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Improving the carbon balance of fermentations by total carbon analyses

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ABSTRACT

Carbon balancing of microbial fermentations is a valuable tool for the evaluation of the process performance and to identify the presence of undesired by-products. In this study, we demonstrate the relevance of total carbon (TC) analysis for carbon balancing in fermentations with the wild-type of *Corynebacterium glutamicum* by (i) quantifying significant amounts of dissolved inorganic carbonic species (TIC) in the culture medium and (ii) determining the effective (mass) carbon content of the biomass fraction (M_{CX}). In principle, TC based carbon balancing yielded at fully matching carbon balances. Thus, the application of our TC approach for the accurate detection of TIC and M_{CX} increased the total carbon recovery in standard batch fermentations with *C. glutamicum* on glucose from about 76% to carbon closures of 94–100% in contrast to conventional approaches. Besides, the origin of the missing 6%-gap could be attributed to incomplete quantification of all carbon sources in the liquid phase. To conclude this study, the concept of TC-based balancing was transferred to an L-lysine production process, successfully quantifying relevant system carbon fractions, which resulted in matched carbon recoveries.

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1. Introduction

Since its discovery by Lavoisier, the law of conservation of mass is an indispensable tool for balancing (bio-) chemical conversion processes. To analyze cultivations, for instance using microorganisms, particularly carbon (C-) balancing turned out to be a powerful approach for studying and evaluating the fate of C-containing educts (e.g. glucose) to C-containing products such as biomass, by-products, and gaseous carbon dioxide ($CO_{2,g}$). Accurate carbon balancing is essential for successful bioprocess development to qualify fermentation results properly. Based on closed C-balances, conclusions for optimizing strains and processes can be drawn, finally establishing high product yields and conversion rates. If carbon balances unravel gaps, they can point to non-balanced, maybe non-expected by-products or reveal the improper functioning of detection devices and analytics. Notably, C-balancing is not only a valuable reflection of the physiological understanding of any given process, but also the basis for advanced metabolic flux studies [1–3].

Recovery calculations are based on mass balancing, consequently assuming that no further mass sinks or sources are considered except for those explicitly balanced. A commonly applied concept is the *black box model* balancing gaseous and liquid streams (containing cells) entering and leaving the boundary system as shown in Fig. 1. Consequently, aerobic conversion of substrate to biomass and extracellular products can be written in C-mole notation as [4–7]:

$$C_sH_{a,s}O_{b,s} + \lambda_n H_{a,n}O_{b,n}N_{c,n} + \lambda O_{2,g} - \lambda_x C_x H_{a,x}O_{b,x}N_{c,x} - \sum_i \lambda_{pj} C_{pj}H_{a,pj}O_{b,pj}N_{c,pj} - \lambda CO_{2,g} - \lambda H_2O = 0 \quad (1)$$

with s, n, x, and p_j encoding substrate, nitrogen source, biomass, and (by)-products, λ_i as (molar) stoichiometric coefficients and the subscripts (a, b, c, and d) as molar proportions of each element.

However, stoichiometric coefficients might be time-dependent, reflecting varying metabolic activities of the cells. Nevertheless, Eq. (1) assumes that the elemental composition of the cell mass stays constant and that no further, unbalanced reactants or products occur [8,9].

Usually, C-balancing is applied such that amounts of C-containing substrates are compared with measurements of liquid phase components (biomass and (by-)products) in addition to CO_2 of the exhaust gas. While this approach is commonly accepted and often applied, our contribution aims at pointing on the important aspect whether this balance fully covers all components.

One sensitive effector is the amount of CO_2 that is produced via cellular respiration. Unlike the rather poor solubility of O_2 in water,

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Nomenclature

C carbon

CDW	cell dry weight
CER	carbon dioxide evolution rate (mmol L^{-1} h^{-1})
CTR	carbon dioxide transfer rate (mmol L^{-1} h^{-1})
K_1,K_2	chemical equilibrium constant (mol L^{-1})
k_1, k_2	reaction rate constant for forward reaction (h^{-1})
k_{-1}, k_{-2}	reaction rate constant for reverse reaction (L mol^{-1} h^{-1})
$M_{C,X}$	mass carbon fraction in biomass in % (g g^{-1})
N	agitation speed (min^{-1})
n	molar quantity (mol)
p	total pressure (bar)
pO_2	dissolved oxygen saturation (%)
q_S	specific substrate uptake rate per unit biomass (g g^{-1} h^{-1})
Q/V_R	volumetric gas flow rate per unit volume liquid, vvm (L L^{-1} min^{-1})
R_C	carbon recoveries in % (mol mol^{-1})
RT	room temperature
RQ	respiratory quotient
T	temperature in reactor (°C)
t	time (h)
TC	total carbon
TIC	total inorganic carbon
TOC	total organic carbon
V_R	reaction volume (L)
WT	wild-type strain
X	biomass concentration in CDW (g L^{-1})
Y_{XS}	yield coefficient of biomass per unit substrate consumed (g g^{-1})

Subscripts

0	process start at t = 0 h
a, b, c and d	molar proportion of respective element
g	gaseous phase
l	liquid phase
lys	L-lysine
s, n, x and pj	substrate, nitrogen source, biomass, and (by-) products j
t	time of sampling (h)

Superscripts

L	liquid phase
S	substrate (glucose)
Sys	system (gaseous + liquid phase)

Greek letters

μ	biomass specific growth rate (h^{-1})
Δ	time interval (h)
λ_i	molar stoichiometric coefficient of chemical formulae i

considerable amounts of CO_2 (which is about 30 times more soluble compared to O_2 at 30 °C [10,11]) are likely to dissolve in the fermentation medium during the cultivation process. Consequently, CO_2 and bicarbonate (HCO_3^-) levels may show significant accumulation and stripping dynamics, dependent on cellular metabolic activity and aeration conditions installed. To be precise, the inherent steady-state assumption of the C-balance may be invalid for a balancing interval due to transiently, non-balanced carbon sinks. Improper C-balance closures may be observed because measured carbon dioxide transfer rates (CTR) derived from exhaust analysis only give an incomplete picture of true carbon dioxide evolution rates (CER) of the biomass [12,13]. This topic was basically addressed by analyzing exhaust gas signal dynamics [14,15] and elucidating physicochemical inter conversions of dissolved CO_2 to HCO_3^- and vice versa [16,17]. On that account, it should be considered that the dissociation equilibrium of CO_2 is complex, with the species carbonic acid (H_2CO_3), HCO_3^-, and the carbonate ion (CO_3^{2-}) being strongly pH dependent [18]:

$$CO_{2,g} \Leftrightarrow CO_{2,l} \quad (2)$$

$$CO_{2,l} + H_2O \Leftrightarrow H_2CO_3 \quad (3)$$

$$H_2CO_3 \Leftrightarrow HCO_3^- + H^+ \quad (4)$$

commonly combining the hydration and protolysis of true H_2CO_3 to:

$$CO_{2,l} + H_2O \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} HCO_3^- + H^+, K_1 = \left(\frac{k_1}{k_{-1}}\right) = 10^{-6.3} \text{ mol } L^{-1} \quad (5)$$

$$HCO_3^- \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} H^+ + CO_3^{2-}, K_2 = \left(\frac{k_2}{k_{-2}}\right) = 10^{-10.3} \text{ mol } L^{-1} \quad (6)$$

Hence, $CO_{2,l}$ forms the major portion at low pH < 5, whereas HCO_3^- is the dominating species at neutral pH 7–8. Consequently, a thorough C-balancing approach should consider all mentioned carbonic species, which can be achieved by total inorganic carbon (TIC) detection [19]:

$$TIC = CO_{2,l} + H_2CO_3 + HCO_3^- + CO_3^{2-} \quad (7)$$

Notably, TIC measurement is an integral part of total carbon determination (TC) thus offering the opportunity to qualify carbon closures for the liquid phase (with and without biomass consideration) independent of additional $CO_{2,g}$ measurements.

Taken together, this contribution evaluates the improvement of TIC and TC measurements for total C-balancing. By studying representative batch fermentations with *Corynebacterium glutamicum* wild-type (WT) and L-lysine producer under highly dynamic conditions, it will be shown that TIC and TOC measurements significantly improve the balancing accuracy outlining that CO_2/HCO_3^- dynamics of the liquid phase and the quantification of the biomass carbon content severely affect carbon closure quality.

2. Materials and methods

2.1. Bacterial strains, pre-culture, media, and bioreactor cultivation

The WT strain *C. glutamicum* ATCC 13032 obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and the L-lysine producer *C. glutamicum* DM1933 [20] were used in this study. Glycerol stock seed cultures of the WT were grown on tryptone-yeast extract (2×TY; [21]) agar plates and used to inoculate first pre-cultures of 5 mL 2×TY medium in glass reaction tubes, whereas 3.7% (w/v) brain heart infusion (BHI) medium was used for the L-lysine producer, respectively. Subsequent seed cultures were grown overnight at 30 °C applying 100 mL modified CGXII minimal salt medium (see below) in 1000 mL baffled shaking flasks at 140 min^{-1} on a bench-top rotary shaker (Infors HT, Bottmingen, Switzerland) and used to inoculate CGXII containing bioreactors, achieving initial biomass concentrations of $X_0 \geq 0.25$ g L^{-1}. The modified CGXII minimal medium was prepared in accordance to Eikmanns et al. [22] containing 10 g L^{-1} $(NH_4)_2SO_4$, 5 g L^{-1} urea, 21 g L^{-1} 3-(*N*-morpholino)propanesulfonic acid (MOPS), 1 g L^{-1} KH_2PO_4, 1 g L^{-1} K_2HPO_4, 0.25 g L^{-1} $MgSO_4 \cdot 7\,H_2O$, 10 mg L^{-1} $CaCl_2 \cdot H_2O$, 10 mg L^{-1} $MnSO_4 \cdot H_2O$, 16.4 mg L^{-1} $Fe(II)SO_4 \cdot 7\,H_2O$, 1 mg L^{-1} $ZnSO_4 \cdot 7\,H_2O$, 0.2 mg L^{-1} $CuSO_4 \cdot 5\,H_2O$, 0.02 mg L^{-1} $NiCl_2 \cdot 6$

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