algorithms which automatically correct OCR measurements from external oxygen diffusion. However, these algorithms are often not accessible and therefore can constitute a black box for biotechnologists. For in-house setups, glass materials are more suited for OCR analysis. Nonetheless, it is very difficult to design an airtight system to enclose the reaction medium without a headspace remaining or creating an overpressure during the closing of the vessel. Such overpressure or a trapped bubble can significantly influence OCR determination and leads to variability between experiments. Moreover, the obligatory use of airtight special oxygen reactors limits the flexibility with respect to the vessel size and the agitation system. Microplate respiratory devices, originally designed for cellular flux analysis, are

valuable tools for high throughput measurements of oxidase activity and are especially suited for the comparison of pH/substrate profiles of many oxidases in parallel [10]. However, the miniaturization (only a few µL of reaction medium are required) is unsuitable for immobilized oxidases, interferences with colored substrates (e.g. syringaldazine) are reported and, finally, devices are still expensive and therefore not widely available.

Another limitation of OCR methods is the presence of endogenous consumption of oxygen within the reaction medium independent of the biocatalyst activity that limits sensitivity and reproducibility. In general this phenomenon occurs due to the oxidation of biological materials within the medium, other enzymatic activities that involve oxygen, oxygen radical formation and subsequent deactivation by antioxidants within the medium, peroxidation of unsaturated fatty acids in membranes of cells or organelles or fractions thereof, oxidation of thiol groups within proteins and other types of oxidation [11]. The sensitivity of the oxygen consumption method is thus dependent upon the background of endogenous oxygen consumption within the medium. All experimentations must therefore record this background consumption (baseline drift) in order to compensate for the phenomenon.

The present work aimed at the development of a straightforward OCR-based methodology for the determination of oxidase activity in *open* systems with a standardized correction for external oxygen diffusion. Estimation of volumetric oxygen mass transfer coefficient (k_La) and OCR in open reaction systems is well established and various methods are described in engineering textbooks [12]. For instance, the dynamic gassing-out method for the measurement of oxygen mass transfer in a stirred vessel is based on the monitoring of the DO concentration of a deoxygenated liquid, either due to microbial respiration in fermentation or due to flushing nitrogen in the liquid, after the start of the air inflow. Thus, the k_La is deduced from the linear regression on the O_2 mass balance equation appropriately plotted [13, 14]. In this work, the gas-out/gas-in method applied to measure the volumetric microbial oxygen uptake rate and k_La in bioreactor systems [15] was modified for the convenient measurement of oxidase activities. Besides the use of an amperometric sensor, a non-

invasive optode O_2 sensor was applied to allow for a low-volume, flexible and possibly high-throughput experimental setup. The principle of optode-based O_2 measurement exploits the dynamic luminescence quenching where the collision between the excited luminophore and molecular oxygen (the quencher) results in a radiationless de-excitation of the luminophore. The relationship between O_2 concentration, luminescence intensity and luminescence lifetime is given by the Stern-Volmer equation [16].

The method presented proposes a fast, convenient and easy-to-use experimental setup which overcomes the aforementioned limitations of conventional methods of measuring oxidase activity and opportunely complements the toolkit available to enzyme technologists regarding immobilized enzyme activity determination. The influx of external oxygen into the medium was determined experimentally and subsequently deconvoluted from the observed OCR. Commercial *Trametes versicolor* laccase, laccase-containing *Coriolopsis polyzona* culture supernatant, *C. polyzona* laccase immobilized on silica support and *AAO* were assayed as a proof-of-concept of the new methodology as well as to assess the impact of enzyme sources (e.g. endogenous consumption of oxygen). The OCRs calculated through the dynamic method for these biocatalysts were compared to OCRs obtained using airtight Clark-type respirometer cells. Calibration curves covering a wide range of *C. polyzona* laccase activity and *Cucurbita* sp. *AAO* were determined in order to correlate activities given by colorimetric assays and OCRs obtained through the proposed dynamic method. Moreover, the effect of environmental factors on the O_2 transfer and on the determination of the oxidase activity has been studied. The aim was to identify an operating window in which O_2 transfer has a low impact on activity determination and can be neglected, thus making the enzymatic activity analysis even easier and faster.

2. Materials and methods

2.1 Enzymes and chemicals

All chemicals, if not specified otherwise, were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were of analytical grade. Laccase from *Trametes versicolor* (*TV*) and L-ascorbate oxidase from *Cucurbita* sp. (*AAO*) were commercial preparations obtained from Sigma-Aldrich. A laccase preparation from *Coriolopsis polyzona* MUCL38443 (*CP*), 0.22 µm filtered culture supernatant, was kindly provided by Wetlands Engineering SPRL (Louvain-la-Neuve, Belgium). This *CP* preparation was 10-kDa filtered and concentrated before use. *CP* laccase-nanobiocatalyst (*LNB*) was prepared as follows: 1% w/v fumed nanosilica particles (aggregates of 7 nm-size particles, Sigma-Aldrich) were suspended in acetone containing 4% v/v 3-aminopropyltriethoxysilane and incubated for 6 hours at 45°C under agitation. The organic phase was discarded (10,000 x g for 10 min), the pellet washed with two equivalents of 0.25 M phosphate buffer pH 7.0 and dried overnight at 80°C. Silanized silica particles (1.5% w/v) were mixed for 4 h at 4°C with 10-kDa filtered *CP* laccase culture supernatant (~80 U_{ABTS} mL^{-1}), the supernatant was discarded and the pellets were gently mixed with glutaraldehyde 0.8 M in 0.25 M phosphate buffer pH 7 for another 4 h at 4°C. The pellets were then washed with 0.25 M phosphate buffer pH 7.0 until no laccase activity was detected in the supernatant. The laccase-nanobiocatalyst aqueous suspension was stored at 4°C before use.

2.2 Spectrophotometric assays

Laccase activities (*TV*, *CP*) were measured photometrically (Powerwave XS, BioTek Instruments Inc., Winooski, VT, USA) by monitoring the oxidation of 1 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) at 420 nm (ε_{420} = 36 mM^{-1} cm^{-1} [17]) in citric acid (Acros Organics, Geel, Belgium)/sodium hydrogen phosphate buffer (M-buffer) pH 3.0, prepared according to McIlvaine (1921) [18], at 25°C. Laccase activity was also assayed by monitoring the oxidative dimerization of 1 mM 2,6-dimethoxyphenol (DMP) at 469 nm (ε_{469} = 27.5 mM^{-1} cm^{-1} [19]) in M-

buffer pH 5.0 at 25°C. Total reaction volume was 200 µL and constituted of 160 µL of buffer, 20 µL of laccase and 20 µL of ABTS or DMP added in this order. All assays were performed in quadruplicate.

For *LNB*, the activity measurements were performed in a stirred beaker (58.97 mL M-buffer pH 3.0, 1 mL ABTS 60 mM and 0.03 mL of sample) and a centrifugation step (10,000 x g for 3 s) was introduced between absorbance measurements of 1 mL-samples taken every minute (Beckman DU-640, Beckman-Coulter, Brea, CA, USA) to avoid light interference due to particles. Activity assays in beakers were performed in triplicate and data processed with a spreadsheet software.

AAO activity was measured spectrophotometrically with a microtiter plate reader (Powerwave XS) according to Oberbacher and Vines (1963) with minor modifications [20]. The oxidation of 0.25 mM L-ascorbic acid was continuously monitored at 265 nm (ε_{265} = 13.4 mM^{-1} cm^{-1}) for 5 min at 25°C in 0.1 M phosphate/EDTA buffer at pH 5.6. Total reaction volume was 200 µL and constituted of 160 µL of buffer, 20 µL of *AAO* dissolved in ice-cold distilled water and 20 µL of substrate (or buffer in case of blank measurements), added in that order. All assays were performed in quadruplicate.

When assays were performed with the microtiter plate reader, Gen 5 1.08 data analysis software (BioTek Instruments) was used to calculate the highest slope of absorbance curves on three consecutive points (one data point every 45 s for 315 s for laccase and one data point every 12 s for 300 s for *AAO*).

One unit of laccase activity (U) was defined as the amount of enzyme necessary to produce 1 micromole of colored product per min under the assay conditions. One unit of *AAO* activity was defined as the amount of enzyme necessary to oxidize 1 micromole of L-ascorbate per min under the assay conditions.

2.3 Clark cell assay – closed system

OCRs were determined in a hermetically closed system using a Clark electrode (Oxytherm, Hansatech Instruments Ltd., King's Lynn, UK). The response time ($\tau_{63.2}$) was typically below 5 seconds according to the manufacturer. 2,300 µL of M-buffer pH 3.0 at 25°C and 50 µL of biocatalyst (*TV*, *CP* or LNB) were added in the reaction chamber and stirred at 100 rpm. Oxygen concentration was monitored until equilibrium was reached ($C^*_{O_2}$), then 50 µL of 48 mM ABTS was added and the signal recorded for 10 min. Data were fitted (linear regression on one-min interval) using the Oxygraph plus software provided by the manufacturer. All measurements were done in triplicates.

2.4 Amperometric O_2 sensor – open system

Laccase OCRs in an open system were determined using an OXI 340® oximeter equipped with a CellOx-325® probe (WTW GmbH, Weilheim, Germany). The CellOx-325® probe includes a temperature sensor which is used to automatically compensate temperature change during assays. The response time (τ_{90}), defined by the manufacturer as 90% of the signal reached within this time period) is typically < 10 seconds at 20°C ($\tau_{63.2}$ < 4.9 s assuming a first-order system). 117.5 mL of M-buffer pH 3.0 (or pH 5.0) and 0.5 mL of samples (*CP*, *TV* or *LNB*) were stirred (100 rpm) and DO and temperature were monitored (data points every 15 s) until equilibrium was reached ($C^*_{O_2}$), then 2 mL of ABTS 60 mM (or BPA 60 mM dissolved in methanol) was added and the DO recorded until 15 min of dissolved oxygen increase was observed. This aeration phase was used to calculate the volumetric mass-transfer coefficient (k_La) as described in section 3.1. After assays, DO and temperature data were further processed with Microsoft Excel 2010® (refer to section 3.1). All measurements were carried out in triplicates.

2.5 Optode O_2 sensor – open system

An OXY-4 mini transmitter with optical fiber sensors and oxygen-sensitive spots (PreSens – Precision Sensing GmbH, Regensburg, Germany) was used for the measurement of OCRs in different configurations. The spots containing the fluorescent dye (silicone-soluble ruthenium diimine complex [21]) were glued inside two different types of reaction vessels, i.e. 20 mL-beakers or standard 1.5-mL HPLC vials. τ_{90} is 30 s in liquid phase ($\tau_{63.2}$ = 10.9 s assuming a first-order system). The temperature was measured by a digital temperature sensor (VWR International, Leuven, Belgium) and the values implemented in the manufacturer software to compensate for deviations from the temperature of calibration. The vessels were placed on a magnetic stirrer to ensure a continuous mixing (Figure 2). For laccase assay, the 15 mL-working volume in the beaker consisted of 13.75 mL of M-buffer at pH 3.0 or 5.0 depending on the substrate used, 0.75 mL of 20 mM substrate solution (ABTS or DMP) and 0.5 mL of enzyme sample. In HPLC vials, the total volume was one mL with the same proportion of reagents (buffer, substrate and sample) as described for the beaker assay. For *AAO*, 800 µL of phosphate/EDTA buffer was first added followed by 100 µL of *AAO* and 100 µL of L-ascorbate in that order.

In beaker or in vial configuration, the assay was allowed to equilibrate at room temperature (temperature was monitored throughout the measurement) until the DO concentration stabilized. Reaction was started by addition of the substrate and the variation of the DO concentration (a decrease followed by a subsequent increase) was recorded until DO concentration increased for 15 min (aeration phase (C) in Figure 3, described in section 3.1 below). In controls containing no enzyme or inactivated enzyme, no decrease in DO was observed.

3. Results and discussion

3.1 Deconvolution of OCR from DO variation

During oxidase activity assays, the O_2 concentration in the reaction medium is affected by two antagonistic processes: the O_2 consumption by the enzymatic reaction and the O_2 diffusion from the atmosphere (Figure 3). The correction model aims at the deconvolution of the O_2 consumption by the oxidase-catalyzed reaction from the observed DO variation ($\frac{\partial C_{O_2}}{\partial t}$). In a glass container, the oxygen transfer from the atmosphere to the liquid occurs only at the air-liquid interface and the main resistance to O_2 transfer from the atmosphere to the bulk reaction medium arises from the thin liquid phase boundary according to the two-film model for mass-transfer across gas-liquid interfaces [22]. The atmospheric diffusion from the air to the liquid can be modeled as a function of the volumetric mass transfer coefficient ($k_L a$) and O_2 gradient (∇C_{O_2} = driving force) (Eq.1):

$$\frac{\partial C_{O_2}}{\partial t} = k_L a \cdot \left(C^*_{O_2} - C_{O_2,t}\right) - OCR_t \qquad (1)$$

where $C_{O_2,t}$ is the DO concentration (µM) at time t, $C^*_{O_2}$ the DO concentration at equilibrium with air, k_L the oxygen-mass transfer coefficient for the liquid boundary layer (min^{-1} m^{-2}), a the interfacial area (m^2) and OCR_t the OCR due to the oxidase activity (µM min^{-1}) at time t in absence of endogenous O_2 consumption.

Before the addition of the enzyme, the oxygen in the reaction medium equilibrates with atmospheric oxygen and $C^*_{O_2}$ can be determined. If stirring is required, for instance to prevent the settling of immobilized biocatalyst particles, the stirring speed needs to be kept constant during experiments.

Right after addition of the substrate, the enzyme starts to convert it into product(s) with the concomitant consumption of O_2 and a decrease of DO is monitored (Figure 3). Once the substrate is

exhausted, the residual variation of DO in the medium is only due to atmospheric diffusion (aeration phase) leading to an increase in DO until it reaches $C^*_{O_2}$. At that time, OCR_t is negligible and k_La can be estimated by integration of Eq. 2 for $OCR_t = 0$ and by introduction of the experimental $C^*_{O_2}$ which yields Eq. 3.

$$\frac{\partial C_{O_2}}{\partial t} = k_La \cdot \left(C^*_{O_2} - C_{O_2,t}\right) \qquad (2)$$

$$ln\left(C^*_{O_2} - C_{O_2,t}\right) = -k_La \cdot t \qquad (3)$$

The oxygen sensor response time (τ) can affect the estimation of k_La, $C^*_{O_2}$ and $C_{O_2,t}$. For accurate values, $\tau_{63.2} << (k_La)^{-1}$ is recommended, with $\tau_{63.2}$ defined as the time to reach 63.2 % of the final value when exposed to a step change in O_2 concentration [15]. This was the case in the current study, where typical volumetric oxygen-mass transfer coefficients were ~5.10^{-4} s^{-1} and $\tau_{63.2}$ < 5-11 s for the tested probes. The deconvolution of the oxygen probe response time was therefore neglected for the estimation of k_La.

Once k_La was determined, the DO intake due to atmospheric diffusion ($\Delta c^{atm}_{O_2,t}$ [µM]) could be calculated at any time *t*. $\Delta c^{atm}_{O_2,t}$ was obtained by discretization of Eq. 2 for its numerical solution (Eq. 4). Replacing Δt by the sampling time (t_s) yielded Eq. 5. The variations of observed and actual DO is usually negligible in the linear portion of the oxygen uptake curve, therefore deconvolution of O_2 probe was also neglected [23].

$$\frac{\Delta c^{atm}_{O_2,t}}{\Delta t} = k_La \cdot \left(C^*_{O_2} - C_{O_2,t}\right) \qquad (4)$$

$$\Delta c^{atm}_{O_2,t} = (k_La \cdot t_s) \cdot \left(C^*_{O_2} - C_{O_2,t}\right) \qquad (5)$$

Finally, the cumulative DO due to atmospheric diffusion was subtracted from $C_{O_2,t}$ to estimate the diffusion-free O_2 concentrations at any time *t* ($C^m_{O_2,t}$ [µM]) i.e. as if there was no diffusion in the reaction medium (hermetically closed vessel) (Eq. 6). *OCR* is estimated through linear regression on the initial slope of the diffusion-free model ($C^m_{O_2,t}(t)$).

$$C^m_{O_2,t} = C_{O_2,t} - \sum_{i=0}^{t} \Delta c^{atm}_{O_2,t} \quad (6)$$

Similarly to other enzymatic activity assays, the interval of the regression analysis in the linear portion of $C^m_{O_2,t}(t)$ needs to be defined according to the user's requirements and equipment capacity (sampling time, τ, *etc.*). In the present study, the initial activity was determined from the highest one-minute slope on the $C^m_{O_2,t}$ curve with a determination coefficient $R^2 \geq 0.99$.

To facilitate the experimenter's acquisition of the method presented, Table 1 details the subsequent steps leading to the determination of the oxidase activity (*OCR*, μM_{O2} min^{-1}).

3.2 Experimental validation

3.2.1 Validation with established OCR methods: closed system versus open system

In order to validate the proposed methodology, OCRs were measured for different laccase sources with the established Clark-type electrodes in closed reaction vessels and compared to OCRs calculated following the dynamic method with correction for O_2 diffusion using an amperometric oximeter in open vessels. OCRs obtained through these two methods were in close agreement with each other (Table 2), supporting the soundness of the proposed method to deconvolute O_2 diffusion.

The addition of the strong oxidase inhibitor sodium azide (NaN_3, 1 mM) at the beginning of the aeration was tested in order to control the presence of oxidase activity which could bias the estimation of k_La. No change in k_La was observed with or without addition of the inhibitor proving that O_2

consumption by the enzyme was negligible during the aeration phase (data not shown). As a consequence, if desired, NaN_3 can be used to shorten the assay by stopping the oxidase activity (e.g. after 2 min of reaction with the substrate) in order to carry out the aeration phase sooner.

OCR assays are mandatory in the particular case of oxidase inhibition studies. For instance, compounds previously categorized as laccase inhibitors using spectrophotometric tests were later discovered to be decolorizing agents of the monitored colored products and therefore not actually inhibiting laccase [17]. Moreover, one advantage of the OCR methods for measuring the oxidase activity is that they can be used with many more reactions contrariwise to conventional spectrophotometric methods which are restricted to UV-Vis absorbing substrates or products. *CP* laccase activity was thus tested with a non-colored substrate (1 mM bisphenol A in M-buffer pH 5.0). *CP* laccase OCRs were similar in closed or open system (Table 2). The closeness of the values shows the applicability, in principle, of the methodology for activity measurements and illustrates the possible extension to non-colored substrates. In addition, the spectrophotometric activity measurement with BPA as a substrate would have been hampered by the presence of various metabolites generated by laccase oxidation [24] and would have required the separation of substrates and products (e.g. by chromatography).

The dynamic method was also applied to the O_2 optode system. This new-generation optical method for DO measurement is non-invasive and allows flexible experimental configurations. For these reasons, the O_2 optode platform was used in order to set up a system for routine activity measurements (Figure 2). This instrument meets the requirements for the determination of k_La (small response time) and allows a flexible choice in the type of reaction vessel as illustrated with experiments in one-mL glass vial (Figure 2) and fifteen-mL reaction vessels (Figure 5). The setup presented in Figure 2 was used to evaluate response linearity, reproducibility, robustness and accuracy of the dynamic method for oxidase activity determination as described in the following sections.

3.2.2 Validation with established methods: Spectrophotometric methods

After showing the proof of concept of the methodology by comparing OCRs determined in open and closed vessels, activity values acquired with the O_2 optode were compared with values obtained with the spectrophotometric method. A good linearity over a wide range of oxidase activity should enable an easy correlation of activities measured through spectrophotometry and through the dynamic OCR method.

Laccase activity is typically determined using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2,6-dimethoxyphenol (DMP), 2-methoxyphenol (guaiacol) or 4-hydroxy-3,5-dimethoxybenzaldehyde azine (syringaldazine) as oxidation substrates [25]. The typical laccase substrates DMP and ABTS were selected to determine *CP* laccase activity both with the conventional colorimetric method and the dynamic OCR method using the O_2 optode sensor in open systems (Figure 4). An excellent linearity was observed in the tested ranges for both substrates as shown by the coefficients of determination. The fast color development hindered the determination of high enzyme activities via the colorimetric method i.e. maximum absorbance was exceeded before three consecutive points were measured ($-\log_{10}\left(\frac{I}{I_0}\right) > 4$)).

In the case of *CP* laccase-mediated catalysis of DMP, the slope of the regression line was 0.16 μM_{O2} $\mu M_{product}^{-1}$ although a theoretical slope of 0.5 μM_{O2} $\mu M_{product}^{-1}$ was expected according to the reported reaction stoichiometry. In this connection, Wan et al. (2008) have observed that *Rhus vernicifera* laccase-mediated catalysis of 2,6-DMP produces para-radical species which subsequently react with each other to yield the colored dimer product [26]. The OCR for this low-redox potential laccase of plant origin is ~½ the dimer product formation rate.

Therefore, there must be other physical and/or chemical interferences in the estimation of *CP* laccase activity either via substrate consumption (colorimetry) or O_2 consumption (OCR dynamic method) or a combination thereof.

As a matter of fact, non-specific dimerization, oligomerization and polymerization were reported for the oxidation of DMP by the *Melanocarpus albomyces* laccase (a high-redox potential fungal laccase) by Kallio et al. (2009) [27]. In accordance with the latter, our observed ~3-fold lower OCR may indicate underlying non-enzymatic, radical-mediated oxidations which yield various colored products - with undefined molecular extinction coefficients - revealed by colorimetry at 469 nm. The observed oxygen/product ratio for the conversion of DMP could be explained by radical-mediated oxidation, as stable radical species produced through laccase oxidation of methoxy substituted phenols has been reported [28, 29].

In the case of using ABTS as a substrate, the single-electron oxidation of ABTS typically leads to one main stable colored ABTS radical ($ABTS^{+\cdot}$) [30], therefore 4 $ABTS^{+\cdot}$ are produced when one O_2 is reduced to water and the OCR should represent ~ $^1/_4$ the $ABTS^{+\cdot}$ production rate. In this case, the slope of the regression line was 0.40 $\mu M_{O2}\ \mu M_{product}^{-1}$. The experimental OCRs were therefore higher than the expected 0.25 $\mu M_{O2}\ \mu M_{product}^{-1}$ for the postulated single electron-oxidation. Nevertheless, the double-oxidation of ABTS by high-redox potential laccases has also been reported [31]. In addition, interferences at 420 nm have been attributed to the absorbance of ABTS-$ABTS^{+\cdot}$ complexes. A multiplication factor of up to 1.33 could be introduced to calculate the actual activity which would yield a slope of 0.30 $\mu M_{O2}\ \mu M_{product}^{-1}$ in this study [17].

Reaction stoichiometry is typically dependent on the type of laccase (among other factors) as more than one oxidation products were reported after oxidation of both illustrative substrates by high-redox potential laccases such as the fungal laccase used in this study [27, 32]. Different molar extinction coefficients for the oxidation products have been occasionally used in the literature for a given

substrate, such as DMP, because the nature of the colored product(s) was still unidentified [33]. Moreover, a variable stoichiometry in the catalytic oxidation of aqueous phenol (1 to 4 $mol_{O2}/mol_{substrate}$) dependent on the substrate concentration (0.15 to 8 mM) was already reported for the fungal laccase from *T. versicolor* [34].

The still not fully elucidated radical reaction upon laccase-catalyzed substrate oxidation underlines the necessity for a reliable method of activity determination which is not influenced by secondary oxidation processes. In their study on fungal laccase activity tests and inhibitors, Johannes and Majcherczyk (2000) concluded by recommending laccase OCR measurements to circumvent interferences in spectrophotometric assays with ABTS and phenolic substrates [17].

In order to avoid such interferences, another type of blue copper oxidase, *AAO*, was also used for the comparison of enzyme activity determined via the spectrophotometric substrate monitoring and by the proposed OCR dynamic method. In this case, a theoretical slope of 0.5 $\mu M_{O2}\,\mu M_{substrate}^{-1}$ was expected since 2 molecules of L-ascorbate are oxidized into 2 molecules dehydroascorbate with the concomitant reduction of one oxygen molecule. However, the experimental slope was 0.80 $\mu M_{O2}\,\mu M_{substrate}^{-1}$. In this case, the deviation was more likely to be caused by limitations of the spectrophotometric assay rather than variations of the reaction stoichiometry.

First, L-ascorbate concentration was limited to 0.4 mM to avoid overflow of absorbance at 265 nm in the spectrophotometric assay. This value was below substrate saturation (~10 K_m) given the reported K_m of *Cucurbita* sp *AAO* ($0.1 < K_m < 2$ mM [35, 36]). In these conditions, the reaction rate was sensitive to substrate concentration variations and therefore more sensitive to experimental variability. This could explain to a certain extent the relative scatter of data points ($R^2 = 0.9371$) (Figure 4).

In addition, a spectrophotometry-based activity assay assumes that the decreasing substrate concentration is the only factor contributing to commensurate absorbance variation even though the product can also contribute to absorbance variation. In the case with *AAO*, the (mono-

)dehydroascorbate produced also absorbed at 265 nm [37] and, given the limiting substrate concentration condition, the product's absorbance might not be negligible and could partially counteract the absorbance decrease due to substrate consumption. An enzymatic reaction where φ moles of substrate are converted into ω moles of product would yield the following expression (Eq.7):

$$\frac{\partial C_s}{\partial t} = \frac{\partial A/\partial t}{\left(\varepsilon_s - (\frac{\varphi}{\omega})\varepsilon_p\right)d} \qquad (7)$$

where *Cs* (µM) is the substrate concentration, $\partial A/\partial t$ is the initial variation of absorbance (usually expressed in (min^{-1})), *d* is the light path (cm) and ε_s and ε_p are the molar extinction coefficients of the substrate and the product respectively (µM cm^{-1}).

In the case where the product extinction coefficient is non-negligible, spectrophotometric activity assays systematically underestimate the actual activity. Moreover, different ε_{265} for the substrate were reported in literature (13,400 – 14,700 µM cm^{-1} [20, 38]) which could also lead to an underestimation of the actual *AAO* activity.

The results suggest that the use of spectrophotometric assays to infer the reaction rate expressed in $\mu M_{product}$ min^{-1} is not accurate in many experimental situations, e.g. not well established reaction stoichiometry, side reactions involving the absorbing substrates, presence of absorbing reaction products, although spectrophotometry stays convenient for benchmarking (relative activity comparison).

Unless the stoichiometry of the reaction is well known and fully described in the literature, calibration curves are required to convert activity measured with an O_2-based assay (μM_{O2} min^{-1}) to activity measured with spectrophotometric methods ($\mu M_{product}$ min^{-1}).

3.2.3 Reproducibility and intra-experimental variability

Reproducibility was assessed by conducting an experiment six times under identical conditions (*CP* laccase, 1 mM ABTS in pH 3.0 M-buffer, in triplicates, O_2 optode system). The activity was 34.8 ± 1.4 μM_{O2} min^{-1} and the obtained precision was in the typical range of established photometric methods with a coefficient of variation below 4.2%.

The intra-experimental precision of activity measurements was assessed by consecutively adding substrate to an activity assay once the substrate was exhausted (Figure 5).

k_La was measured during each aeration phase. The DO concentration was always higher than 150 µM (> 3-9 fold the reported Michaelis-Menten constant for O_2 (K_{mO2}) of fungal laccases [39]). The coefficient of variation of OCRs after 10 additions was 3.5%, confirming the good precision of the proposed methodology.

3.2.4 Influence of k_La on OCR determination

If the oxygen diffusion rate from the atmosphere is low compared to the biocatalyst OCR, the diffusion correction for OCR calculation might be omitted in subsequent assays with the same system (O_2 optode setup, for instance) to save time without dramatically affecting the accuracy of the assay. Here, the error due to neglecting the diffusion correction was characterized with respect to experimental conditions (e.g. k_La, pressure, temperature, etc.). Under a user-defined threshold (e.g. 5%), the determination of k_La might not be necessary in order to save time.

With this aim, Φ was defined as the percent of error on the estimation of OCR (Eq. 8). Using Φ, the experimenter can decide whether to determine systematically k_La for the subsequent assays or possibly do "shortcut" assays with a reasonable approximation of the oxidase activity.

$$\Phi = \frac{k_L a \cdot \nabla C_{O_2} - OCR}{OCR} \qquad (8)$$

where ∇C_{O_2} is the maximal O_2 gradient during the time interval for the determination of *OCR*.

Considering the oxygen diffusion OCR and the biocatalytic OCR as the only two factors affecting the variation of DO in the reaction medium, overlooking the diffusion would always lead to an underestimation of the OCR given that $|OCR| \geq \left|k_L a \cdot \nabla C_{O_2}\right|$. Figure 6 illustrates the up to 5% error ($-0.05 < \Phi < 0$) introduced by omitting the diffusion correction in the determination of OCRs for a range of $k_L a$ and for a given ∇C_{O_2} of 31.5 µM min^{-1} (1 mg L^{-1}). The diagram may be generalized to any ∇C_{O_2} according to Eq. 8 e.g. doubling $k_L a$ is equivalent to doubling ∇C_{O_2}.

For assays characterized by small volumetric mass transfer coefficients (e.g. ≤ 0.005 min^{-1}), OCRs as low as 3 μM_{O2} min^{-1} can be estimated without correction for diffusion (Figure 6). On the other hand, if $k_L a$ is high (e.g. large interfacial area or high turbulence agitation), the impact of the diffusion on the OCR calculation soon becomes significant, and only high OCR can be evaluated without correction (e.g. for Φ = -5% and $k_L a$ = 0.08 min^{-1}, minimum acceptable OCR is ~ 50 μM_{O2} min^{-1}). A vessel with a large liquid column relative to the surface of the opening is then most appropriate. Agitation will obviously lead to an increased O_2 influx, but without agitation an oxygen gradient can be formed within the liquid column which makes the measurements dependent upon the placement of the oxygen sensor.

Open-system OCR measurements are therefore well-suited for the evaluation of highly active immobilized biocatalysts since a direct measurement of activity can be obtained without correction for diffusion.

The factors that influence $k_L a$ estimation by the dynamic method – and, by extension, enzyme activity determination – are the interfacial area, the temperature, the ionic strength of the medium, the atmospheric pressure, the agitation and the medium viscosity. These factors either directly affect the volumetric mass transfer coefficient $k_L a$ of the system or its estimation by the proposed method due to

a change of $C^*_{O_2}$ during the test (the linear regression on Eq. 3 for the determination of k_La assumed that $C^*_{O_2}$ was conserved during the test). Interfacial area, agitation and viscosity can be either controlled or considered as being constant within and between activity assays. The remaining factors - pressure, temperature and ionic strength - which can vary over time and assay conditions might significantly affect the oxygen transfer and therefore the activity determination. Hereafter, the effect of these environmental factors on oxygen transfer (k_La) and resulting error on the determination of oxidase activity (Φ) were examined.

In some cases, the estimation of enzyme activity is not significantly impacted (-5% < Φ < 5%) although the variation of these factors has an effect on the oxygen transfer (k_La) or on the DO at equilibrium ($C^*_{O_2}$). In these cases, the deconvolution of atmospheric diffusion can be avoided and the activity assay can be substantially shortened because the aeration phase is no longer required. Under 100% air-saturated atmosphere (normal conditions), saturated DO concentration ($c^*_{O_2}$, µM) is given by Eq. 9 with P_N equal to 1013 mbar, P_{atm} the actual atmospheric pressure and V_M the molar volume of oxygen. The vapor pressure of water (P_w) at a given temperature (in Kelvin) was obtained using the Campbell expression (Eq. 10). The Bunsen solubility coefficient (α) as a function of T was derived from thermodynamic correlations (Eq. 11). Eq. 10-11 and constants a, b and c were recommended by the O_2 optode manufacturer (PreSens GmbH) and can be found in handbooks of chemistry and physics [40]. Eq. 9-11 were used to investigate the effect of atmospheric pressure and temperature variation on k_La and OCR determinations through the dynamic method.

$$c^*_{O_2} = 0.2095 \cdot \left(\frac{P_{atm} - P_w}{P_N} \cdot \alpha \cdot \frac{10^6}{V_M}\right) \qquad (9)$$

$$P_w = e^{\left(a - \frac{b}{T} - c \cdot \ln(T)\right)} \qquad (10)$$

$$ln(10^3 \cdot \alpha) = \frac{a}{T} + b \cdot \ln(T) + c \qquad (11)$$

The atmospheric pressure affects the saturated oxygen concentration ($c^*_{O_2}$, Eq. 9). Figure 7A shows that this effect is negligible (< 0.5% variation over a wide range of atmospheric pressures) when evaluating high activity, illustrated here with OCR = 79.5 µM min^{-1} (actual P_{atm} = 988 mbar) while the effect can be more pronounced, but still limited (up to 5% deviation), for lower-activity determination, as shown with OCR = 8.4 µM min^{-1} (actual P_{atm} = 1020 mbar). An incorrect k_La would be determined following the method (Eq. 2, step n°5 in Table 1) if this effect was not taken into account. $c^*_{O_2}$ increases with an increasing atmospheric pressure following (Eq. 9); as a consequence the k_La values estimated through linear regression on Eq. 2 tended to decrease to compensate for the increasing ∇C_{O_2} (Figure 7A).

In the same way, temperature variations can affect the estimation of k_La due to variation of $c^*_{O_2}$ during the assay, the saturated oxygen concentration being a function of the vapor pressure of water (P_w) and the Bunsen solubility coefficient (α) which are both functions of temperature. The estimated k_La tended to increase with temperature by compensation of a decreasing $c^*_{O_2}$(decreasing ∇C_{O_2}) (Figure 7B). Typical variations of ± 2 °C in non-thermostated assays had a limited effect on the determination of OCR as illustrated with OCR = 79.5 µM min^{-1} (actual T = 303 K) (solid black line in Figure 7B,) and OCR = 8.4 µM min^{-1} (actual T = 295 K) (solid grey line in Figure 7B) where deviations from actual OCR were limited to 0.2-0.3% and 0.9-6% respectively. On the other hand, if activity assays are intended to be performed at different temperatures, in studies aiming to determine e.g. optimal process temperature, k_La and $c^*_{O_2}$ need to be properly determined for each tested temperature.

In studies of optimal pH and of inhibition effects, the ionic strength of the reaction medium can change due the use of different buffers or presence of inhibitors (e.g. salts). The Bunsen solubility coefficient is indeed a function of both T and ionic strength ([Cl^-]) (Eq. 12 [40]).

$$ln(10^3 \cdot \alpha) = \left(a + \frac{b}{T} + c \cdot ln(T) + d \cdot T\right) - [\mathrm{Cl}^-] \cdot \left(e + \frac{f}{T} + g \cdot \ln(T) + h \cdot T\right) \qquad (12)$$

Eq. 12 is valid for $0 \leq [Cl^-] \leq 30‰$ (18‰ Cl^- is equivalent to seawater salinity (35 g_{salts} L^{-1})), $273.1 \leq T \leq 308.2$ K, and a-h are regression constants based on O_2 measurements with varying salt concentrations (given by the manufacturer PreSens GmbH in this case).

An increase in medium ionic strength reduces O_2 solubility ($c^*_{O_2}$). k_La values estimated by the dynamic method tended to increase with the ionic strength to compensate the apparent decreasing O_2 gradient (Figure 7C). The impact on high-activity determination, OCR = 79.5 µM min^{-1} (actual $[Cl^-]$ = 0‰) (solid black line in Figure 7C) was again limited while the impact was very pronounced for low OCR = 8.4 µM min^{-1} (actual $[Cl^-]$ = 0‰) (solid grey line in Figure 7C). A measurement of k_La is therefore required as soon as ions are introduced at concentration above 8‰ into the reaction medium (e.g. enzyme inhibitors) or when a buffer with a different ionic strength is used.

4. Concluding remarks

The presented work reports an alternative straightforward methodology for oxidase activity determination based on oxygen consumption especially suited to high oxidase activity (e.g. immobilized enzymes). The equipment required for applying the methodology (a short response-time O_2 sensor) is basic, flexible, widely available and inexpensive. In principle, the method is not affected by limitations inherent in spectrophotometric assays (e.g. light scattering, limited range of substrates and substrate concentrations). The working volume (cm^3- to dm^3-scale) may be chosen as needed, unlike Clark-type cells (respirometry), and the use of an optode O_2 sensor reduces the required volume for activity assay (e.g. one-mL vial). Furthermore, the method allows the measurement of oxidase activity against non-absorbing compounds and is not perturbed by non-enzymatic secondary reactions, known to interfere in some spectrophotometric assays. Our results indicate that a change of the pressure, temperature or salinity in a range that is expectable during normal operation of the equipment had a relatively low impact on the determination of OCRs via the dynamic method.

The method proposed is not well suited for the measurement of low enzyme activities – i.e. when OCR is of similar magnitude as diffusion rate – and is low-throughput compared to spectrophotometric assays, but is particularly advantageous (i) in cases where an exact measurement of reaction rates is required (contrary to enzyme activity in arbitrary units), (ii) in studies of inhibitors that may react with the monitored absorbing compounds, (iii) whenever high concentrations (> 10-fold K_m) of substrates with a high molar extinction coefficient are required, (iv) whenever the use of substrates not adsorbing in the UV-Vis spectrum is required, (v) whenever the use of substrate with interfering biocatalytic product(s) or medium in the target UV-Vis spectrum is required, or (vi) for the measurement of highly active immobilized enzymes avoiding problems of substrate/product adsorption on the solid carriers and light scattering.

Finally, the dynamic method can be potentially applied to other oxidases such as cytochrome c oxidases, cytochrome P450 oxidases (monooxygenase activity), NADH oxidases, glucose oxidases, tyrosinases, etc.

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5. References

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Table 1. Sequence of operations to run the oxidase activity assay and to process the data for the determination of *OCR* by linear regression on the diffusion-free model.

Steps	Actions
1	The reaction medium (buffer + enzyme) is stirred and C_{O_2} and temperature (T) are monitored.
2	Once C_{O_2} is constant for one minute, $C^*_{O_2}$ is assumed.
3	The substrate is added and C_{O_2} is monitored up to 15 min during the aeration phase.
4	The data are processed in a standardized spreadsheet containing the following columns: **C1**: t; **C2**: $C_{O_2,t}$; **C3**: T; **C4**: $ln(C^*_{O_2} - C_{O_2,t})$; **C5**: $\Delta c^{atm}_{O_2,t}$; **C6**: $\sum_{i=0}^{t} \Delta c^{atm}_{O_2,t}$; **C7**: $C^m_{O_2,t}$ The time course of columns C2, C4 and C7 are plotted for data check and linear regression analyses.
5	$k_L a$ is determined by linear regression (C4 versus time) during the aeration phase, starting one minute after the C_{O_2} minimum is reached.
6	Using the experimental $k_L a$, the atmospheric diffusion (C5) is calculated throughout the assay.
7	The cumulative atmospheric diffusion (C6) is subtracted from $C_{O_2,t}$ (C2) to obtain the diffusion-free O_2 concentration (C7).
8	OCR is determined by linear fitting over a one-minute interval (highest slope) on the diffusion-free model (C7 versus time).

Table 2. OCRs of different enzyme sources with 1 mM ABTS as substrate in M-buffer pH 3.0 and 1 mM BPA in M-buffer pH 5.0. OCR values calculated following the dynamic method in an open system were dilution-corrected (1.25 x) to enable comparison with OCR values measured with Clark electrodes (closed system). Given are means of triplicates ± S.D.

Substrate	Laccase source	Activity - Clark electrode [μM_{O2} min^{-1}]	Activity - Dynamic method [μM_{O2} min^{-1}]
ABTS	*CP*	86 ± 6	90 ± 2
	TV	161 ± 10	160 ± 10
	LNB	134 ± 1	122 ± 2
BPA	*CP*	43 ± 4	37 ± 7

CP: *C. polyzona* culture supernatant, *TV*: *T. versicolor* laccase (Sigma), *LNB*: immobilized laccase.

Table 3. Dilution-corrected OCRs calculated by the dynamic method and estimated k_La after the repeated addition of substrates as illustrated in Figure 5 (—).

N° injection	OCR (μM_{O2} min^{-1})	k_La (min^{-1})
1	20.1	0.027
2	20.0	0.031
3	19.6	0.031
4	19.2	0.030
5	18.9	0.030
6	19.8	0.030
7	19.6	0.031
8	18.3	0.030
9	18.6	0.028
10	18.3	0.030
Mean	19.2	0.03
S.D.	0.7	0.001
CV (%)	3.50	4.39

S.D.: standard variation, CV: relative standard variation

Figure legends

Figure 1.

Simplified laccase catalytic cycle. Oxidized laccase transfers four electrons from four phenolic substrate molecules to one oxygen molecule as final electron acceptor. To determine laccase activity, the phenolic substrate (dis)appearance can be monitored through photometry (λ, right-hand side), or, alternatively, the oxygen consumption rate (OCR) can be monitored by a dissolved oxygen probe (left-hand side).

Figure 2.

The setup presented is designed for routine OCR measurements using an oxygen optode with 1.5 mL-HPLC vials as reaction vessels. The spots containing the fluorescent dye are glued on the side of the vials and the optical fibers are positioned in front of them by a holder which maintains the vial on top of a magnetic stirrer.

Figure 3.

Schematic representation of the dynamic method for the determination of oxidase activity. Before addition of the substrate, the reaction medium is equilibrated in presence of the enzyme and $C^*_{O_2}$ is determined (A). After addition of the substrate, oxygen concentration decreases till the substrate is exhausted (B) and atmospheric oxygen diffusion exactly balances oxygen consumption by the enzymatic reaction for a short period of time. Next, the oxygen concentration rises to reach $C^*_{O_2}$(C). During this aeration phase, the volumetric mass transfer coefficient (k_La) is estimated.

Figure 4.

Calibration curves of the *OCR* for *CP*-laccase and *AAO* calculated through the dynamic method versus *CP* laccase activity measured photometrically with 1 mM ABTS and 1 mM DMP and *AAO* activity measured with 0.25 mM L-ascorbate. The data are means of triplicates ± S.D.

Figure 5.

Time course of oxygen concentration (▫) in M-buffer pH 3 containing *CP* laccase after repeated additions of 0.1 mM ABTS (final concentration). The diffusion-free modeled O_2 concentration ($C^m_{O_2,t}$) is shown in grey (—). OCRs and k_La were calculated after each substrate addition and listed in Table 3.

Figure 6.

Assay conditions (k_La) for which the correction for atmospheric diffusion can be optional i.e. for -5% of introduced error ($\Phi \geq -0.05$). The diagram is valid for ∇C_{O_2} = 31.5 μM_{O2} with k_La values ranging from 0.005 to 0.08 min^{-1}.

Figure 7.

Effects of pressure (top, A), temperature (middle, B) and salinity (bottom, C) variations on the determination of oxidase activities. Error (Φ) on the estimation of high oxidase activity (actual conditions: OCR = 79.5 μM_{O2} min^{-1}, P_{atm} = 988 mbar, T= 303 K, $[Cl^-]$ = 0‰) is shown in black line (—) and low oxidase activity (actual conditions: OCR = 8.4 μM_{O2} min^{-1}, P_{atm} = 1020 mbar, T= 295 K, $[Cl^-]$ = 0‰) is shown in grey line (—) and estimations of k_La are illustrated by dashed lines for high (- -) and low (- -) OCR.

Table 1. Sequence of operations to run the oxidase activity assay and to process the data for the determination of *OCR* by linear regression on the diffusion-free model.

Steps	Actions
1	The reaction medium (buffer + enzyme) is stirred and C_{O_2} and temperature (T) are monitored.
2	Once C_{O_2} is constant for one minute, $C^*_{O_2}$ is assumed.
3	The substrate is added and C_{O_2} is monitored up to 15 min during the aeration phase.
4	The data are processed in a standardized spreadsheet containing the following columns: **C1**: t **C2**: $C_{O_2,t}$ **C3**: T **C4**: $ln(C^*_{O_2} - C_{O_2,t})$ **C5**: $\Delta c^{atm}_{O_2,t}$ **C6**: $\sum_{i=0}^{t} \Delta c^{atm}_{O_2,t}$ **C7**: $C^m_{O_2,t}$ The time course of columns C2, C4 and C7 are plotted for data check and linear regression analyses.
5	$k_L a$ is determined by linear regression (C4 versus time) during the aeration phase, starting one minute after the C_{O_2} minimum is reached.
6	Using the experimental $k_L a$, the atmospheric diffusion (C5) is calculated throughout the assay.
7	The cumulative atmospheric diffusion (C6) is subtracted from $C_{O_2,t}$ (C2) to obtain the diffusion-free O_2 concentration (C7).
8	OCR is determined by linear fitting over a one-minute interval (highest slope) on the diffusion-free model (C7 versus time).

Table 2. OCRs of different enzyme sources with 1 mM ABTS as substrate in M-buffer pH 3.0 and 1 mM BPA in M-buffer pH 5.0. OCR values calculated following the dynamic method in an open system were dilution-corrected (1.25 x) to enable comparison with OCR values measured with Clark electrodes (closed system). Given are means of triplicates ± S.D.

Substrate	Laccase source	Activity - Clark electrode [μM_{O2} min^{-1}]	Activity - Dynamic method [μM_{O2} min^{-1}]
ABTS	*CP*	86 ± 6	90 ± 2
	TV	161 ± 10	160 ± 10
	LNB	134 ± 1	122 ± 2
BPA	*CP*	43 ± 4	37 ± 7

CP: *C. polyzona* culture supernatant, *TV*: *T. versicolor* laccase (Sigma), *LNB*: immobilized laccase.

Table 3. Dilution-corrected OCRs calculated by the dynamic method and estimated k_La after the repeated addition of substrates as illustrated in Figure 5 (—).

N° injection	OCR (μM_{O2} min^{-1})	k_La (min^{-1})
1	20.1	0.027
2	20.0	0.031
3	19.6	0.031
4	19.2	0.030
5	18.9	0.030
6	19.8	0.030
7	19.6	0.031
8	18.3	0.030
9	18.6	0.028
10	18.3	0.030
Mean	19.2	0.03
S.D.	0.7	0.001
CV (%)	3.50	4.39

S.D.: standard variation, CV: relative standard variation

Figure 1.

Simplified laccase catalytic cycle. Oxidized laccase transfers four electrons from four phenolic substrate molecules to one oxygen molecule as final electron acceptor. To determine laccase activity, the phenolic substrate (dis)appearance can be monitored through photometry (λ, right-hand side), or, alternatively, the oxygen consumption rate (OCR) can be monitored by a dissolved oxygen probe (left-hand side).

Figure 2.

The setup presented is designed for routine OCR measurements using an oxygen optode with 1.5 mL-HPLC vials as reaction vessels. The spots containing the fluorescent dye are glued on the side of the vials and the optical fibers are positioned in front of them by a holder which maintains the vial on top of a magnetic stirrer.

Figure 3.

Schematic representation of the dynamic method for the determination of oxidase activity. Before addition of the substrate, the reaction medium is equilibrated in presence of the enzyme and $C^*_{O_2}$ is determined (A). After addition of the substrate, oxygen concentration decreases till the substrate is exhausted (B) and atmospheric oxygen diffusion exactly balances oxygen consumption by the enzymatic reaction for a short period of time. Next, the oxygen concentration rises to reach $C^*_{O_2}$(C). During this aeration phase, the volumetric mass transfer coefficient (k_La) is estimated.

Figure 4.

Calibration curves of the *OCR* for *CP*-laccase and *AAO* calculated through the dynamic method versus *CP* laccase activity measured photometrically with 1 mM ABTS and 1 mM DMP and *AAO* activity measured with 0.25 mM L-ascorbate. The data are means of triplicates ± S.D.

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Figure 1

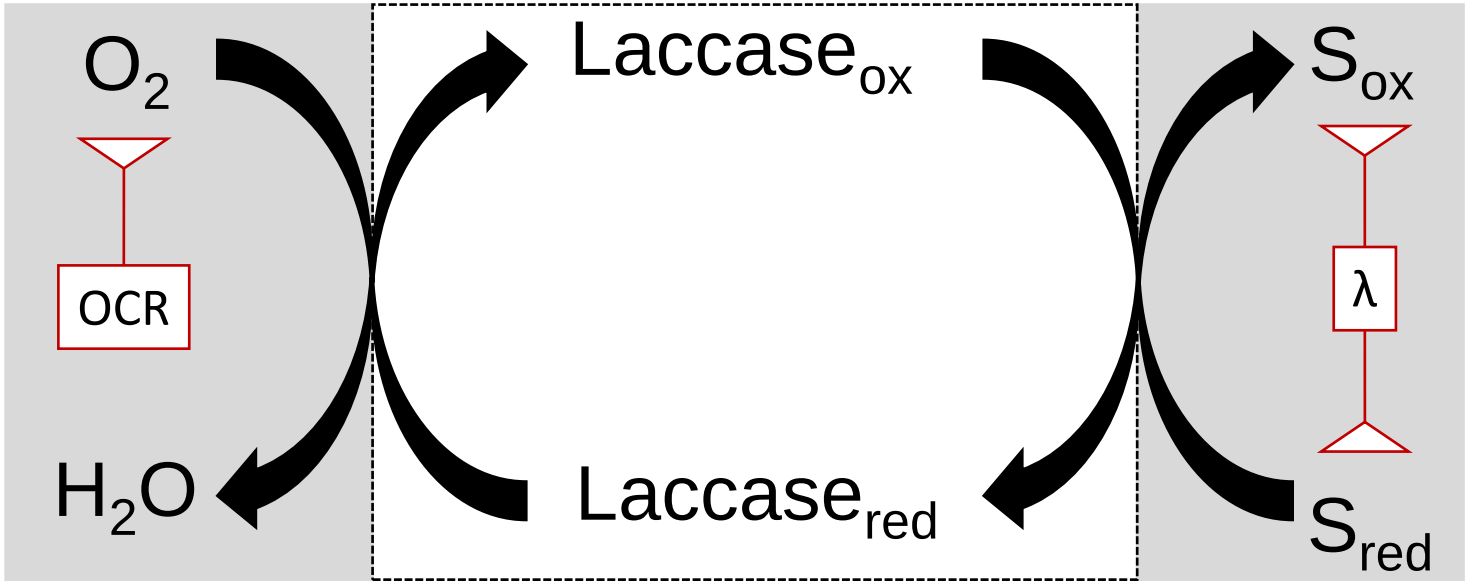


Figure 3

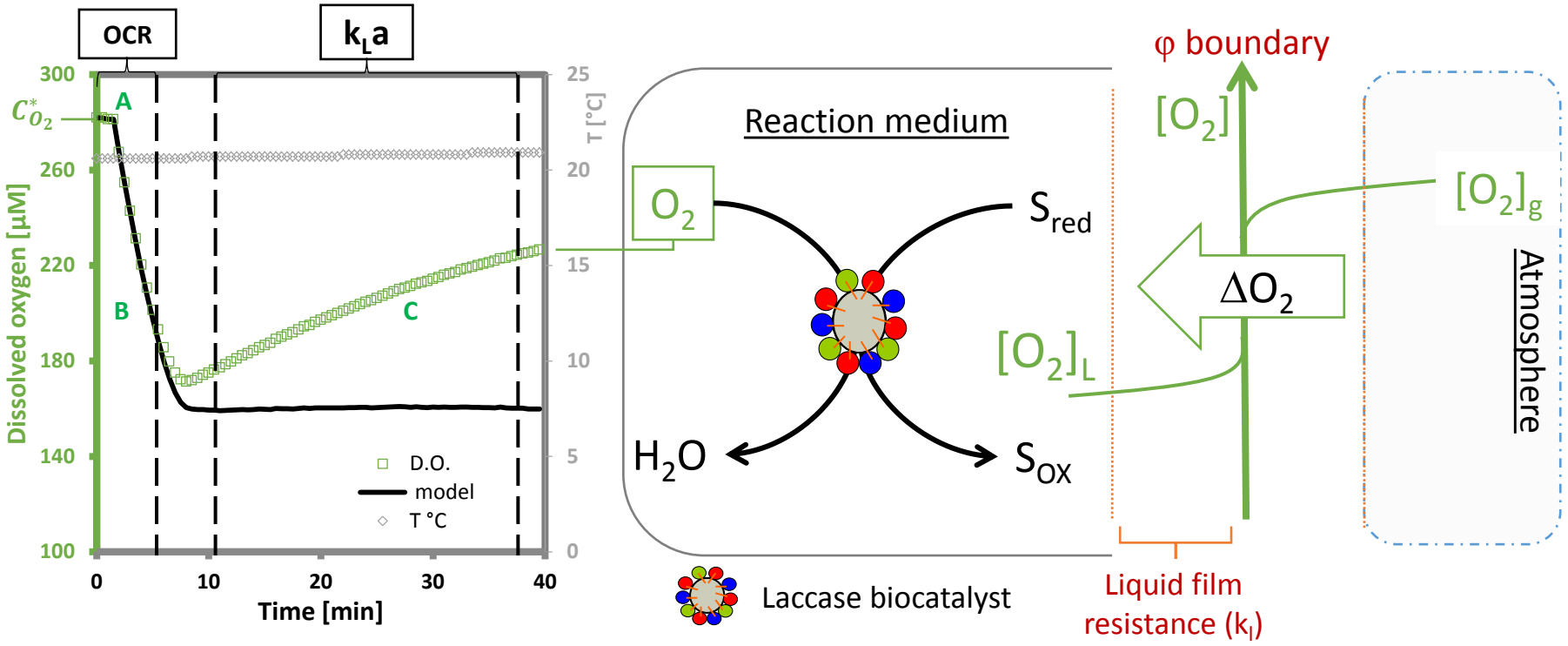

OCR
k_La
$C^*_{O_2}$
A
B
C
Dissolved oxygen [µM]
T [°C]
Time [min]
D.O.
model
T °C
Reaction medium
O_2
S_{red}
H_2O
S_{OX}
Laccase biocatalyst
φ boundary
$[O_2]$
ΔO_2
$[O_2]_L$
$[O_2]_g$
Atmosphere
Liquid film resistance (k_l)

Figure 4

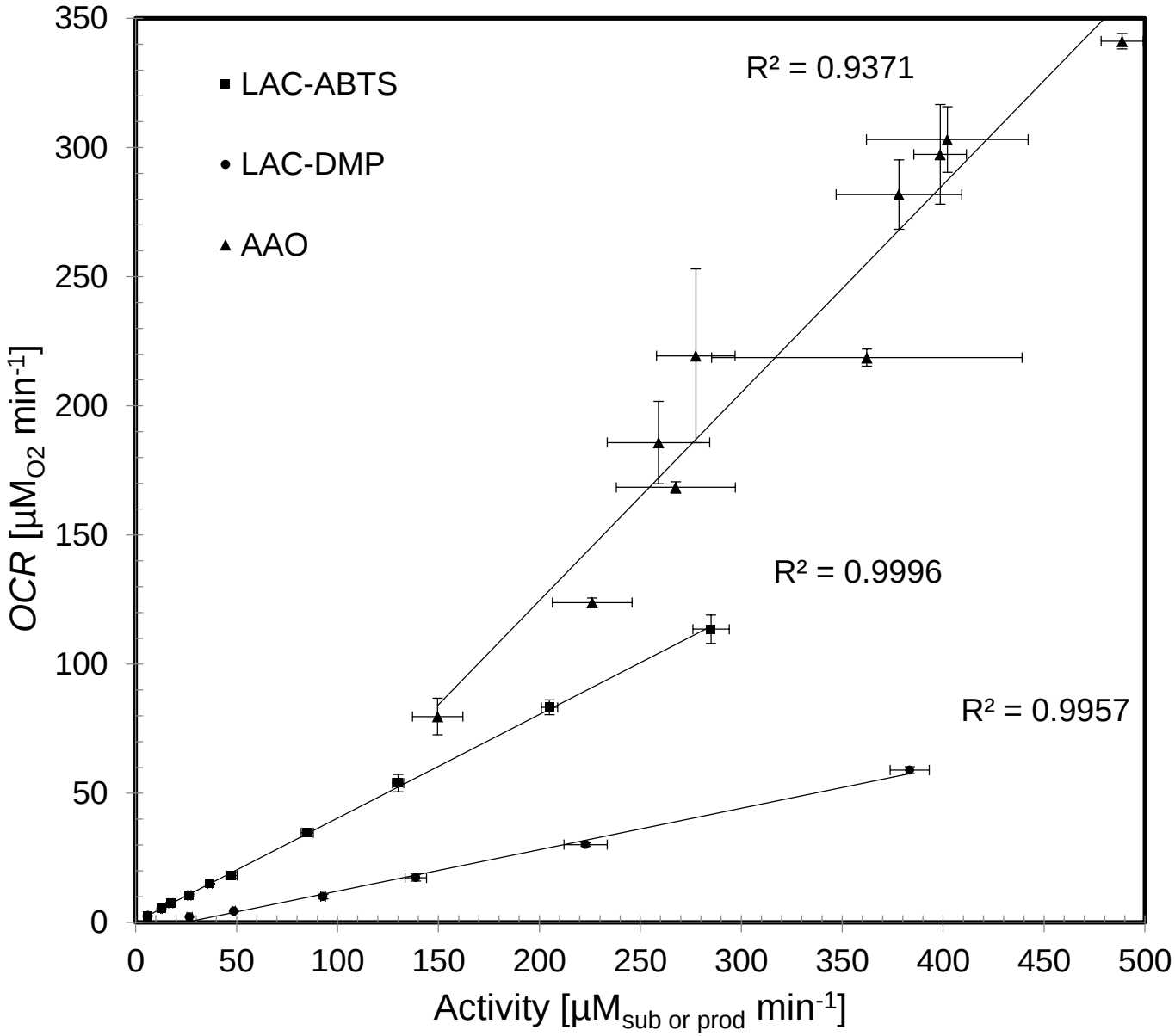

LAC-ABTS
LAC-DMP
AAO
R² = 0.9371
R² = 0.9996
R² = 0.9957
OCR [µM$_{O2}$ min^{-1}]
Activity [µM$_{sub\ or\ prod}$ min^{-1}]
0
50
100
150
200
250
300
350
400
450
500

Figure 5

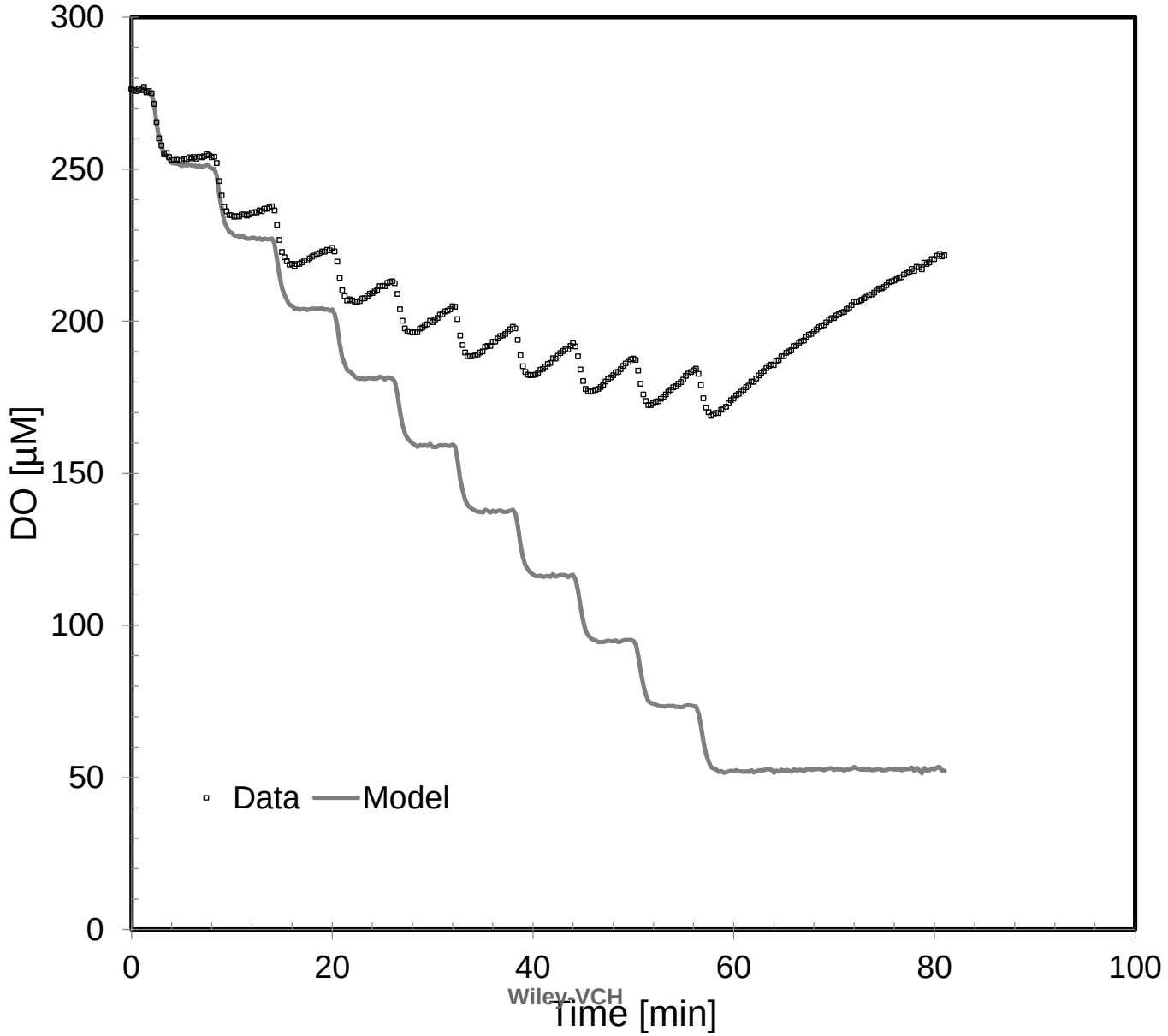


Figure 6

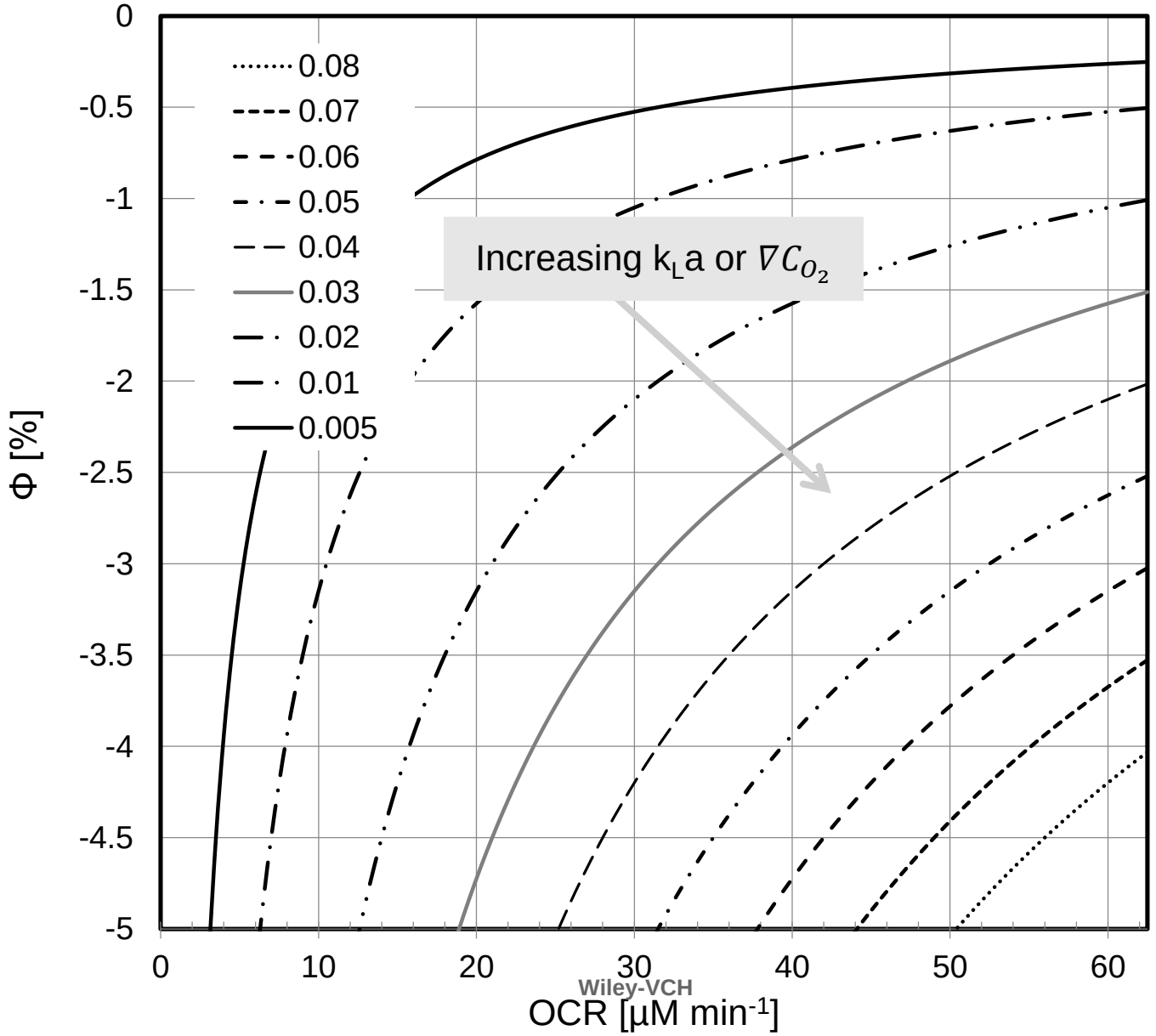
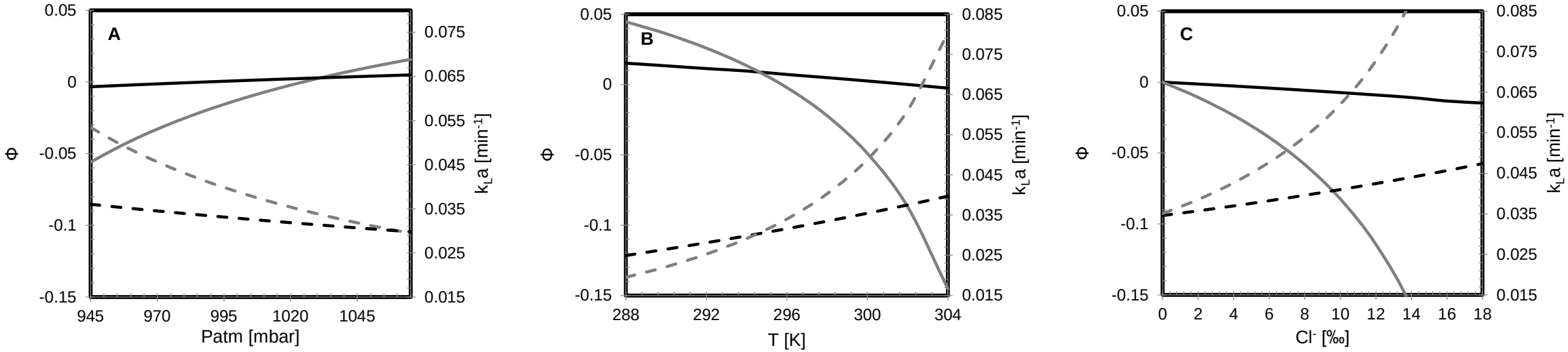


Figure 7


A
B
C
Φ
k_La [min^{-1}]
Patm [mbar]
T [K]
Cl^- [‰]
0.05
0
-0.05
-0.1
-0.15
0.085
0.075
0.065
0.055
0.045
0.035
0.025
0.015
945
970
995
1020
1045
288
292
296
300
304
0
2
4
6
8
10
12
14
16
18

134x48mm (150 x 150 DPI)