Monitoring Biomass Concentration and Specific Growth Rate Using a Dissolved Oxygen Soft-Sensor

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Application note

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Nomenclature

c_{O2}^{Aer}	%	molar fraction of oxygen in aeration supply
c_{O2}	mol L^{-1}	concentration of dissolved oxygen
c_{O2}^{*}	mol L^{-1}	saturated concentration of oxygen
$c_{O2,cal}^{*}$	mol L^{-1}	saturated concentration of oxygen during calibration of p_{O2} probe
$C_sH_sN_sO_s$	–	composition of a substrate molecule
$C_xH_xN_xO_x$	–	relative elemental composition of the biomass
k_La	h^{-1}	oxygen mass-transfer coefficient
M_s	g mol^{-1}	molar mass of substrate
m_s	g g^{-1} h^{-1}	specific rate of substrate consumption for cell maintenance
m_s^{mol}	mol mol^{-1} h^{-1}	specific rate of substrate consumption for cell maintenance
m_{O2}^{mol}	mol mol^{-1} h^{-1}	specific rate of oxygen consumption for cell maintenance
M_x	g mol^{-1}	molar mass of biomass
OTR	mol L^{-1} h^{-1}	oxygen transfer rate
p	bar	pressure in the bioreactor
p_{O2}	%	dissolved oxygen concentration in the media
t	h	time
T	°C	temperature
V	L	working volume
x	g L^{-1}	concentration of biomass (cell dry weight)
$Y_{x/CO2}^{mol}$	mol mol^{-1}	yield biomass/carbon dioxide
$Y_{x/O2}^{mol}$	mol mol^{-1}	yield biomass/oxygen
$Y_{x/s}^{mol}$	mol mol^{-1}	yield biomass/substrate
$Y_{x/s,max}$	g g^{-1}	maximum yield biomass/substrate
$Y_{x/s,max}^{mol}$	mol mol^{-1}	maximum yield biomass/substrate
μ	h^{-1}	specific growth rate
μ_{max}	h^{-1}	maximal specific growth rate

Introduction

In biotechnological manufacturing, the quality and quantity of an end product depend significantly on the biomass concentration (x) and the specific growth rate (μ). The biomass concentration is usually measured offline, either by detecting the optical density (OD) of the cultivation broth or by gravimetric determination of the cell dry weight (CDW). Subsequently, the specific growth rate is calculated from adjacent biomass concentrations and the elapsed time between them.

Since OD and CDW measurements are typically conducted in offline mode, the values of x and μ are not obtained in real-time. By using soft-sensors, the biomass concentration and specific growth rate can be monitored online instead. Since this allows for live supervision of each bioprocess, the FDA recommends online solutions for process analytics (PAT-Initiative).

An oxygen soft-sensor for online monitoring of x and μ was programmed into eve®, and applied during the cultivation of a recombinant *Pichia pastoris* strain. The sensor used the dissolved oxygen concentration (p_{O2}) in the media as input value. The p_{O2} signal is a pertinent parameter, considering the majority of bioreactors is equipped with a p_{O2} probe.

Experimental specifications

Strain

For the study, a *Pichia pastoris* strain *(Komagataella phaffii)* based on the wild type strain CBS 7435 was used. The recombinant strain secretes *Candida antarctica* lipase B (CALB), under the control of a novel methanol-independent promotor. This allows to conduct the whole bioprocess, consisting of a batch and a subsequent fed-batch, with glucose as the sole substrate. The elementary composition of the biomass was assumed to be $CH_{1.761}N_{0.143}O_{0.636}$. In a previous batch experiment with glucose as the sole substrate, the strain showed a maximum specific growth rate (μ_{max}) of (0.20 ± 0.02) h^{-1} and a maximum yield ($Y_{x/s,max}$) of (0.57 ± 0.04) g_{CDW} g_{Glc}^{-1}. The specific maintenance rate (m_s) was assumed to be 0.009 g_{Glc} g_{CDW} h^{-1} (Looser *et al.*, 2015).

Growth media and bioreactor setup

Preparation of inoculum and culture media was made according to Hyka *et al.* (2010). The batch media contained 30 g L^{-1} glucose and the feed media 575 g L^{-1} glucose as the sole carbon source.

The cultivation was performed at constant conditions: airflow of 18 L min^{-1} (2 L L^{-1} h^{-1}) with atmospheric air (20.95 % O_2 were assumed) and 0.5 bar overpressure, temperature of 28 °C, agitator speed of 1100 min^{-1} and pH of 6 (regulated by ammonia (25 %) and phosphoric acid (8.5 %)). Under these conditions, the oxygen mass-transfer coefficient (k_La) of the used bioreactor amounts 795 h^{-1}.

The cultivations were initiated with a working volume of 5.8 L after inoculation. The processes were divided into an initial batch phase for biomass growth and a subsequent fed-batch phase for the production of CALB. The biomass concentration after inoculation was planned as 2 g_{CDW} L^{-1} and reached in average 18.02 g_{CDW} L^{-1} at the end of the batch phase. The fed-batch was started when the substrate from the batch media was depleted, which was after 10.2 h on average. During the fed-batch the feed media was added with an exponentially increasing feed rate.

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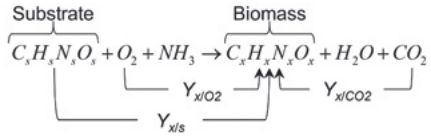
Measurements

The concentrations of CO_2 and O_2 in the exhaust gas, oxygen partial pressure in the media, pH, cultivation temperature, and reactor weight were all measured online and monitored using eve®. The online values were determined with a time increment of 30 s. The biomass concentration was measured as cell dry weight. For this purpose, 2 mL of sample were transferred to tared Eppendorf tubes, centrifuged at 14'000 min^{-1} (RCF: 21'342 · g) for 5 min, washed with 1 mL PBS, re-centrifuged and dried at 105 °C to a constant weight.

Analysis

If product formation is neglected, the reaction from substrate to biomass can be described as follows:



The proportion of substrate which becomes biomass corresponds to the yield biomass/substrate ($Y_{x/s}^{mol}$). Since a part of the substrate is used for maintenance (m_s^{mol}) rather than growth, $Y_{x/s}^{mol}$ is not constant but is dependent on the specific growth rate (μ):

$$Y_{x/s,max}^{mol} = Y_{x/s,max} \cdot \frac{M_s}{M_x}$$

$$m_s^{mol} = m_s \cdot \frac{M_x}{M_s}$$

$$Y_{x/s}^{mol}(\mu) = \frac{\mu \cdot \mu_{max} \cdot Y_{x/s,max}^{mol}}{\mu \cdot \mu_{max} + m_s^{mol}{}_s \cdot (\mu_{max} - \mu) \cdot Y_{x/s,max}^{mol}}$$

With known $Y_{x/s}^{mol}$ (μ) dependency, the amount of consumed oxygen required to produce one mole of biomass ($Y_{x/O2}^{mol}$) can be determined (Fig. 1):

$$Y_{x/CO2,max}^{mol} = \frac{Y_{x/s,max}^{mol}}{c_s - c_x \cdot Y_{x/s,max}^{mol}}$$

$$Y_{x/O2,max}^{mol} = \frac{\frac{Y_{x/s,max}^{mol}}{O_x \cdot Y_{x/s,max}^{mol} + \frac{H_s + 3 \cdot (N_x \cdot Y_{x/s,max}^{mol} - N_s) - H_x \cdot Y_{x/s,max}^{mol}}{2} + \frac{2 \cdot Y_{x/s,max}^{mol}}{Y_{x/CO2,max}^{mol}} - O_s}}{2}$$

$$m_{O2}^{mol} = \frac{m_s^{mol} \cdot (H_s \cdot 0.5 + C_s \cdot 2 - O_s - N_s \cdot 1.5)}{2}$$

$$Y_{x/O2}^{mol}(\mu) = \frac{\mu \cdot \mu_{max} \cdot Y_{x/O2,max}^{mol}}{\mu \cdot \mu_{max} + m_{O2}^{mol} \cdot (\mu_{max} - \mu) \cdot Y_{x/O2,max}^{mol}}$$

Therefore, the increase of biomass between two measurements equals the amount of O_2 consumed during the same timespan multiplied by $Y_{x/O2}^{mol}$. However, the oxygen uptake must be known first.

The oxygen transfer rate (OTR) from the sparged air into the media can be described as follows; where c_{O2}* is the saturated concentration of oxygen in water, determined by Henry's law:

$$c_{O2}^* = \frac{p \cdot c_{O2}^{Aer}}{100\%} \cdot 0.0013\ mol\ L^{-1}\ bar^{-1} \cdot e^{1700°K \cdot \left(\frac{1}{T+273.15°K} - \frac{1}{298.25°K}\right)}$$

$$OTR = k_L a \cdot (c_{O2}^* - c_{O2})$$

Because the measured p_{O2} values are given as a percentage of the conditions during the calibration of the probe, the equation is adjusted accordingly:

$$OTR(t) = k_L a \cdot \left(c_{O2}^*(t) - \frac{p_{O2}(t) \cdot c_{O2,cal}^*}{100\%}\right)$$

Without any biomass in the bioreactor c_{O2}* is reached within minutes and the OTR drops to zero. The O_2 consumption of the biomass, however, will lead to a lower equilibrium value for c_{O2}. It can therefore be assumed that all oxygen described by the OTR is used for the biomass growth:

$$\frac{dx(t)}{dt} = Y_{x/O2}^{mol}(\mu) \cdot M_x \cdot k_L a \cdot \left(c_{O2}^*(t) - \frac{p_{O2}(t) \cdot c_{O2,cal}^*}{100\%}\right)$$

Following these considerations, a soft-sensor was introduced. With each new measured value, the next μ is calculated between the two last adjacent biomass concentrations:

$$\mu_i = \frac{ln(x_i \cdot V_i) - ln(x_{i-1} \cdot V_{i-1})}{t_i - t_{i-1}}$$

$$\mu_i = \lim_{\Delta V \to 0} \frac{ln(x_i \cdot V_i) - ln(x_{i-1} \cdot V_{i-1})}{t_i - t_{i-1}} = \frac{ln(x_i) - ln(x_{i-1})}{t_i - t_{i-1}}$$

To calculate $Y_{x/O2}^{mol}$, the last determined μ is used. The yield leads to the next biomass concentration and the next μ, which than is used to determine the next $Y_{x/O2}^{mol}$, and so on.

$$x_i = x_{i-1} + Y_{x/O2}^{mol}(\mu_{i-1}) \cdot M_x \cdot \int_{t_{i-1}}^{t_i} \left(k_L a \cdot \left(c_{O2}^*(t) - \frac{p_{O2}(t) \cdot c_{O2,cal}^*}{100\%}\right)\right) \cdot dt$$

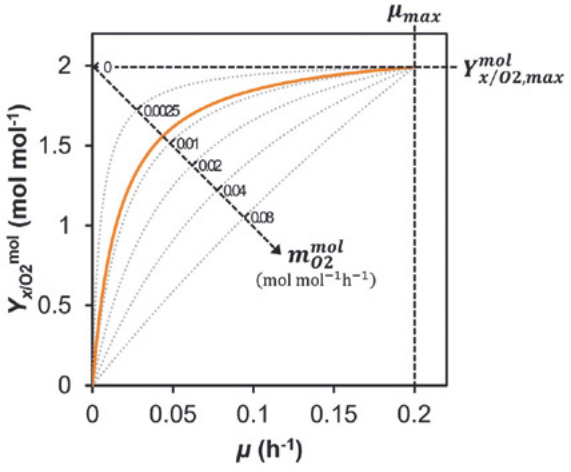


Fig. 1: Yield Biomass/Oxygen ($Y_{x/O2}$) dependency on the specific growth rate (μ). The orange line (–) correspond to the characteristics assumed for the used *P. pastoris* strain. The gray dotted line (•••) shows the $Y_{x/O2}^{mol}(\mu)$ dependency at varying values for the specific rate of oxygen consumption for maintenance (m_{O2}^{mol}).

Results

The soft-sensor achieved deviations of less than ± 27% between gravimetrically determined and sensor-determined values of *x*. On average, the deviation amounts ± (8.3 ± 2.3)(1 – α = 0.95)%.

During the batch phase, some values for μ were greater than the determined μ_{max} (Fig. 2). This suggests that a value of μ_{max} determined by linear regression is to be considered as an average value for batch phases and the actual values can fluctuate around it. The same applies to the μ from the fed-batch phase.

As the composition of the substrate *(s)* of complex media is not always known, the soft-sensors should only be used with chemically defined media. If different substrates are used for different process phases, separate values for $Y_{x/O2,max}^{mol}$ and m_{O2}^{mol} must be given for each phase.

In the analysed processes, stable cultivation conditions (stirring, aeration, etc.) were applied. However, this will not be the case for many other processes. For example, often the p_{O2} is controlled by a cascade including stirrer speed, aeration flow and O_2 content of the sparged gas. For such cultivations, a second soft-sensor to determine an online k_La value will be required. In order to establish a k_La model, we recommend proceeding as described by DECHEMA (Meusel *et al.*, 2016), to either experimentally determine a model (by Design of experiments) or to use the generalist van't Riet model.

There are many dependencies that have not been considered in the soft-sensor. For example, Carnicer et al. (2009) showed a connection between the elementary composition of biomass and the molar fraction of oxygen in the air supply. Therefore, the benefit of the described soft-sensors lies predominantly in monitoring repeated production processes, which have already been shown to work for the soft-sensor.

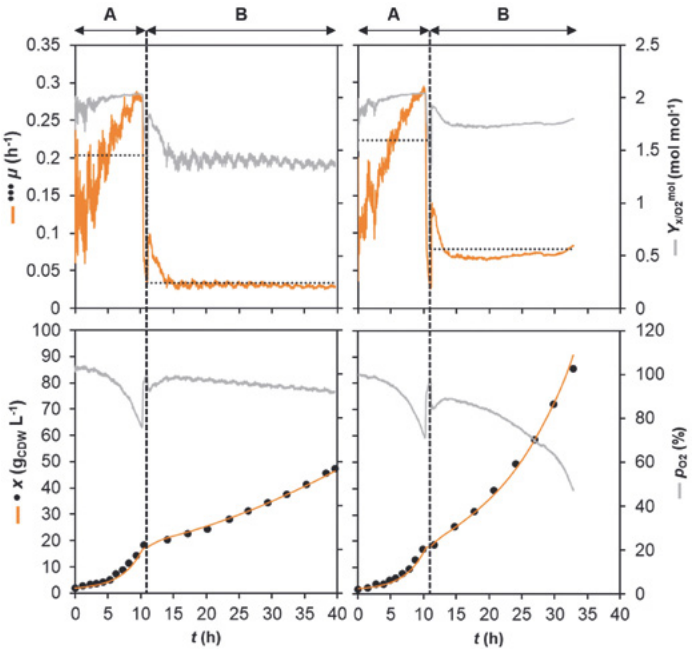


Fig. 2: Biomass concentration *(x)* and specific growth rate *(μ)* estimated with the soft-sensors. Both sides show an independent cultivation, each consisting of a batch (A) and a fed-batch (B) phase. The biomass concentration was measured gravimetrically (lower graph: •) and was used to calculate the average *μ* (upper graph: •••) by linear regression. The soft-sensor was used to estimate *x* (lower graph: –), *μ* (upper graph: –) and the yield biomass/O2 ($Y_{x/O2}^{mol}$; upper graph: –) using the measured dissolved oxygen concentration values (*p*O2; lower graph: –).

Summary

- On average, there was less than ± 10% deviation between *x* determined gravimetrically and by soft-sensor.
- Process control for x and μ in real time, is achievable without additional hardware since most bioreactors are equipped with a dissolved oxygen probe.
- To a certain degree, the soft-sensor is self-regulating. For example, if the biomass concentration is overestimated, the resulting μ will be underestimated until both values are in balance. This makes the soft-sensor more stable against measurement deviations or incorrect entries of starting values (e.g. biomass concentration at the inoculation)
- Theoretically, this method is suitable for all cultivation species using aerobic respiration as long as the elemental composition of biomass is known, and a chemically defined media is used.
- $Y_{x/s,max}$ and μ_{max} must be known, and may be determined by a preliminary batch cultivation. The needed value for m_s may be gained by preliminary trials or from publications.

References

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Script for the soft-sensor in eve®

Name:	BiomassP02		
Description:	Calculates the biomass concentration and the specific growth rate from the measured dissolved oxygen signal		
Inputs:	p02	DO	
	p	Pressure	
	O2GasMix	Gas Mix	
	T	Temperature	
Outputs:	x	$g\ l^{-1}$	Biomass CDW
	mu	h^{-1}	Metabolic
	YxO2Mol	$mol\ mol^{-1}$	Metabolic
Constants:	muMax	h^{-1}	0.2
	YxsMax	$g\ g^{-1}$	0.57
	ms	$g\ g^{-1}\ h^{-1}$	0.009
	Cs	None	6
	Hs	None	12
	Ns	None	0
	Os	None	6
	Cx	None	1
	Hx	None	1.761
	Nx	None	0.143
	Ox	None	0.636
	kLa	h^{-1}	795
	pCalibration	bar	1.5
	O2GasMixCalibration	$\%O_2$	20.95
	TCalibration	°C	28
	x0	$g\ l^{-1}$	2
Sampling Time:	30 s		

Expression

```
//Calculate molar masses for substrate and biomass
double Ms = Cs * 12.0107 + Hs * 1.00794 + Ns * 14.0067 + Os * 15.999;          //substrate
double Mx = Cx * 12.0107 + Hx * 1.00794 + Nx * 14.0067 + Ox * 15.999;          //biomass

//Calculate the maximum yield biomass/O2
double YxsMaxMol = YxsMax * Ms / Mx;
double YxCO2MaxMol = YxsMaxMol / (Cs - Cx * YxsMaxMol);
double YxO2MaxMol = YxsMaxMol / ((Ox * YxsMaxMol + (Hs + 3 * (Nx * YxsMaxMol - Ns) - Hx * YxsMaxMol) / 2 + 2 * YxsMaxMol / YxCO2MaxMol - Os) / 2);

//Calculate the O2 maintenance
double msMol = ms * Mx / Ms;
double mO2Mol = msMol * (Hs * 0.5 + Cs * 2 - Os) / 2;

//Calculate the O2 saturation concentration at the calibration
double cO2SatCalib = pCalibration * O2GasMixCalibration / 100 * 0.0013 * Math.Exp(1700 * (1 / (TCalibration + 273.15) - 1 / 298.15));

//Calculate the current O2 saturation concentration
double cO2Sat = p.Value * O2GasMix.Value / 100 * 0.0013 * Math.Exp(1700 * (1 / (T.Value + 273.15) - 1 / 298.15));

//Adjust maximum pO2 (account for drifts in the calibration)
if (double.IsNaN(batch["pO2Max"]))
{
        batch["pO2Max"] = 100;
}
if (pO2.Value > batch["pO2Max"])
{
        batch["pO2Max"] = pO2.Value * 1.01;
}

//Calculate current OTR
double OTR = kLa * ( cO2Sat - pO2.Value * cO2SatCalib / batch["pO2Max"]);

//For the first iteration...
if (double.IsNaN(batch["xLast"]))
{
        x.Value = x0;                                   //... set biomass to inoculation concentration x0
        mu.Value = muMax / 2;                           //... set specific growth rate to half the maximum value
}
//... For any further iteration...
else
{
        //... calculate time span between last and actual measurement
        double dt = batch.TimeSinceInoculation.TotalHours - batch["tLast"];
        //... integrate the OTR between the last and the actual measurement
        double intOTR = Math.Min(OTR, batch["OTRLast"]) * dt + (Math.Max(OTR, batch["OTRLast"]) - Math.Min(OTR, batch["OTRLast"])) * dt / 2;

        //... calculate the actual biomass concentration and actual specific growth rate
        x.Value = batch["xLast"] + batch["YxO2MolLast"] * Mx * intOTR;
        mu.Value = (Math.Log(x.Value) - Math.Log(batch["xLast"])) / dt;
}

//Calculate the current yield biomass/O2
YxO2Mol.Value = mu.Value * muMax * YxO2MaxMol / (mu.Value * muMax + mO2Mol * (muMax - mu.Value) * YxO2MaxMol);

//Save global variables for next iteration
batch["OTRLast"] = OTR;
batch["xLast"] = x.Value;
batch["YxO2MolLast"] = YxO2Mol.Value;
batch["tLast"] = batch.TimeSinceInoculation.TotalHours;
```