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A combined application of tunable diode laser absorption spectroscopy and isothermal micro-calorimetry for calorespirometric analysis

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ABSTRACT

Calorespirometry is the simultaneous analysis of the rate of heat emission (R_q), O_2 consumption (R_{O2}) and CO_2 production (R_{CO2}) by living systems such as tissues or organism cultures. The analysis provides useful knowledge about thermodynamic parameters relevant for e.g. biotechnology where parameter based yield maximization (fermentation) is relevant. The determination of metabolism related heat emission is easy and normally done by a calorimeter. However, measuring the amount of consumed O_2 and produced CO_2 can be more challenging, as additional preparation or instrumentation might be needed. Therefore, tunable diode laser absorption spectroscopy (TDLAS) was investigated as an alternative approach for respirometric analysis in order to facilitate the data collection procedure. The method determines by a spectroscopic laser non-invasively CO_2 and O_2 gas concentration changes in the respective vial headspaces. The gathered growth data from *Pseudomonas aeruginosa* cultured in two different scarce media was used to compute respiratory quotient (RQ) and calorespirometric ratios (CR_{CO2} [R_q/R_{CO2}], CR_{O2} [R_q/R_{O2}]). A comparison of the computed (experimental) values (for RQ, CR_{CO2} and CR_{O2}) with values reported in the literature confirmed the appropriateness of TDLAS in calorespirometric studies. Thus, it could be demonstrated that TDLAS is a well-performing and convenient way to evaluate non-invasively respiratory rates during calorespirometric studies. Therefore, the technique is definitively worth to be investigated further for its potential use in research and in diverse productive environments.

1. Introduction

Calorespirometry is the simultaneous analysis of the rates of heat emission (R_q), O_2 consumption (R_{O2}) and CO_2 production (R_{CO2}) by living systems (single cells, tissues, or organism cultures from culture or environmental samples). Calorespirometry provides useful information about efficiency in metabolic turnover and potentially on corresponding metabolic pathways (aerobe or anaerobe). Especially in biotechnology where yield maximization of biomass or alternatively of a certain product is of interest, calorespirometry helps to understand system thermodynamics, enabling an optimization of culturing conditions (Hansen et al., 2004; Schuler and Marison, 2012; Schuler et al., 2012). R_q is normally determined using an isothermal heat conduction or power compensation calorimeter (Wadsö and Hansen, 2015). A thermoelectric module located between the sample and the calorimeter heat sink measures the emitted heat and transforms it into an electronic signal. An accurate determination of R_{CO2} (and R_{O2}) is more difficult but has been described (Criddle et al., 1991; Criddle et al., 1990). The approach was based on the determination of the heat produced by the reaction of CO_2 with a 0.4 M NaOH solution (absorbent) contained in the reaction container as described by $CO_{2\,(g)} + 2OH^-_{(aq)} \rightarrow CO_3^{2-}{}_{(aq)} + H_2O_{(aq)}$ with $\Delta H_{abs} = -108.5\ kJ{\cdot}mol^{-1}$ (Wadsö and Hansen, 2015).

By subtracting heat rate values of samples lacking the absorbent from those including the absorbent, the amount of produced CO_2 can be determined and conclusions on metabolic efficiencies drawn. The described approach is used as a gold standard in works of calorespirometric analysis such as feeding control of biopolymer production, evaluations of soil organic matter decomposition, and metabolic assessments of microorganisms, plants, animals, fruits, and other living systems (Barros et al., 2015; Criddle et al., 1990; Hansen et al., 2002; Nogales et al., 2013; Regan et al., 2013; Rohde et al., 2016; Wadsö, 2002). The technique is limited by its invasive nature, leading to the absorption of the entire CO_2 in the system. The effect of CO_2 degassing in combination with the absorbent removes the entire CO_2 from the medium, generating a shift in the carbonate equilibrium ($HCO_3^- \rightarrow CO_2 + OH^-$). Hydroxide ions ($OH^-$) arise therefrom resulting in a pH increase potentially influencing the cell growth potential

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in weakly buffered systems. A more convenient approach to perform such experiments is the use of optrodes. Several calorespirometric studies used modified calorimeters with optrodes included in the measurement vessel to determine oxygen consumption. For example, moth metabolism or oxygen consumption by Maple tree leaf buds were studied using such modified instruments (Itoga and Hansen, 2009; Neven et al., 2014). Tunable diode laser absorption spectroscopy (TDLAS) is discussed in this work as an alternative approach capable to determine CO_2 production and O_2 consumption rates non-invasively and easily. The technology uses a laser to measure relative CO_2 (λ = 2000 nm) and O_2 (λ = 760 nm) concentrations in the sample headspace. Of major interest is the investigation of a combined use of IMC and TDLAS for calorespirometric studies based on one single model organism (*Pseudomonas aeruginosa*). Measurements were run in parallel and in different media (M9 and M9 + nitrate) to compare calorespirometric ratios under different metabolic conditions (respiration and denitrification [$NO_3^- \rightarrow NO_2^- \rightarrow NO + N_2O \rightarrow N_2$ (g)]). Biomass (cell count per ml medium, optical density, and protein concentration), substrate consumption (glucose concentration), gas concentrations and metabolic byproducts (nitrite concentration) were used to interpret the obtained respiratory quotient R_{CO2}/R_{O2} (RQ) and calorespirometric ratios R_q/R_{CO2} (CR_{CO2}) and R_q/R_{O2} (CR_{O2}). Those results were compared to values found in the literature to gain insight into reliability and applicability of TDLAS in the field of calorespirometry.

2. Materials and methods

Pseudomonas aeruginosa was chosen as a model organism for calorespirometric studies due to its aerobe and denitrifying metabolic characteristics. The microorganism was purchased from ThermoScientific™ (Quanti-Cult Plus, ATCC 9027, < 100 colony forming units/0.1 ml) and maintained at 37 °C in minimal salt medium (M9, Glucose concentration ~3.9 millimolar [mM]) enriched with 1% BME vitamins. In addition the same medium was supplemented with 100 mM nitrate (M9N) (Williams et al., 1978), which allowed a calorespirometric assessment of denitrification pathways.

Two sample sets (4 ml glass vials, TA Instruments) were prepared for the study filled with either 0.9 ml M9 (50 samples) or 0.9 ml M9N (80 samples). Samples were inoculated each with 0.1 ml media containing 1,000–2,000 CFUs (used microbial units were cultured in their respective media). Inoculated vials were capped under laminar flow and transferred into an isothermal micro-calorimeter or an incubator set at 37 °C. The M9 and M9N sample sets were further split in two groups for calorimetry and TDLAS measurements, made in parallel (i.e., 1 M9 + 1 M9N set for calorimetry and 1 M9 + 1 M9N set for TDLAS).

The metabolic heat emission rate was monitored by a 48 channel isothermal micro-calorimeter (TAM III, TA Instruments, Delaware, USA) set to an incubation temperature of 37 °C. Two sets of 10 inoculated vials and non-inoculated control samples (2 ×) filled with either M9 or M9N were tested. At the same time point, tunable diode laser absorption spectroscopy (TDLAS) analyzed the respiratory activity of growing bacteria by measuring O_2 (λ = 760 nm) and CO_2 (λ = 2000 nm) concentration changes in the headspaces of vials kept outside the calorimeter but within a thermostatic oven at the same temperature (i.e., 37 °C). Experiments were started with preheating the TDLAS analyzers (Lighthouse Instruments, Charlottesville) for minimally 30 min followed by a calibration with certified standards of known concentrations (0%, 4%, 8% and 20%). Flushing the TDLAS measurement channels with nitrogen (4 l/min) allowed a headspace analysis with improved signal to noise ratio. For both sample sets incubated in parallel in the oven (M9 and M9N) CO_2 and O_2 concentrations of 15 inoculated and 2 reference vials were tested three times every 2 to 3 h during ~100 h. Care was taken that the measurement time per sample was as short as possible to keep the sample temperature as close as possible to 37 °C. In addition, optical density (Bio Photometer plus, Eppendorf, λ = 595 nm) and CFU counts (per 0.1 ml media) were performed on tryptic soy agar (TSA) after each TDLAS measurement, with samples taken from an additionally prepared sample stock. To monitor nitrate reduction, glucose consumption, and protein aggregation, every 2 to 4 h one inoculated sample filled with M9N was taken from the sample stock and frozen at − 82 °C. At the end of the experiment the respective glucose content was determined for all thawed samples with an Accu-Chek Aviva diabetes device (detection range 0.6 mM–33.3 mM) from Roche Diagnostics. The device was calibrated with glucose standard dilutions of 1, 5, 10 and 25 mM prepared in M9 and M9N. Nitrite concentrations of the same samples were determined by colorimetric analysis using the Griess reagent (Sigma Aldrich). Assay calibration was performed with 0, 15, 40, 60 and 80 nmol/ml nitrite and absorption measured at a wavelength of 500 nm. Protein concentration changes in the medium were identified by the Bradford assay. Dependent on the protein concentration a respective colorimetric change occurred measurable at a wavelength of 586 nm. A standard curve for the assay was performed with 0, 20, 40, 100, 500 and 1000 µg/ml of bovine serum albumin (BSA) per assay.

The data analysis included integration of the measured heat flow data resulting in sigmoidal heat profiles. The respective medians of CO_2 production, O_2 consumption (inverse values) and heat were fitted using the Gompertz function in R ("grofit" package). Molar amounts of CO_2 and O_2 were then determined, respecting the size of the vial headspace (3.31 ml) and assuming that CO_2 and O_2 behaved as ideal gases. Using the ideal gas law, we estimated that in our condition (vials closed at 22.5 °C and 1.029 bar, followed by an increase of temperature to 37 °C) 1 mol of gas had a volume of 23.89 l. Calculations relating to the respiratory quotient (RQ: R_{CO2}/R_{O2}) and calorespirometric ratios for CO_2 (CR_{CO2}: R_q/R_{CO2} [kJ·mol^{-1}]) and O_2 (CR_{O2}: R_q/R_{O2} [kJ·mol^{-1}]) were performed for each data point between 0 and ~100 h by using the fitted values. Finally, standard deviations were determined for all relevant values.

3. Results

Pseudomonas aeruginosa cultured in M9 produced after ~100 h and in vials with a headspace volume of 3.31 ml, at the maximum (fitted data) 4.68 ± 0.08% CO_2 (6.49 ± 0.11 µmol), consumed 8.70 ± 0.22% O_2 (12.06 ± 0.31 µmol) and emitted 5.48 ± 0.19 J (Fig. 1A–B). Optical density (OD) and bacterial cell number (BCN/ml) reached their respective maxima after 47 h (absorption = 0.476) and 43 h ($2.1 \cdot 10^9$ cells) respectively (Fig. 1C–D, consistent with the development of the CO_2, O_2 and heat profiles. Growth of *P. aeruginosa* in nitrate supplemented M9 medium (M9N) was, in comparison, marginally lagged and reached after 100 h CO_2, O_2 and heat values of maximally 5.43 ± 0.07% (7.53 ± 0.10 µmol), 9.06 ± 0.22% (12.56 ± 0.31 µmol) and 6.38 ± 0.06 J (Fig. 1A–B) respectively. The related OD and BCN/ml values attained the top after 61 h (absorption = 0.295) and 69 h ($3.8 \cdot 10^9$ cells). A slight but steady drop in biomass after reaching the maximum was observed in both media (Fig. 1C–D).

A first decrease of the glucose concentration in the medium (~3.9 mM) could be observed after 17 h whereas after 26 h the carbon source was entirely depleted (Fig. 1E). The protein concentration started to increase after 17 h reaching a maximum amount of 59 µg/ml after 40 h incubation (Fig. 1E). Nitrite concentrations (resulting from denitrification) increased intensively after 20 h, reached a maximum of 201 nmol/ml within 26 h and equilibrated 20 h later at a level of approximately 19 nmol/ml (Fig. 1 F, dashed triangles). In addition, the main peak of the heat flow profile of samples containing M9N (Fig. 1 F brown curve) was observed at 16 h (65.2 ± 1.2 µW) whereas a second smaller peak was detected after 25 h (39.6 ± 0.9 µW). For growth in M9, two heat flow peaks were detected after 15 h (35.5 ± 5.6 µW) and 40 h (43.8 ± 1.7 µW) (Fig. 1F, orange curve). Flat heat flow profiles at baseline level (0 ± 0.2 µW) were observed for all tested references. Similarly, gas profiles remained flat varying very little around their