A method to compute instantaneous oxygen evolution rates in cyanobacterial cultures grown in shake flasks

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Cyanobacteria have been attracting attention for various biotechnological applications due to their ability to carry out oxygenic photosynthesis. The oxygen evolution rate (OER) of cyanobacterial cultures is a key physiological parameter, which is typically measured offline by using oxygen electrodes. In situ measurement of OER has been reported in a photobioreactor fitted with a dissolved oxygen (DO) probe. Here, we show that the OER can be estimated from online measurements of the DO levels by using sensors fitted in shake flasks, which are widely used for preliminary characterization of microbial strains. We study how the DO levels change under diurnal light regime and different growth conditions in two strains of *Synechococcus elongatus*, namely, PCC 7942 and PCC 11801, in shake flasks. We use the DO levels coupled with the knowledge of the overall gas-liquid mass transfer coefficient for oxygen ($k_{La}$) to compute the OER.

For the growth conditions tested, the cumulative oxygen evolved in a day correlated with the increase in biomass suggesting that the calculated OER, in turn, might provide an estimate of the organism’s growth rate. We believe that the method may be widely applicable for the growth characterization of organisms that perform oxygenic photosynthesis.

KEYWORDS
abiotic factors, cyanobacteria, dissolved oxygen profile, oxygen evolution rates, PreSens technology, shake flask

1 | INTRODUCTION

Cyanobacteria are photosynthetic prokaryotes that are capable of harvesting natural light and atmospheric CO₂ as the sole sources of energy and carbon. These photoautotrophs are being genetically engineered to develop carbon-neutral-green cell factories for the production of various platform chemicals, fuels, and sensors. Metabolic models and ¹³C flux analysis studies have helped gain a deeper understanding of cyanobacterial metabolism. Understanding the physiological characteristics of these strains would help optimize their productivity. Among others, the oxygen evolution rate (OER) is an important health parameter of the culture and its monitoring is desirable even during the initial studies. The OERs of cyanobacterial cultures can be measured offline by using Clark-type oxygen electrodes. Some photobioreactors enable in situ measurement of OER by using similar principles but with a series of automated steps that may involve brief dark adaptation and switching off the aeration. Other bioreactors are instrumented with probes for online monitoring.
of dissolved oxygen (DO) levels although estimation of OER from such data is not reported. Initial screening and characterization of strains are performed in shake flasks. However, there are no reports on real-time, in situ measurements of OER in shake flasks.

In this study, we explored the possibility of determining the OER by measuring the DO level in shake flasks. We have monitored the instantaneous DO levels of two cyanobacterial cultures, a model strain *Synechococcus elongatus* PCC 7942 and its fast growing neighbor *S. elongatus* PCC 11801 under different growth conditions in flasks, having optical probes to study the effect of growth conditions on the OER.

## 2 | MATERIALS AND METHOD

The *S. elongatus* strains PCC 7942 and PCC 11801 were cultivated in 50-mL BG11 media at 38°C under ambient (0.04% CO₂) or 0.5% CO₂ in a shaker incubator (Model: LT-X, Adolf Kuhner AG, Switzerland) at different rpm (80 and 60 rpm) having a pitch of 5 cm and illuminated with sinusoidal light. The light used for the experiments was a combination of red and blue LEDs (2,1) adjusted to two light intensities of 120 and 300 μE·m⁻²·s⁻¹ subjected to 14 hours sinusoidal light pattern.

The DO levels were measured in 250-mL specialized baffled flasks with optical probes, which was combined with a module for online data acquisition (SP-PSt3, PreSens GmbH, Regensburg, Germany). The DO levels were recorded every 2 minutes using the proprietary software from the vendor, SFRS. The cell pellet of the known value of OD 730nm was harvested and dried overnight at 80°C to measure the dried weight of the cell.

## 3 | RESULTS AND DISCUSSION

Cyanobacteria generate oxygen by the process of oxygenic photosynthesis and consume it during respiration. Several reports on cyanobacterial cultures have shown above-saturation DO levels during light phase and below-saturation during dark for light-dark cycles. These studies were conducted under constant light intensity unlike natural sunlight that approximates a sine function. Therefore, we conducted the growth experiments in specialized 250-mL baffled flask (Erlenmeyer flasks) embedded with DO optical sensors under sinusoidal light-dark cycle (Figure 1C). During the day, we observed a gradual rise in DO levels that followed the trajectory of the light profile peaking at mid-day for both the *S. elongatus* strains (Figure 1). *S. elongatus* PCC 11801 exhibits faster growth phenotype than its closest neighbor *S. elongatus* PCC 7942 and a representative OD profile of the strains under similar growth conditions has been shown in Figure 2. The higher growth rate of *S. elongatus* PCC 11801 was reflected in a greater amplitude of DO oscillations for this strain (Figure 1A,B). Increased growth rate is a result of increased photosynthetic activity of the organism, thereby evolving greater amount of oxygen. Since cyanobacterial growth is dependent on the incident light intensity and CO₂ levels, we monitored the DO profiles under different light and CO₂ concentrations for the strains *S. elongatus* PCC 7942 and PCC11801 in shake flasks. Increase in light and CO₂ concentrations increased the amplitude of DO level for the strains (Figure 1A,B). It is well known that these strains show higher growth rates at higher light and CO₂ levels. Interestingly, an opposite effect was observed for both strains when grown at different agitation rates, and the amplitude of DO oscillation was lower for higher rpm (Figure 1A,B). This might be due to higher kLa under increased agitation but relatively unaltered OER (Equation 1). We ignored the effect of biomass on kLa as the reported change in kLa is under 8% for cell OD of 8.0. The cultures in the present study rarely cross OD 730nm of 1.0.

The cyanobacterial DO profile during the dark phase remained near saturating levels (Figure 1A,B), indicating no oxygen limitation in the cultures and further suggesting low respiration rate. The DO profiles of photoautotrophs are very different from that of heterotrophs. While the rise in DO profile is an indicator of photosynthesis for cyanobacteria, it signifies cell death in heterotrophic organism. Heterotrophs utilize oxygen via respiration during growth exhibiting significant reduction in DO levels, unlike cyanobacterial cultures. The O₂ uptake rate (OUR) for heterotrophs is calculated based on kLa and concentration difference driving force (C*O₂ - C₀₂). Therefore, assuming pseudosteady state for the DO levels, we propose that the instantaneous O₂ evolution rate (OER) for photoautotrophs can be estimated from Equation (1)

\[
\text{OER} = -k_{La} \times (C_{O_2}^* - C_{O_2}),
\]
**FIGURE 1** Online dissolved oxygen (DO) profiles of the cyanobacterial strains *S. elongatus* PCC 7942 and PCC 11801. A, The effect of peak light intensity (120 or 300 μEm⁻² s⁻¹) and agitation (60 or 80 rpm) on the DO levels for *S. elongatus* PCC 7942 cultures grown under ambient CO₂. B, The effect of CO₂ concentrations (ambient or 0.5% CO₂) and agitation (60 or 80 rpm) on the DO levels of *S. elongatus* PCC 11801 under 300 μEm⁻² s⁻¹ light intensity. C, Light used for the experiments was in a combination of red and blue LED (2,1) illuminated with two different light intensities following a light phase of 14 hours in sinusoidal curve and 10 hours of dark. DO levels averaged over moving window of 20 minutes were plotted. The experiments were performed in duplicates.

**FIGURE 2** The growth profile of *S. elongatus* PCC 7942 and PCC 11801 strains grown under 300 μEm⁻² s⁻¹ light intensity in shaker incubator aerated at 80 rpm with ambient air in chamber. The error bars are based on experiments performed in triplicate.

where \( k_L a \) is the overall mass transfer coefficient for O₂ and \( C_{O_2}^* \) and \( C_{O_2} \) are the saturation and actual oxygen concentrations, respectively. For the chosen shake flask geometry, liquid levels, and the agitation conditions, the \( k_L a \) values were 4.2 and 8.9 hours⁻¹ for 60 and 80 rpm, respectively, for a pitch of 5 cm. The \( k_L a \) was measured by the gassing out-gassing in method as described previously.²¹,²²

It has been observed that during the day, \( C_{O_2} \) is supersaturated for cyanobacteria and the peak OER, calculated using Equation (1), was in the range of 0.38 to 3.5 mg/L/h, depending on growth conditions. The instantaneous OER peaked at mid-day, coinciding with the peak light intensity. Since the calculated OER was observed to be related with the growth phenotype, the OER might act as an indirect measure for growth. To that end, it was of interest to examine if a direct correlation exists between the biomass accumulation and the cumulative O₂ evolved (cOER) in a day. The biomass was estimated offline and the cOER was computed for a specified time period \((t_1 - t_2)\) from the area under the curve (Figure 3A) using Equation (2). A correlation was observed with \( R^2 = 0.99 \) (Figure 3B), suggesting that the instantaneous DO data might provide a rough estimate of the organism’s growth. Although the correlation holds good under the tested conditions, it might deviate when the culture attains a higher biomass due to self-shading and unequal exposure to light intensity. Moreover, a prolonged cultivation without CO₂ supplementation might change the pH of the media and thereby affect the DO levels. Since the main focus of this study was to determine instantaneous OER under sinusoidal conditions.
light condition in shake flasks, we have not considered the continuous illumination condition that might have a different dynamics, which needs to be investigated in future

\[
O_2 \text{ evolved between } t_1 \text{ and } t_2 = k_L a \times \left( \frac{A(t_1 - t_2)}{100} \right) \times C^*_O_2,
\]

where \(C^*_O_2\) is 7 mg \(O_2/L\) (or 0.21 mmole of \(O_2/L\)) under ambient air.

4 | CONCLUSION

This work presents a novel method to estimate instantaneous OER for cyanobacterial cultures grown in shake flasks. The method requires the knowledge of \(k_L a\) for oxygen under the pertinent growth conditions, which can be readily measured experimentally by the gassing-out, gassing-in method, as described previously.\(^ {21,22}\) The \(k_L a\) values from the published literature can be used when available for the matching conditions including agitation speed and pitch, flask geometry, and liquid levels in flask.\(^ {15,23}\) Furthermore, the excellent correlation observed between the biomass accumulation and cumulative oxygen evolution provides an alternative, noninvasive method to estimate growth and will improve the extent of preliminary characterization of cyanobacteria in shake flasks. The proposed method will eliminate the sudden change in growth environment that the culture goes through while collecting an offline sample. Furthermore, this method is likely to be more sensitive than the conventional, offline method of measuring optical density (\(OD_{730nm}\)). We argue that the method can be applied widely to characterize growth of cyanobacteria and microalgae under different cultivation conditions and abiotic stress factors. While the OER rates appear to be within measurable limits for the growth conditions in this proof-of-concept study, the lower and upper limits of quantitation of instantaneous OER and cumulative oxygen evolution need to be determined systematically. Furthermore, the effect of various chemicals and other stressors on the \textit{in situ} oxygen sensors needs to be investigated. Finally, the ability to quantify OER is subject to reliable measurement of the degree of supersaturation, \(C_{O_2} - C^*_O_2\). From Equation (1), we see that for a given value of OER (and in turn for a given growth condition), the degree of supersaturation is inversely proportional to the value of \(k_L a\). Thus, the difference \(C_{O_2} - C^*_O_2\) may be relatively small and may not be measured reliably at high agitation rates, which result in high \(k_L a\).

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CONFLICT OF INTERESTS
The authors have no conflict of interest relevant to this article.

AUTHOR CONTRIBUTION
Annesha Sengupta and Pramod P. Wangikar designed the research, analyzed the data, and wrote the manuscript. Annesha Sengupta performed the experiments. The authors have reviewed the manuscript and have agreed to the order of the authorship.

ETHICS STATEMENT
This article does not contain any studies with human participants or animals performed by any of the authors.

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