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Short Communication

Quantification of oxygen production and respiration rates in mixotrophic cultivation of microalgae in nonstirred photobioreactors

Online monitoring and controlling of different cellular parameters are key issues in aerobic bioprocesses. Since mixotrophic cultivation, in which we observe a mixture of cellular respiration and oxygen production has gained more popularity, there is a need for an on-process quantification of these parameters. The presented and adapted double gassing-out method applied to a mixotrophic cultivation of *Galdieria sulphuraria*, will be a tool for monitoring and further optimization of algal fermentation in nonstirred photobioreactors (PBR). We measured the highest net specific oxygen production rate (opr_{net}) as $5.73 \cdot 10^{-3} \text{ mol}_{O_2} \text{ g}^{-1} \text{ h}^{-1}$ at the lowest oxygen uptake rate (OUR) of $1.00 \cdot 10^{-4} \text{ mol}_{O_2} \text{ L}^{-1} \text{ h}^{-1}$. Due to higher cell densities, we also demonstrated the increasing shading effect by a decrease of opr_{net} , reaching the lowest value of $1.25 \cdot 10^{-5} \text{ mol}_{O_2} \text{ g}^{-1} \text{ h}^{-1}$. Nevertheless, with this on process measurement, we can predict the relation between the zone in which oxygen is net produced to the area where cell respiration dominates in a PBR, which has a major impact to optimize cell growth along with the formation of different products of interest such as pigments.

Keywords: Microalgae / Mixotrophic cultivation / Oxygen production rate / Oxygen uptake rate / Photobioreactor

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1 Introduction

Providing oxygen is one important key in aerobic bioprocesses, because oxygen is the final electron acceptor in the electron transport system and therefore, is directly related to growth, maintenance, and product synthesis of cells [1]. Since oxygen has a low solubility in culture broths, a deep knowledge as well as the possibility to measure the oxygen transfer rate, the OUR, and the volumetric mass transfer coefficient is needed for the design of effective cultivation processes. For optimization or scaling-up of such processes, the determination of these factors is necessary for every reactor design and microorganism [2]. The most widely used technique to determine the OUR in cell

cultures is the dynamic method, which is usually used for measuring the oxygen respiration rate in stirred reactors [3,4]. But this method does not work in bioreactors which are only mixed by the aeration itself, since switching off the air supply immediately results in a lack of mixing which is necessary for accurate measurement of the OUR. Without proper mixing, settlement of the microorganism as well as the development of an oxygen concentration gradient prevents accurate measurement. This in turn, results in the need to use alternative methods. In bubble columns, one possibility to measure the respiration rate is the double gassing-out method. It is based on the measurement of OUR under nitrogen gas application instead of pressurized air during the cultivation process [4].

In contrast to the cultivation of heterotrophic cells, e.g. bacteria or yeasts, light saturation of a cell culture plays a crucial role in phototrophic and mixotrophic microalgae fermentation. Due to increasing shading effect caused by the cells, the light is less homogeneously distributed in PBR. At high cell densities, the light penetration consequently is very limited [5]. Since photosynthesis ensures the energy balance during phototrophic growth and thus contributes to formation of biomass and different pigments, it is desirable to quantify the photosynthesis during photo- and mixotrophic cultivations in PBRs to be able

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Abbreviations: DO, dissolved oxygen; $OD_{750 \text{ nm}}$, optical density at 750 nm; ODR, oxygen displacement rate; our , specific oxygen uptake rate; OUR, oxygen uptake rate; opr_{net} , specific oxygen production rate; OPR_{net} , oxygen production rate; PBR, photobioreactor; PSM, photobioreactor screening module

to design an effective process [6]. Bubble column, airlift, and flat panel reactors are the most used closed PBRs in outdoor as well as indoor cultivations nowadays [7, 8]. Thus, the aim of this work was to establish an improved double gassing-out method applicable for microalgae cultivation, which ensures the quantification of the net oxygen production rate (OPR_{net}) and OUR during the process. Our method is demonstrated for a mixotrophic cultivation of *Galdierira sulphuraria*, which is able to grow under photo-, mixo- and heterotrophic conditions accumulating a variety of different pigments [9, 10]. Furthermore, this new method enables precise conclusions about the share of photoactive zones and zones where cell respiration dominates in the PBR, which have a major impact on cell growth as well as pigment formation or the synthesis of other photosynthetic metabolites [11, 12].

2 Materials and methods

The measurements of OPR_{net} and OUR were performed with cultures of *G. sulphuraria* SAG 108.79, which was provided by the Culture Collection of Algae at Goettingen University in Germany (SAG). The stock cultures were maintained in 200 mL Erlenmeyer flasks with a culture volume of 100 mL. The flasks were placed in an incubator (Multitron AJ190, FA. HT Infors) under continuous and external illumination with a photon flux density of $75 \mu\text{mol m}^{-2} \text{s}^{-1}$, measured with a planar light sensor (LI190, FA LiCOR, Germany). The temperature was kept constant at 40°C . The microalgal cultivation was undertaken in a sterilizable bubble column, the photobioreactor screening module (PSM), with a working volume of 700 mL, as previously described earlier [13–15]. The PSM consists of a glass cylinder, which is sealed with a stainless steel flange and an ethylene propylene diene gasket at the bottom and the top. The length of the glass cylinder is 500 mm and the diameter is 50 mm resulting in a surface to area ratio of $80 \text{ m}^2 \text{m}^{-3}$ [14, 15]. The cultivation temperature was 45°C and the photon flux density was constant at $300 \mu\text{mol m}^{-2} \text{s}^{-1}$, which was measured with a submersible spherical light sensor (US-SQS/L, ULM-500, FA Walz, Germany) in the center of the PSM (filled with deionized water).

The medium for stock cultures and cultivations in the PSM was prepared according to Gross and Schnarrenberger (1995), in which the concentrations of $(\text{NH}_4)_2\text{SO}_4$ was 32.4 g/L, the amount of KH_2PO_4 and $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ were increased fivefold and glycerol was added with 20 g/L [9]. The pH was adjusted to 2.0 with 6.0 M HCl. The air gas flow rate in the PSM was 2.0 L/min, controlled by a mass flow controller (FA Bronkhorst High-Tech B.V.), with a corresponding volumetric oxygen transfer coefficients of $17.02/\text{h} \pm 0.10$ and a gas hold up of $55.29 \text{ mL} \pm 0.08$, which results in a headspace of 233 mL after deducting the volumes of the cooling device, sample port, and DO-probe. The dissolved oxygen (DO) was measured with the optical oxygen sensor VisiPerm 225 DO (FA Hamilton, Germany). All experiments were performed in triplicates.

In the following equations every oxygen displacement, volumetric and specific uptake as well production rate are by definition positive. According to the double gassing-out method, the oxygen displacement rate by nitrogen (ODR_{N_2}) of every reactor needs to be known and is measured by change of oxygen

concentration over time under abiotic conditions ($dC_{O_2A} dt^{-1}$) within the medium before inoculation as described earlier [4]:

$$\frac{dC_{O_2A}}{dt} = - ODR_{N_2} \quad (1)$$

During the fermentation of microalgae and while replacing the air supply by gaseous nitrogen, the change in oxygen concentration is described under conditions of light supply ($dC_{O_2L} dt^{-1}$) and under conditions without illumination ($dC_{O_2D} dt^{-1}$), respectively, with the following equations:

$$\frac{dC_{O_2L}}{dt} = - OUR_L - ODR_{N_2} + OPR_{net} \quad (2)$$

$$\frac{dC_{O_2D}}{dt} = - OUR_D - ODR_{N_2} \quad (3)$$

The corresponding OPR_{net} is created in the light saturated zone of the PBR in mixotrophic or photoautotrophic processes. The OPR_{net} is the difference in the slopes of the descending curves with and without illumination (4). The $OUR_{L/D}$ consists of the cell respiration in the presence of an organic carbon source and the photorespiration, which occurs predominately at high temperatures as well as at high oxygen to carbon dioxide concentrations [16]:

$$OPR_{net} = \left| \frac{dC_{O_2D}}{dt} \right| - \left| \frac{dC_{O_2L}}{dt} \right| \quad (4)$$

In addition to the volumetric oxygen production and consumption rates, the determination of their specific rates ($our_{L/D}$ and opr_{net}) can be calculated with a known biomass concentration (X) during the process (Eq. [5 and 6]):

$$OUR_{L/D} = our_{L/D} \cdot X \quad (5)$$

$$OPR_{net} = opr_{net} \cdot X \quad (6)$$

The values of the biomass concentration for this calculation were computed using the following linear dependence, which was figured out in preliminary experiments in our institute with a coefficient of determination as 0.98:

$$X = 0.4705 \cdot OD_{750 \text{ nm}} + 0.0716 \quad (7)$$

The Henry-coefficient for oxygen was calculated according to Sander for aqueous solutions [17] for a temperature of 45°C . The volume fraction in the supplied air was assumed as 20.94 % at ambient pressure. Thus the Henry-coefficient was determined with $8.97 \cdot 10^{-4} \text{ mol L}^{-1} \text{ bar}^{-1}$.

3 Results and discussion

The air supply via pressurized air is switched to pure gaseous nitrogen during illumination of mixotrophic cultivation of the acidophilic red algae *G. sulphuraria*. As a result, the DO signal decreased over time as shown in Fig. 1A (for an $OD_{750 \text{ nm}}$ of 6.49 which is equal to $3.13 \text{ g}_x/\text{L}$) at the position of I_a , which can be described mathematically by Eq. (2). After 1.5 min., when the PBR was supplied with gaseous nitrogen, the aeration was reset to pressurized air increasing the DO again to equilibrium concentration at $t = 0 \text{ min}$ and this was subsequently performed twice

Figure 1. (A) Measurement of the OPR_{net} and OUR during illumination (L, I_{a-c}) and without illumination (D, II_{a-c}) at an $OD_{750\text{ nm}} = 6.49$, which is equal to $3.13\text{ g}_x/\text{L}$; (B) the ODR as slope III (B) under abiotic conditions and the decrease in oxygen concentration represented by the slopes I_{a-c} during illumination and the steepest slopes II_{a-c} without illumination.

(I_b and I_c). After 22 min. the illumination was switched off resulting in a decrease of DO signal by almost 7.4 % during further incubation due to complete change from mixotrophic cultivation to heterotrophic cultivation conditions. After the equilibrium of oxygen concentration was achieved in the culture broth, the measurement of OUR_D was again performed three times, as described previously for submerged fixed bed reactors [4]. Thereafter, the illumination was turned on at $t = 46\text{ min.}$ in order to achieve the equilibrium state of dissolved oxygen signal related to the start of measurement. The drop in DO signal without illumination is described by Eq. (3) and represents the real OUR in the process ($OUR_D = OUR$). The slopes of the curve I_{a-c} , II_{a-c} , and III were evaluated for oxygen concentrations between $1.25 \cdot 10^{-4}\text{ mol L}^{-1}$ and $5.00 \cdot 10^{-5}\text{ mol L}^{-1}$ (Fig. 1B). Since the ODR_{N_2} is constant in this range, changes in the slopes of curves representing oxygen concentrations are exclusively based on mitochondrial respiration in case of cultivation performed without illumination. This is shown by the difference of slopes between ODR_{N_2} and the sum of OUR_D and ODR_{N_2} . In contrast to that, the overall absolute values of slopes during illumination I_{a-c} (Fig. 1B) are lower due to photosynthesis. During this condition the cells are continuously producing oxygen even if the air supply was switched over to pure nitrogen in presence of an organic carbon source. Therefore, the oxygen concentration was shifted to higher values over time compared to the measured values of II_{a-c} , which have a steeper slope in comparison (Fig. 1B).

The values of OUR and the corresponding our are based on sum of mitochondrial respiration and photorespiration. But it is reasonable to assume that these values result predominantly from mitochondrial respiration independently of light saturation in the PBR, since mitochondrial respiration is not affected by light [18]. Furthermore the complex process of photorespiration

Figure 2. The OPR_{net} , OUR , opr_{net} and our as a function of the $OD_{750\text{ nm}}$ in a mixotrophic cultivation of *G. sulphuraria* using glycerol as an organic carbon source. Black dots – OUR , black circle – OPR_{net} , red dots – our , red circle – opr_{net} , gray triangles – PFD , error bars indicate the standard deviation of $n = 3$ experiments.

takes place under certain circumstances like high oxygen concentration, which may not be the case in our experiments, and is many times smaller than the mitochondrial oxygen respiration rate [6]. In addition this is especially valid under mixotrophic conditions when there is a surplus of organic carbon source in the media or may be produced by the organism itself [19]. Under such circumstances, the possible fraction of photorespiration to the respiration of the cells during the measurement can be neglected.

In Fig. 2, the OUR and OPR_{net} as well as the our and opr_{net} , which were calculated using equations 5–7, were

plotted as a function of cell density represented by the $OD_{750\text{ nm}}$. The OUR increases in a linear way between an $OD_{750\text{ nm}}$ of 0.1 and 3.0. Above an optical density of 5.0, the OUR is constant at $2.59 \cdot 10^{-3} \text{ mol O}_2 \text{ L}^{-1} \text{ h}^{-1}$. This is also displayed by a corresponding decrease of opr from $1.44 \cdot 10^{-3} \text{ mol O}_2 \text{ g}^{-1} \text{ h}^{-1}$ to the lowest value of $3.58 \cdot 10^{-4} \text{ mol O}_2 \text{ g}^{-1} \text{ h}^{-1}$ at an $OD_{750\text{ nm}}$ of 14.43. This is based on the fact that at this cell concentration, the microalgae are not sufficiently supplied by oxygen any more for their facultative heterotrophic growth, exercised in dark zones of the PBR [20]. The OPR_{net} is relatively constant at an $OD_{750\text{ nm}}$ of 5.0 with values around $4.45 \cdot 10^{-4} \text{ mol O}_2 \text{ L}^{-1} \text{ h}^{-1}$ and further increase in $OD_{750\text{ nm}}$ results in a slight decrease of OPR_{net} . In contrast, the opr_{net} starts with $2.73 \cdot 10^{-3} \text{ mol O}_2 \text{ g}^{-1} \text{ h}^{-1}$ at lowest $OD_{750\text{ nm}}$ and decreases exponentially with increasing $OD_{750\text{ nm}}$. This results in the lowest opr_{net} of $1.29 \cdot 10^{-5} \text{ mol O}_2 \text{ g}^{-1} \text{ h}^{-1}$ at an $OD_{750\text{ nm}}$ of 14.43 probably due to increased shading effects resulting in the reduction of photosynthetically active volume of the PBR with higher cell densities [15, 21]. The amount of values of OUR and OPR_{net} is identical around an $OD_{750\text{ nm}}$ of 1.56, which is in good correlation to a remaining PFD of $83 \mu\text{mol m}^{-2} \text{ s}^{-1}$ measured in the center of the PSM (Fig. 2). Nevertheless, we can just quantify the OPR_{net} , since in mixotrophic cultivation, cellular respiration, and production of oxygen through uptake of organic as well as inorganic carbon sources take place concurrently and it is not possible to measure the gross volumetric OPR [22, 23].

4 Concluding remarks

The present study investigated an applicable on-process method for the quantification of the OPR_{net} and OUR in nonstirred PBR. Therefore, information about the increasing shading effect, metabolic state, and occurring limitations of the culture is quickly accessible during the mixotrophic fermentation of microalgae. With this tool, it will be possible to intervene in the process, if it is already known which relation is necessary between OUR and OPR_{net} in the PBR for the product of interest. This could be done by an increase in the light intensity in order to get a higher OPR_{net} or raising the gas flow rate and therefore, the volumetric oxygen transfer coefficient which is necessary to fulfill the oxygen demand at higher cell densities. Further information about the relation of gross OPR, net OPR, and OUR at a cellular level could lead to precise calculation of the light saturated and dark zones in a PBR with the established method. But it should be noted that apart from its benefits, the method has its limitations similar to the dynamic method applied to stirred tank reactors. It is not applicable if the DO concentration is too low [24]. Furthermore, we can only measure the OPR_{net} and not the total OPR due to concurrent oxygen production and consumption by microalga. To further evaluate the significance of the new method, the relation between OPR_{net} and OUR can be investigated using different microalgae, which have different amounts of pigments per cell resulting in changes in opr_{net} , and also by applying this method to closed PBRs with different shapes as well under diverse light intensities.

Practical application

This new method for the quantification of the OPR and OUR in mixotrophic fermentations of microalgae could help maximize the productivity of cell dry weight as well as the formation of different products, which are dependent on a sufficient light supply to microalgal cultures. Furthermore, the adapted double gassing-out method is even applicable to phototrophic cultivations. Since growth and product formation are highly dependent on the light saturation in a PBR, there is an urgent need to know the relation between photosynthesis and cellular respiration. By applying this method, one can understand the limitations during growth with an easy and fast on-process measurement. It will be even possible to correlate the results of this measurement with data achieved by mathematical modeling showing the limitations of microalgae fermentation as a function of OPR_{net} and OUR.

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Nomenclature

C_{O_2A}	[mol L ⁻¹]	concentration of oxygen in the medium under abiotic condition
C_{O_2L}	[mol L ⁻¹]	concentration of oxygen in the medium during illumination
C_{O_2D}	[mol L ⁻¹]	concentration of oxygen in the medium without illumination
ODR_{N_2}	[mol L ⁻¹ h ⁻¹]	oxygen displacement rate by nitrogen
OUR_L	[mol L ⁻¹ h ⁻¹]	volumetric oxygen uptake rate during illumination
OUR_D	[mol L ⁻¹ h ⁻¹]	volumetric oxygen uptake rate without illumination
our	[mol g ⁻¹ h ⁻¹]	specific oxygen uptake rate
OPR_{net}	[mol L ⁻¹ h ⁻¹]	net volumetric oxygen production rate
opr_{net}	[mol g ⁻¹ h ⁻¹]	net specific oxygen production rate
X	[g L ⁻¹]	biomass concentration
$OD_{750\text{ nm}}$	[-]	optical density at a wavelength of 750 nm

5 References

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