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A Generic Biomass Soft Sensor and Its Application in Bioprocess Development

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Abstract

Biomass concentration is a key process variable, which is used to identify trends and initiate process events in microbial bioprocesses. Real-time data on the biomass is essential to implement advanced control strategies, including the control of biomass-specific nutrient uptake rates.

Christoph Herwig's group at Technische Universität Wien (TU Wien) has developed a biomass soft sensor based on readily available measurements and process parameters. They used the soft sensor to control various substrate uptake rates in parallel small-scale bioreactors.

Introduction

In developing a bioprocess, researchers incur significant costs to determine the optimal culture conditions and install the process control technology to maintain them [1, 2, 3]. They routinely monitor and control the temperature, pH, and availability of oxygen in the culture, but these are not the only relevant process parameters. A time-resolved determination of the biomass concentration is also of great importance, enabling them to not only monitor cell growth, but also to establish an optimal supply of substances, like nutrients, co-factors, and inducers. The demand for them changes over time as the culture grows. To manage the availability of nutrients we must know how much of them is consumed. The nutrient supply can be described by the biomass-specific nutrient uptake rate at a given point in time ($qs_{(t)}$) (Box 1).

There are several methods to determine the biomass concentration in a microbial bioprocess. The choice of the best method for high-cell-density fermentations depends strongly on the demands of the application. At the production stage, robustness and accuracy are the main demands, while cost and transferability are of limited concern [4]. Methods based on light scattering

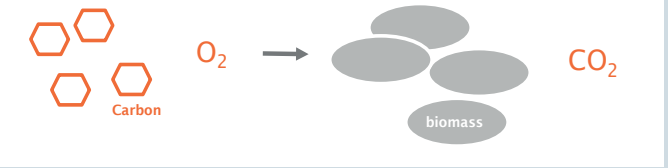

Fig. 1: In many bioprocesses carbon is metabolized to biomass and CO_2 by oxidative transformation. Out of known or measured amounts of carbon (substrate), O_2, and CO_2 that are introduced to and leave the bioreactor, a soft sensor can calculate the biomass.

and transmittance, like optical density measurement, are widely used. They are inexpensive and easy to implement, but their application range is limited to certain biomass concentrations.

Researchers at TU Wien developed a biomass soft sensor as an alternative method for biomass determination. The term soft sensor combines the words software and sensor. The idea behind it is to use easy-to-determine parameters to calculate variables that are more difficult to obtain by direct measurement. The soft sensor delivers readings like a conventional hardware sensor, but uses a mathematical model to determine the sensor signal based on other measurements [5].

The soft sensor for biomass determination described here uses mass balancing. It is based on the hypothesis that in a bioprocess carbon is metabolized to biomass and CO_2 by oxidative transformation. This applies to the most industrially used bacterial, mammalian, and yeast cell lines. From the amount of carbon that goes into the bioreactor (quantified, for example, by measuring the amount of substrate that is fed in) and leaves the bioreactor (quantified, for example, by measuring the CO_2 concentration in the exhaust), we can estimate the biomass concentration (Fig. 1). Feeds and outflows of the bioreactor are quantified using standard online measurements, and the software calculates the biomass based on a data model.

This application note describes the successful implementation of such a biomass soft sensor in an Eppendorf DASbox® Mini Bioreactor System using the DASware® control software. The biomass estimate was used to evaluate and control the biomass-specific substrate uptake rates of different nutrients in high-density *E. coli* fermentations. The combination of the biomass soft sensor with an optical density sensor enables full automation of the multi-bioreactor system for bacterial processes.

Box 1: Biomass-specific nutrient uptake rate qs

A way to describe the substrate consumption of organisms is to calculate the biomass-specific nutrient uptake rate at a given time point ($qs_{(t)}$). It indicates how many grams of substrate are consumed per gram of biomass per hour. The amount of substrate consumed by the culture equals the amount of substrate fed in, as long as the nutrients are limited and thus are completely taken up by the culture. The nutrient supply, and thus the biomass specific substrate uptake rate, can be controlled with the feed pump rate [9]. To calculate $qs_{(t)}$ the biomass concentration must be measured.

$$qs_{(t)} = \frac{F_{(t)}\, C_s}{x_{(t)}\, V_{(t)}} \quad [g/(g*h)]$$

$qs_{(t)}$: Biomass specific substrate uptake rate [g/g] at time point (t)
$F_{(t)}$: Feed flow rate [L/h] at time (t)
C_s: Substrate concentration in feed [g/L]
$x_{(t)}$: Cell dry weight concentration [g/L] at time (t)
$V_{(t)}$: Bioreactor volume [L] at time (t)

Material and Methods

The project comprised two steps. First, the researchers implemented the soft sensor. Then they used it to set up control loops to control the biomass-specific uptake rates of different substrates in *E. coli* high-density fermentation processes (Fig. 3). For example, they show results on the simultaneous control of the biomass-specific uptake rates of glucose and lactose.

Soft sensor implementation

Setup of data model

As the first step of the soft sensor implementation the

	S	O_2	x	CO_2				
C-balance	+1	0	+1	+1	*	r_s r_{O2} r_x r_{CO2}	=	0 0
DoR-balance	γ_s	γ_{O2}	γ_x	0				
	E					X		ε

Fig. 2: Stoichiometric matrix of the considered system where E is the stoichiometric matrix, X contains the reaction rates and ε the residuals which in a perfect system are 0. Both the material balances of carbon (C balance) and free electrons (DoR balance) can be used to determine missing reaction rates, such as the biomass formation rate r_x.

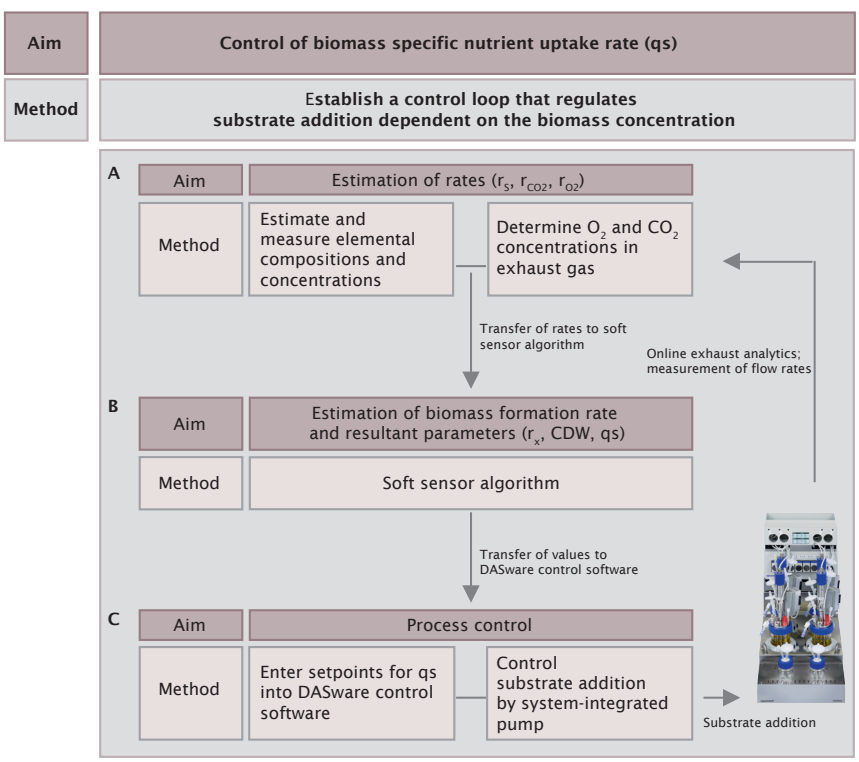

Fig. 3: Process monitoring and control loop for control of the biomass-specific nutrient uptake rate.
A: Rate estimation: The amounts of carbon (substrate), O_2, and CO_2 that are introduced to and leave the bioreactor are known or measured. From these data the substrate uptake rate, carbon evolution rate an oxygen uptake rate are calculated.
B: Using these rates the soft sensor estimates the biomass and the biomass-specific nutrient uptake rate qs.
C: The setpoint for qs is entered in the DASware control software. Using the biomass estimate the required amount of feed solution to maintain qs at the setpoint is pumped into the bioreactor.

researchers set up a data model that describes the oxidative transformation of carbon to biomass and CO_2. The mass balance system is depicted in Figure 2. One can solve the material balances of carbon and free electrons (C balance and DoR balance). The rates of substrate uptake, carbon evolution, oxygen uptake, and biomass formation have to be estimated [6]. Table 1 summarizes the measurements and constants which are used to do so. The substrate uptake rate is determined from the pump flow rate. The rates of carbon evolution and oxygen uptake are calculated from exhaust composition and mass flow.

The additional system redundancy allows for system reconciliation. The residuals ε are assigned to the rates according to their measurement error. A statistical test (h-value) evaluates the system integrity, which applies if the residuals can be explained solely by measurement errors [7].

In this application, the researchers designed the soft sensor to work in strictly C-source-limited fed-batches or

Table 1: Needed measurements and constants for rate estimation.
*error propagation [8]. sL: Standard liter

Rate	Description	Measurement	Constant parameter	Assumed error
r_s [C-mol/h]	Substrate uptake rate	Pump flow rate [L/h]	C-concentration [C-mol/L]	2 %
r_{co_2} [mol/h]	Carbon evolution rate (CER)	CO_2 and O_2 concentrations (off gas analyzer [%] and mass flow [sL/h]	In-gas composition, evaporation	3 %
r_{o_2} [mol/h]	Oxygen uptake rate (OUR)			3 %
r_x [C-mol/h]	Biomass formation rate	Calculated by soft sensor	Elemental composition of biomass [g/C-mol]	≈10 %*

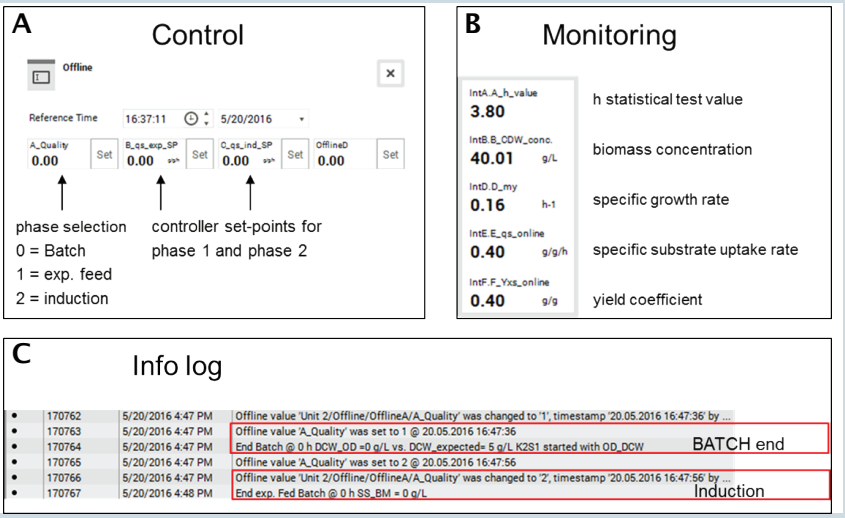

Fig. 4: Soft sensor integration into DASware control software. The setpoints of the biomass specific substrate uptake rates can be entered (A) and the biomass estimates are visualized (B) directly in the DASware control software. Parameter changes are automatically logged (C).

continuous cultures where the product and possible by-products can be neglected.

Creation and integration of soft sensor algorithm

Once they had established the data model, the researchers created the soft sensor algorithm in MATLAB®. They incorporated it into the DASware control software through a Visual Basic control script that accesses the function. The results are stored, and can be visualized directly, within the DASware control software (Fig. 4) without the need for further customization or the establishment of OPC connections. Additional features like automated phase transition from batch to fed batch and induction as well as control options were included.

Figure 3 summarizes how the the soft sensor is integrated into the bioprocessing environment.

Soft sensor calibration

The bioprocesses were run in two phases. In the initial batch phase the cultures grew to a cell dry weight (CDW) of up to 10 g/L. During this phase, the biomass concentration can be measured accurately with optical density sensors. At the end of the batch phase, the actual biomass concentration measured with the OD sensor was used to start the soft sensor.

High-density fermentation

Culture conditions

The researchers conducted the fermentations in a DASbox® Mini Bioreactor System (Eppendorf AG, Germany) equipped with glass vessels or BioBLU® 0.3f Single-Use Vessels. Both vessel types have a maximum working volume of 250 mL.

They used a recombinant *E. coli* strain, which bears a green fluorescent protein (GFP) gene under the control of the LAC promoter. The bacteria were cultivated in a chemically defined medium with a limited amount of C-source (10 g/L glucose).

After C-source depletion, glucose and inductor (lactose) were fed to the culture with a DASGIP® MP8 multi pump module (Eppendorf AG, Germany).

The pH was maintained at 7.2 by NH_3 addition, which served as an additional N-source. Air saturation was kept over 30 % by increasing the stirrer speed and oxygen concentration in the inflow air, which was supplied with 2 VVM through an L-sparger. The five M18 ports were occupied by pH, DO, and OD sensors (Eppendorf DASGIP OD4 Module, 880 nm), gas inlet and gas outlet. Three dip tubes were used for feed, base addition, and offline sampling.

The exhausted gas was analyzed by a DASGIP GA4 gas sensor module (Eppendorf AG, Germany) with a ZrO_2 sensor for O_2 and infrared CO_2 sensor technology.

Offline measurements

To determine the cell dry weight (CDW) the researchers centrifuged 2 mL of culture broth (4,500 x g, 4°C, 10 min), washed the cell pellet with a 0.1 % NaCl solution, and subsequently dried it at 105°C for 48 h.

Cell-free samples of the cultivation broth were analyzed

for concentrations of substrates and metabolites using HPLC (Agilent Technologies, USA) with a SUPELCOGEL™ C-610 H ion exchange column (Sigma-Aldrich, USA) and a refractive-index detector (Agilent Technologies, USA). The mobile phase was 0.1 % H_3PO_4 with a constant flow rate of 0.5 mL/min, and the system was run isocratically at 30°C.

Results

To demonstrate the functionality of the system, the researchers carried out experiments with a recombinant *E. coli* strain bearing a GFP gene under the control of the LAC promoter. They aimed to simultaneously control the availability of lactose and glucose, to equilibrate the bacterial growth, energy maintenance, and productivity.

Lactose was used instead of IPTG to induce the GFP hosting LAC operon. Lactose is metabolized by the host strain, so for continuous protein production it must be continuously added to the culture.

The researchers tested different glucose-to-lactose ratios in four parallel fermentations. Each fermentation run used the same setpoint for the glucose uptake rate qs_{Glu} but varied the lactose uptake rate qs_{Lac}. To control qs, the biomass concentration was estimated online using the soft sensor. Based on this data, the pump flow rate was automatically adjusted to control the supply of a mixed feed containing lactose and glucose.

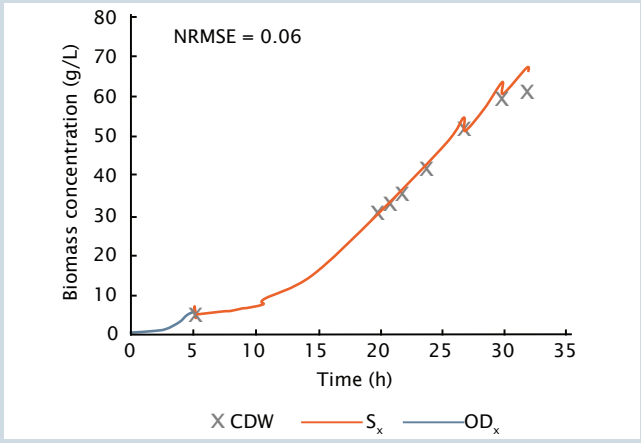

Fig. 5: Biomass concentration estimates.
Crosses: Biomass concentration (cell dry weight, CDW) deriving from offline measurements.
Blue line: OD correlation.
Orange line: Results from soft sensor.
NRMSE: Normalized root mean square error

Figure 5 shows optical density and the soft sensor-based biomass concentration estimates of one fermentation. The biomass concentration deriving from offline measurements (black crosses), OD correlation (blue line) and soft sensor (orange line) are depicted.

The data derived from the biomass soft sensor showed good alignment with the reference measurements. The biomass soft sensor application achieved an accuracy of < 10%, which is within its working range [5]. The soft

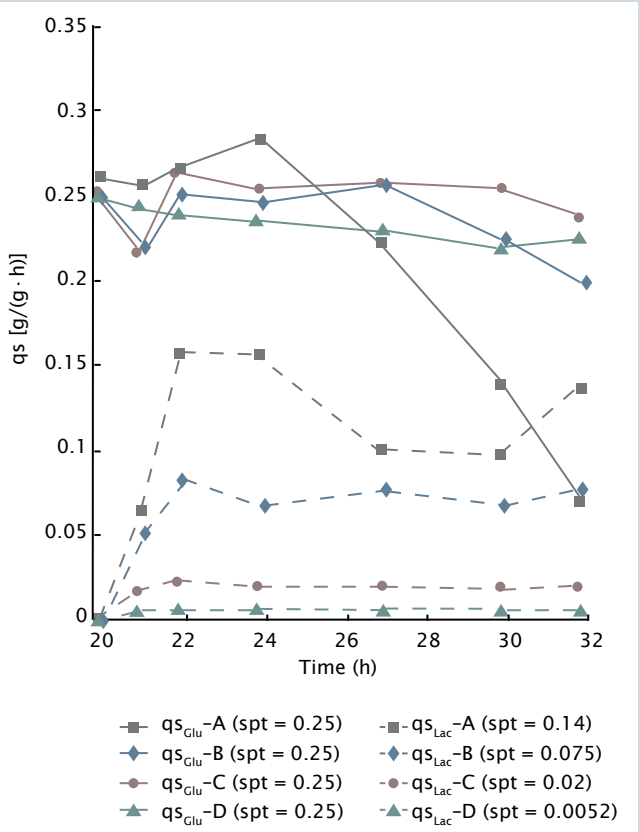

Fig. 6: Setpoints and time courses of qs_{Glu} (solid lines) and qs_{Lac} (dashed lines) in the four fermentation runs (bioreactor A-D). spt: setpoint

sensor overestimated the biomass concentration only at the end of the induction phase, because the uptake capacity of the cells was overstressed and the substrate accumulated. This was more pronounced in experiments with a higher lactose supply.

Figure 6 displays the specific glucose and lactose uptake rates during induction. The setpoint for qs_{Glu} was kept at 0.25 g/(g·h) in all experiments, whereas the one for qs_{Lac} was varied (0.14/0.75/0.02/0.0052 g/(g·h)).

The setpoints were reached. Only at the highest qs_{Lac} was the uptake capacity of the cells overstressed, causing accumulation of the substrate. Evaluation of the uptake dynamics showed that high qs_{Lac} impeded the glucose uptake. Therefore, the qs_{Glu} of Reactor A (grey line) dropped significantly. The glucose-to-lactose ratio affected product formation and product quality. The induction could be controlled by continuous lactose addition, and product quality and productivity were improved compared to induction with a one shot IPTG addition. The latter led to higher inclusion body (inactive product) formation, making the cheaper, non-toxic lactose a reasonable alternative to IPTG.

Conclusion

The researchers implemented a relatively simple and straightforward method for biomass estimation in a small-scale parallel bioreactor system. The soft sensor works for strictly C-source-limited fed-batch and continuous cultures. The researchers used it to simultaneously control the uptake rates of a substrate and an inducer. The results obtained are fully scalable and applicable in industry without the need for expensive additional measuring equipment. Besides manual offline sampling and sample volume reduction, the described fermentations could run fully automatically.

In bioprocess development, the control of biomass-specific substrate uptake rates is a valuable tool to determine the optimal supply of nutrients and/or inducers. The researchers performed similar studies with *Pichia pastoris* strains to tune the pAOX promoter. Repressing glycerol was used as the C-source and methanol as the inducer.

The Eppendorf bioprocess control software DASware control 5 (former version DASGIP control 4.5) offers the interface for the integration of soft sensors. The soft sensor algorithm and process control strategies are developed by the end-user.

Literature

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Ordering information

Description	Order no.
DASbox® Mini Bioreactor System for Microbial Applications, max. 25 sL/h gassing	
4-fold system	76DX04MB
8-fold system	76DX08MB
16-fold system	76DX16MB
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DASbox® Mini Bioreactor, 2 Rushton-type impellers, 60 – 250 mL, overhead drive	76SR0250ODLS
BioBLU® 0.3f Single-Use Vessel, fermentation, 2 Rushton-type impellers, sterile, 4-pack	78903509
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