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Calorespirometric Feeding Control Enhances Bioproduction From Toxic Feedstocks—Demonstration for Biopolymer Production Out of Methanol

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ABSTRACT: The sustainable production of fuels and industrial bulk chemicals by microorganisms in biotechnological processes is promising but still facing various challenges. In particular, toxic substrates require an efficient process control strategy. Methanol, as an example, has the potential to become a major future feedstock due to its availability from fossil and renewable resources. However, besides being toxic, methanol is highly volatile. To optimize its dosage during microbial cultivations, an innovative, predictive process control strategy based on calorespirometry, i.e., simultaneous measurements of heat and CO_2 emission rates, was developed. This rarely used technique allows an online-estimation of growth parameters such as the specific growth rate and substrate consumption rate as well as a detection of shifts in microbial metabolism thus enabling an adapted feeding for different phases of growth. The calorespirometric control strategy is demonstrated exemplarily for growth of the methylotrophic bacterium *Methylobacterium extorquens* on methanol and compared to alternative control strategies. Applying the new approach, the methanol concentration could be maintained far below a critical limit, while increased growth rates of *M. extorquens* and higher final contents of the biopolymer polyhydroxybutyrate were obtained.

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KEYWORDS: biocalorimetry; respirometry; process control; methylotrophy; *Methylobacterium extorquens*

Introduction

The increasing demand for fuels and bulk chemicals and the progressing depletion of fossil resources motivate the exploration of alternative energy carriers and raw materials as well as the development of new biotechnological strategies for their conversion. Due to a high selectivity and mild reaction conditions, bioproduction processes by genetically engineered microorganisms promise environmental and economic benefits (Gavrilescu and Chisti, 2005). However, most established bioprocesses rely on sugar or other carbohydrates and thus potentially compete with food and fodder production.

To resolve this conflict, industrial biotechnology is searching for alternative substrates that can be obtained from sustainable and reliable sources. One very promising feedstock is methanol. This single-carbon compound can be synthesized from fossil oil, synthetic gas, and natural gas, but also from renewable resources such as biogas, wood, or agricultural waste materials (Olah, 2005). In addition to its relatively low and stable price over the recent years, advantages of methanol include its liquid form and unlimited miscibility with water, which make it easy to handle, store, and transport. And, in contrast to many other resources, it can be produced worldwide and independently from seasonal variations.

The ability of microbes to use methanol as the sole source of carbon and energy is quite widespread in nature (Chistoserdova et al., 2009). During the past decades, a variety of methylotrophic bacteria and yeasts applying different metabolic pathways for methanol assimilation were identified and the processes of methanol utilization are widely understood (Anthony, 1982). Moreover, several bioprocess technologies for the production of bulk chemicals with methylotrophic bacteria are potentially economically competitive (Schrader et al., 2009).

A methylotrophic model bacterium is the strictly aerobic, facultative methylotroph *Methylobacterium extorquens* AM1. This strain was first isolated in 1961 in Oxford (Peel and Quayle, 1961). *M. extorquens* has been shown to assimilate methanol and other single carbon compounds (e.g., formate, methylamine) via the serine cycle (Large and Quayle, 1963) and to produce the biopolymer polyhydroxybutyrate (PHB) under nutrient-limited conditions (Föllner et al., 1995). Due to the knowledge of its whole genome (Vuilleumier et al., 2009), the establishment of a complete metabolic network (Peyraud et al., 2011), and adequate genomic

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tools (Bélanger et al., 2004; Marx and Lidstrom, 2001), this bacterium is a promising candidate for biotechnological applications. Indeed, it has already been used for recombinant protein expression and the production of bulk and fine chemicals such as polymers, amino acids, and dicarboxylic acids (Ochsner et al., 2015). First steps were even made to introduce a pathway for the production of biofuels in *M. extorquens* (Hu and Lidstrom, 2014).

Methanol as a substrate in biotechnological processes has two main shortcomings: its high volatility and toxicity for microorganisms. Although many methylotrophs are able to tolerate methanol concentrations up to 50 g L^{-1} (Dijkhuizen et al., 1988; Kim et al., 2003), the even more toxic formaldehyde generated from excess methanol is problematic. Furthermore, the next oxidation product, formic acid, is known to uncouple energy generation from ATP synthesis resulting in reduced yield coefficients (Pilat and Prokop, 1975). Therefore, methanol-based microbial production processes require an appropriate control of the substrate dosage to adjust methanol concentrations high enough for rapid growth and product formation and low enough to avoid intoxication and uncoupling. Online sensors providing real time information about substrates, biomass, and product concentration appear most suited for controlling the feeding rate. Unfortunately, most of the existing techniques used for direct measurements or indirect calculations of metabolic conversions, such as capacitance readings, near-infrared spectroscopy, respirometry, online microscopy, and measurements of C- or N-consumption rates, are restricted to certain kinds of processes and do not provide information on the bioproducts. In contrast, biocalorimetry, a rather rarely used technique, can be applied independently of the nature of the process.

As every metabolic flux is quantitatively related to the heat production rate via the law of Hess, the heat signal can provide a global real time insight into the stoichiometry and kinetics of bioprocesses (von Stockar and Marison, 1989). Biocalorimetry is applicable to any microbial system, for instance, bacteria (Ishikawa and Shoda, 1983), yeasts (Larsson et al., 1991), animal cell cultures (Guan et al., 1998), and even for monitoring phage-bacteria interactions (Maskow et al., 2010). Other advantages of this technique are its non-invasiveness and high sensitivity, and the facts that it delivers real time signals, works in optically opaque solutions, and does not require labeling or specific reactants. The most important advantage of calorimetry for process control is scalability. The ratio of heat producing volume to heat exchanging surface is increasing with any scale up and thereby improving the accuracy and stability of the calorimetric signal.

Until now, only few attempts were made to establish a calorimetry-driven process control despite the pioneering works of Larsson et al. (1991) and Randolph et al. (1990) demonstrating the control of glucose feeding. Only recently, the potential of biocalorimetry as a tool for controlling microbial growth processes was further investigated by Biener et al. (2010, 2012) and Schuler et al. (2012). In these approaches, feedback control strategies were applied that allowed to maintain a constant specific growth rate on glucose.

However, the conversion of toxic substrates into bioproducts may require different approaches to process control. This was first demonstrated for the calorimetrically controlled bioconversion of phenol into polyhydroxyalkanoate or ectoine using a sequence of batch processes (Maskow and Babel, 2001) or continuous processes (Maskow and Kleinsteuber, 2004; Maskow et al., 2006). For the industrially much more important fed-batch processes, a control strategy for the conversion of toxic substrates into bioproducts is still a challenge. Therefore, we aimed to develop a feeding strategy for fed-batch processes which is applicable to toxic feedstocks. Furthermore, the targeted control strategy is not only supposed to optimize the growth rate but also the product formation rate. The latter is important, because growth and product formation are often sequential processes. To distinguish between these metabolic stages, a further online signal (i.e., respirometry) has to be included. The combined measurement of heat and CO_2 emission rates, referred to as calorespirometry, thus serves as the basis for an improved feeding strategy.

Materials and Methods

Bacterial Strain and Growth Media

M. extorquens AM1 (DSM 1338) was routinely grown in mineral salt medium containing 4 g L^{-1} of methanol as described by Choi et al. (1989). For fermentation experiments, media were composed as follows: $(NH_4)_2SO_4$, 5 g L^{-1}; KH_2PO_4, 1.305 g L^{-1}; Na_2HPO_4 *7 H_2O, 4.02 g L^{-1}; $MgSO_4$ *7 H_2O, 0.45 g L^{-1}; $CaCl_2$ *2 H_2O, 20 mg L^{-1}; $FeSO_4$ *7 H_2O, 2.6 mg L^{-1}; $MnSO_4$ *H_2O, 200 μg L^{-1}; $ZnSO_4$ *7 H_2O, 260 μg L^{-1}; $CuSO_4$ *5 H_2O, 80 μg L^{-1}; Na_2MoO_4 *2 H_2O, 80 μg L^{-1}; $CoCl_2$ *6 H_2O, 80 μg L^{-1}; H_3BO_3, 60 μg L^{-1}; pH 7.0. Fermentation media were supplied with 0.5 g L^{-1} of methanol prior to inoculation.

Culture Conditions and Reactor Design

All pre-cultures were inoculated with a frozen stock containing 1.5 mg of cells in 10% glycerol and grown in shaking flasks with 50 mL mineral salt medium at 30°C and 150 rpm for 72 h. These cultures were used to inoculate the 2 L bioreactor of reaction calorimeter RC1e (Mettler Toledo, Greifensee, Switzerland) with an initial working volume of 1.4 L. The RC1e has all features of a typical bioreactor (stirrer, baffles, in- and outlet ports, probes for pH and dissolved oxygen). For fed-batch cultivation, the reactor was run with a constant temperature of 30 ± 0.0003°C and constant stirring at 800 ± 1 rpm. pH was maintained at 7.0 ± 0.05 by addition of 1 M sodium hydroxide. The air flowing through the bioreactor (1.0 ± 0.01 L min^{-1}) was sterilized by passing through a 0.2 μM pore filter, water saturated, and heated to reactor temperature in a washing bottle. The outlet gas passed a condenser and another 0.2 μM pore filter before entering the gas analyzer Ultramat 23 (Siemens AG, Nürnberg, Germany). The signal noise of the Ultramat was 0.05% for oxygen and 0.005% for CO_2. Feeding was carried out by weighed addition of 500 g L^{-1} methanol in nitrogen-free culture medium.

Online Analytics and Data Processing

The working principle of reaction calorimeter RC1e has been described elsewhere (Marison et al., 1998). All process parameters (temperature difference, pH, dissolved oxygen level, off-gas oxygen

and carbon dioxide levels, mass of substrate added) were collected and recorded in 2 s intervals by the iControl software (Mettler Toledo, Greifensee, Switzerland). The software also performed temperature and pH control. The data for temperature difference and CO_2 level were directly transmitted via icDataShare software (Mettler Toledo) to a MS-Excel document (Microsoft, Redmond, WA) containing the process control unit implemented in Visual Basic for Applications (VBA). This acted as a soft sensor by performing a moving average of raw data over 5 min and calculating the heat per CO_2 ratio which was further smoothed by a recursive filter. From these data, various growth parameters such as the specific growth rate, biomass concentration, and substrate consumption rate were estimated (all equations and derivations are given in the supplementary material, section A) and the actual feeding rate of methanol was calculated. Feeding was subsequently carried out by a remote-controlled peristaltic pump. For growth simulations, additional code was integrated into the process control unit (see supplementary material, section B).

Off-Line Analytics

After adding the pre-culture, samples from the reactor were taken regularly over a total period of 3 days. The concentrations of methanol and other metabolites were quantified by high-performance liquid chromatography with refractive index detection after separation on a Rezex ROA-Organic Acid H+ column (300 × 7.8 mm, Phenomenex, Torrance, CA) using an eluent of 0.01 N sulfuric acid at 0.5 mL per min. Bacterial dry mass was determined gravimetrically after oven-drying at 105°C for 5 h. The amount of intracellular polyhydroxybutyrate was quantified by gas chromatography (GC) after hydrochloric acid propanolysis as described before (Riis and Mai, 1988). GC measurements were carried out with a GC-14B instrument (Shimadzu, Kyoto, Japan) using a zebron ZB-35 HT Inferno capillary (20 m *0.18 mm, Phenomenex).

Results and Discussion

Simulating Growth of *M. extorquens* on Methanol

Recently, a feedback control strategy based on biocalorimetry was developed by Schuler et al. (2012). They applied a proportional-integral-derivative (PID) controller to maintain a constant specific growth rate of yeast cultures on glucose. However, our own experiments and an *in silico* simulation (see supplementary material, section B) illustrated the weaknesses of the PID controller when applied to growth of *M. extorquens* on methanol (Fig. 1). In particular the approach was inappropriate due to: (i) the toxic properties of methanol; (ii) disregard of the change in the metabolism from growth to product formation and an associated decrease in the specific growth rate (see supplementary material, Fig. S1); and (iii) the extremely low affinity constant (K_S) of bacteria for methanol in comparison to the tested yeasts.

In the feeding strategy of Schuler et al. (2012), the specific growth rate was chosen as the set parameter for PID control. In case of toxic feedstocks, however, a decrease in growth rate may be the result of either a low substrate concentration or an intoxication of the

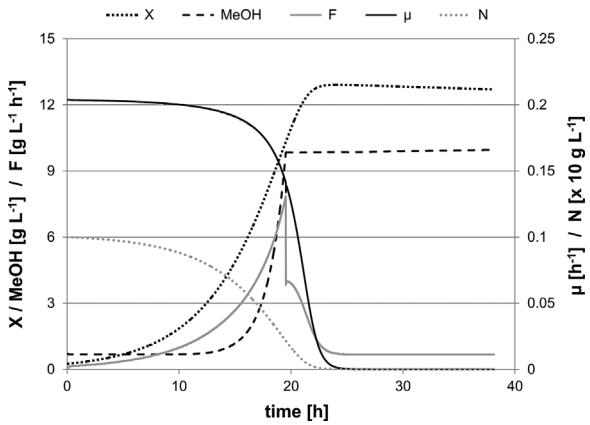

Figure 1. Simulating growth of *M. extorquens* on methanol with a PID controller. The MS-Excel VBA script simulates growth with previously determined growth parameters and calculates the actual biomass concentration *X*, methanol concentration (MeOH), feeding rate *F*, specific growth rate μ, and nitrogen concentration *N*. The simulation starts at the beginning of exponential growth phase and is stopped in the product formation phase. Apart from the targeted specific growth rate being set to 0.2 h^{-1}, the software was programmed to keep a maximum methanol concentration of 10 g L^{-1} to avoid an infinite increase of methanol.

culture. As the controller is not able to distinguish between both scenarios, it will always react by increasing the feeding rate which may lead to a self-enforcing destabilization of the process.

A second difference from the yeast example is the fact that *M. extorquens* forms PHB when the nitrogen in the culture medium becomes limited. Exponential growth will thus inevitably cease over time as cells pass into a kind of stationary or product formation phase, which is characterized by PHB formation and energy spilling (Russell, 2007). In this phase, PID control will also not work properly as it will try to maintain a constant growth rate by increasing substrate feed. This can be seen in the simulation as a massive overfeeding of the culture after 19 h (Fig. 1).

Furthermore, while for yeasts like *S. cerevisiae* the half-saturation constant K_S of glucose is in the range of 900 mg L^{-1} (Kotyk and Janáček, 1975), the equivalent value of methanol for *M. extorquens* is 0.3 mg L^{-1} (Page and Anthony, 1986). This did not visibly affect growth during simulation, because the methanol concentration in the reactor was always at least 500 mg L^{-1} and thus several orders above the K_S value. However, if the concentration decreases into the range of K_S, the specific growth rate will decline very suddenly and provoke a fierce controller reaction. This would lead to pulsed feeding not resulting in constant growth. For these reasons, the control strategy described by Schuler et al. (2012) is not suited for growth of *M. extorquens* on methanol.

Development of an Alternative Feeding Strategy

In contrast to the feedback regime via a PID controller, we developed an alternative feeding strategy based on a feedforward system, that is the controller output is based on an evaluation of all available sensory information. This controller does also include an exponential growth model, but performs a periodic adaptation of

growth parameters. The core of the new feeding strategy is a MS-Excel VBA script receiving the online process data from the reaction calorimeter and calculating the respective feeding rate of methanol which is added via a pump (see Fig. 2).

The first step of the control strategy consists of the calculation of the actual specific growth rate from the heat production rate, similar to the approach of Schuler et al. (2012). In the next step, the specific growth rate is used to calculate the actual substrate consumption and to adjust the feeding rate to compensate for the consumption. Thus, the residual methanol concentration in the reactor is estimated at any time. To verify the accuracy of these calculations, a special control procedure was developed: after defined time intervals, the control software stops methanol addition and waits until methanol is completely consumed by the culture. This is detected immediately as a sudden drop of the heat signal. The program then rapidly feeds a pre-defined amount of methanol into the reactor. This ensures that at that time, the methanol concentration is mainly controlled by pumping and only insignificantly by consumption. Subsequently, substrate addition is stopped again. Depending on the progressing biomass concentration X, the specific growth rate μ, the combined yield coefficient for biomass and product formation $Y_{CO/S}$, and the observed time interval until methanol is consumed again, the program calculates a correction factor for $Y_{CO/S}$, which is implemented into the equation for methanol consumption (for detailed information see supplementary material, Equation (A21) and derivations). Thus, if the real measurements do not absolutely coincide with the estimations, the feeding rate is adjusted appropriately. A detailed flow diagram of the process control software and the exemplary course of an adjustment cycle are shown in supplementary Figure S2 and S3, respectively.

The correction factor for $Y_{CO/S}$ is maintained until the next adjustment cycle where it is calculated anew. In this way, the software continuously revises itself and an accumulation of errors is minimized. The methanol concentration will always stay below a critical limit that can be defined in advance by specifying the amount of methanol added after depletion. If, due to measuring errors, substrate feed were too low estimated and thus methanol is consumed before the onset of the next cycle, the system will recognize this by a decline in the heat signal and start a new adjustment cycle with $Y_{CO/S}$ reset to its initial value.

Prior to testing the process control, several experiments were conducted to obtain basic values for the yield coefficients $Y_{Q/CO}$ and $Y_{CO/S}$. The combined heat yield coefficient for biomass and product formation $Y_{Q/CO}$ was determined by plotting the heat production rate $\dot{Q}$ against the formation rate of PHB-free biomass r_X for a whole cultivation process (Fig. 3). Thereby, the resulting curve shows an abrupt change which can be explained by the different stages of growth of *M. extorquens*. Each fermentation starts with a lag phase which is characterized by very low heat and biomass production rates. During subsequent exponential growth, heat production is proportional to biomass formation as expected. The measured value of $-50{,}300\,J\,g^{-1}$ is near the balanced heat yield coefficient of $-45{,}227\,J\,g^{-1}$ (see supplementary material, section C). As soon as the nitrogen in the medium is consumed, the culture shifts into the product formation phase. During this stage, the biomass formation rate decreases while the heat production rate remains almost stable. For that reason, the value for $Y_{Q/CO}$ drops to $-5{,}400\,J\,g^{-1}$ and an intercept term of $-4.91\,J\,g^{-1}\,s^{-1}$ is added. This can be correlated with the coefficient for heat production for biomass maintenance $m_{Q/CO}$ and is well in accordance with calculated values between -3.6 and $-6.1\,J\,g^{-1}\,s^{-1}$. In theory, if there is no growth, $\dot{Q}$ is no

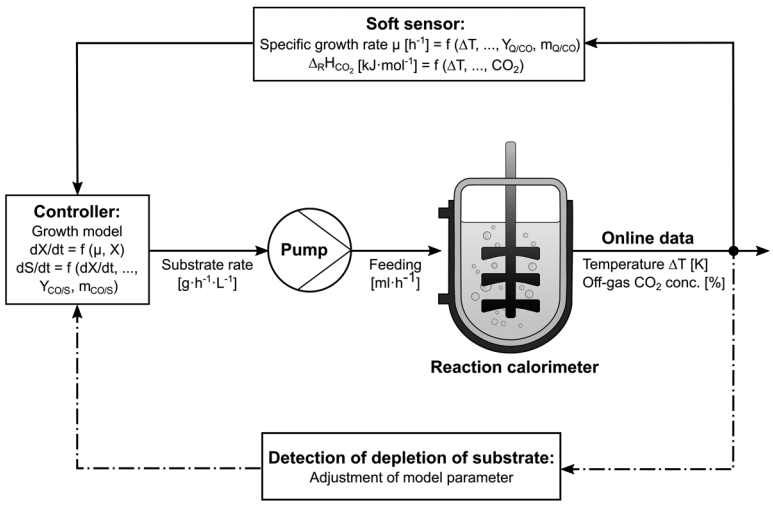

Figure 2. Schematic view of the calorespirometric process control strategy. Online data of the reaction calorimeter RC1e for temperature difference ΔT and off-gas CO_2 level are transmitted to the MS-Excel VBA script serving as soft sensor and process control unit. Depending on the estimated growth parameters and a possible detection of substrate depletion, the feeding rate is calculated and adjusted as necessary. Substrate feeding is executed by a remote-controlled pump. The previously determined coefficients ($Y_{Q/CO}$, $Y_{CO/S}$, $m_{Q/CO}$, $m_{CO/S}$) used for estimation of specific growth rate μ and change in substrate concentration dS/dt, are indicated. For detailed calculations, see supplementary material, section A.

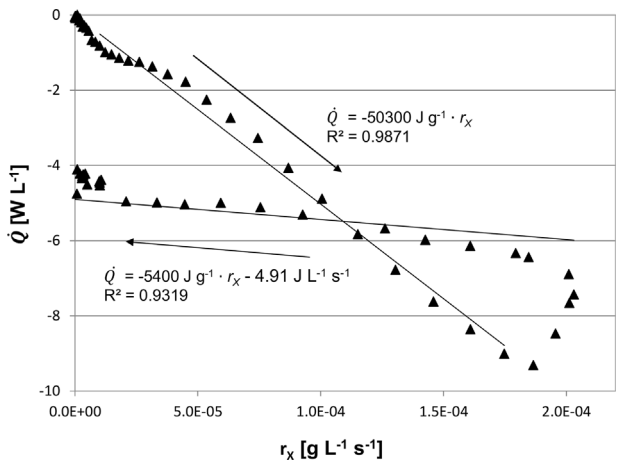

Figure 3. Heat production rate as function of biomass formation rate. The heat production rate $\dot{Q}$ versus biomass formation rate r_X is plotted for the course of a cultivation of *M. extorquens* on methanol. Equations and R squared values of linear fitting for exponential growth phase and product formation phase are indicated.

longer a function of r_X and the curve is expected to be parallel to the x-axis. In our experiment, this seemed to be almost the case.

For the combined yield coefficient for biomass and product formation per substrate $Y_{CO/S}$, an average value of 0.35 g biomass per g methanol was obtained by plotting the methanol consumption rate against the biomass formation rate (not shown). Similar to $m_{Q/CO}$, a coefficient of substrate consumption for biomass maintenance m_S of $1.5 \times 10^{-5}\,\mathrm{g\,g^{-1}\,s^{-1}}$ could be calculated for the product formation phase.

During the transition from exponential growth to product formation phase, the yield coefficients are changing gradually. These changes must be taken into account to attain an accurate estimation of specific growth rate and substrate consumption for the whole process. This requires that the control software reliably recognizes the different stages of growth.

Differentiation Between Growth Phases Via Calorespirometry

After inoculation of the bioreactor with the pre-culture, growth of *M. extorquens* usually shows a lag phase of 8–12 h. As during this stage online data for ΔT and CO_2 level are of similar size as the instrument noise, the process control is not yet started. Only when the heat signal exceeds a signal-to-noise ratio (SNR) of about 5, the first adjustment cycle is initiated.

The differentiation between exponential growth and product formation phase uses the detected shifts in metabolism. If only microbial growth is considered, five yield coefficients describe the total growth stoichiometry and can be calculated using four balances for the elements (C, H, O, N) and the heat balance (Maskow and Harms, 2006; von Stockar et al., 1993). However, if a product is formed, an additional online measure is required. Therefore, we took advantage of calorespirometry, a rarely used, but highly informative technique (Wadsö and Hansen, 2015). Many metabolic events, for example, shifts from one substrate to another, or occurrence of nutrient limitations, lead to a characteristic change in the calorespirometric ratios, heat per CO_2 and heat per O_2. Hence, these ratios can contain information about the coupling of catabolic and anabolic processes and can be used to distinguish between these processes or to quantify product formation (Maskow et al., 2011). For

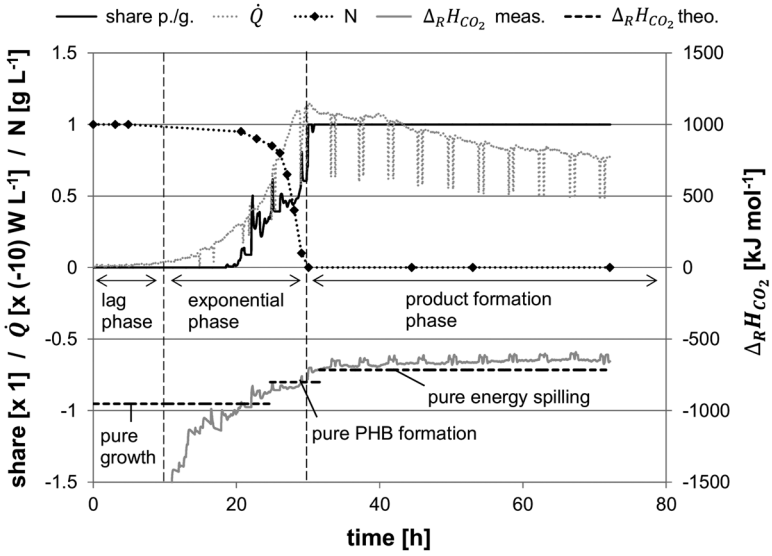

Figure 4. Influence of the shift from growth to product formation phase on the heat production rate and the heat per CO_2 value. The measured heat production rate $\dot{Q}$, heat per CO_2 value, and nitrogen concentration *N* during growth of *M. extorquens* on methanol by applying the calorespirometric process control strategy are shown. The share of product formation phase to growth (share p./g.) was calculated by the control software from $\Delta_R H_{CO_2}$. Thereby, a value of 0 stands for pure growth and a value of 1 for pure product formation and energy spilling. Additionally, the theoretical values of heat per CO_2 (theo.) for pure growth, pure PHB formation, and pure energy spilling are displayed.

M. extorquens, a theoretical value of heat per CO_2 ($\Delta_R H_{CO_2}$) can be calculated for every share of product formation or energy spilling to growth from the respective enthalpy balances and the molar ratio of CO_2 release (see supplementary material, section C).

Indeed, $\Delta_R H_{CO_2}$ changed notably during cultivation of *M. extorquens* and the control software was able to calculate the actual share of product formation phase (consisting of product formation and energy spilling) to growth (share p./g., see Fig. 4). This value is already increasing during exponential growth and reaches 1 when nitrogen is entirely consumed, which is also marked by a significant maximum in the heat signal. Comparing the measured $\Delta_R H_{CO_2}$ data with the theoretical values calculated for pure growth, pure PHB formation and pure energy spilling, the signal shows a slow transition from one metabolic state to the other. The deviation between measured and theoretical values is only significant in the beginning, which can be explained by ΔT as well as CO_2 level being still very low. For the later stages of the process, $\Delta_R H_{CO_2}$ data could be used successfully to estimate the actual share of product formation phase and thus enable the control software to modulate the yield coefficients for an accurate calculation of specific growth rate and substrate consumption.

Application of the Calorespirometric Control Strategy

A typical cultivation of *M. extorquens* on methanol applying the improved control strategy is shown in Figure 5. As described above,

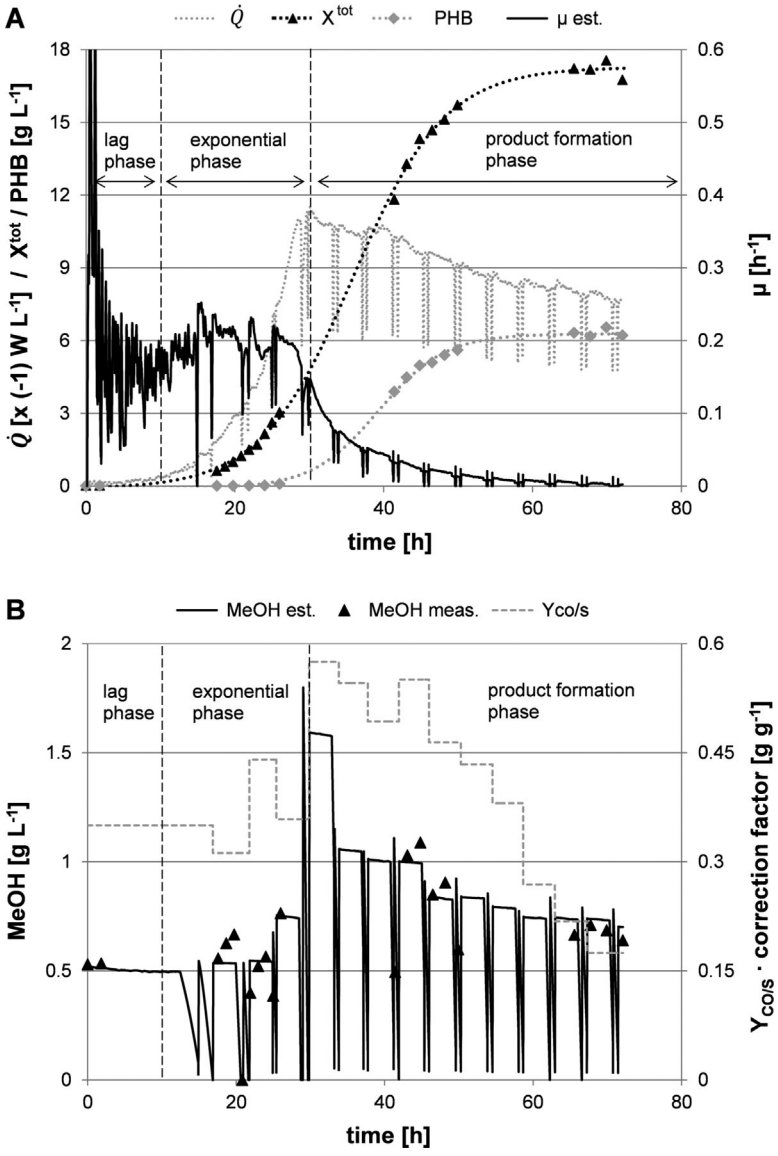

Figure 5. Growth pattern and product formation of *M. extorquens* on methanol by applying the calorespirometric process control strategy. The heat production rate $\dot{Q}$, total biomass concentration X^{tot}, PHB concentration (PHB), and estimated specific growth rate (μ est.) (**A**) as well as the methanol concentration estimated by the control software (MeOH est.) and quantified via HPLC (MeOH meas.), and the modulated combined yield coefficient $Y_{CO/S}$ (**B**) are displayed as a function of time.

the heat signal serves as the basis to calculate the specific growth rate μ (Fig. 5A). After about 10 h, the culture enters the exponential growth phase and the process control software initiates the first adjustment cycles. The substrate depletion induced thereby is immediately recognized by a fast decline in the heat signal and methanol is added allowing growth to continue steadily. Off-line measurements of methanol concentration showed that the estimations of the software were quite accurate (Fig. 5B). Small deviations could be corrected within the next adjustment cycle. This can be seen as changes in the corrected combined yield coefficient $Y_{CO/S}$, leading to an inverse change in the feeding rate. Using $\Delta_R H_{CO_2}$ data for calculating the specific growth rate, the estimation of substrate consumption by the program was very precise and the corrected $Y_{CO/S}$ lay between 0.18 and 0.58 g g^{-1} during the whole process. This is in accordance with final yields ranging from 0.19 to 0.33 g g^{-1} (Bélanger et al., 2004; Bourque et al., 1995). After 3 days of cultivation, *M. extorquens* reached total biomass and PHB concentrations of 17.55 and 6.54 g L^{-1}, respectively. The cells thus contained on average 37.3% PHB. This is perfectly in the range of previously reported maximal PHB contents (34–42%) for *M. extorquens* AM1 (Korotkova and Lidstrom, 2001). The observed maximal specific growth rate during exponential growth (0.209 h^{-1}) was close to the theoretical maximum of 0.21 h^{-1} (Peyraud et al., 2011). The methanol concentration on the other hand was never higher than 1.8 g L^{-1} and thus far below the critical toxic level of 8 g L^{-1} (Bourque et al., 1992).

To evaluate the efficiency of the calorespirometric control strategy, it was tested in three independent growth experiments and compared to cultivations using a process control aiming at maintaining a constant specific growth rate μ as described by Schuler et al. (2012), and to cultivations with manually adjusted feeding. The results are summarized in Table I. In the experiments performed with process control for μ, the software always increased methanol feed in response to a declining μ and thus enhanced the methanol concentration above a toxic level. In cultivations without process control, the methanol consumption rate could only be roughly estimated and thus the feeding rate was often either too high or too low, resulting in overfeeding or extended starvation. Applying the calorespirometric process control strategy, these inefficiencies could be avoided and the cultures exhibited optimal growth. This was reflected in the highest maximal growth rates and biomass concentrations. The PHB content, which particularly is an indicator of appropriate methanol dosage, was considerably higher with the new strategy.

Table I. Demonstration of the effectiveness of the new calorespirometry-derived process control strategy by comparison with differently controlled processes.

Control strategy	μ_{max} [h^{-1}]	Max. biomass conc. X^{tot} [g L^{-1}]	Max. PHB conc. [g L^{-1}]	Max. MeOH conc. [g L^{-1}]
Without process control	0.185	8.83	1.39	56.47
	0.180	10.37	2.89	5.17
	0.194	8.30	1.31	7.60
Process control for μ	0.180	12.70	0.80	18.80
	0.196	11.94	1.86	17.16
	0.171	13.98	3.78	16.79
Calorespirometric process control	0.212	15.57	5.23	1.43
	0.211	14.46	4.72	1.64
	0.209	17.55	6.54	1.79

The maximal specific growth rates μ_{max} and the maximum concentrations of total biomass, PHB, and methanol are given for growth of *M. extorquens* on methanol by applying either the calorespirometric process control strategy, a control strategy with a PID controller aiming at maintaining a constant specific growth rate (process control for μ), or by manually adjusting methanol feed (without process control).

The new control software offers several settings for process variation. The time interval between adjustment cycles can be chosen freely, depending on the desired degree of precision. For cultivation of *M. extorquens*, the interval was 3 h, but for a very exact adjustment of feeding rate, intervals as small as 30 min can be selected. Furthermore, the amount of substrate added during cycles can be defined to ensure that the concentration remains below a critical level. In case of more toxic feedstocks than methanol, concentrations can be limited to 0.1 g L^{-1} or even lower.

These parameters however also define the limits of the new control software. The longer the interval between adjustment cycles is set, the higher is the probability of error accumulation leading to miscalculations of the feeding rate. Also, if a low substrate concentration is fed to a high amount of bacterial biomass, the time of substrate consumption is very short and minor deviations can cause a high variation in the $Y_{CO/S}$ value which manifests itself as process oscillation. For this reason, an accurate estimation of growth parameters like $Y_{Q/CO,}$ e.g. from respirometric ratios is very important.

Conclusions

This study reveals the high potential of biocalorimetry as a tool for qualifying toxic substrates for biotechnological processes. The newly developed process control strategy enabled an optimal growth of *M. extorquens* on methanol and a considerably improved formation of PHB compared to other strategies. In addition, calorespirometric data were used successfully to independently recognize different stages of growth.

In contrast to other techniques, the calorimetric approach is completely independent of the chosen substrate and can be applied for even more toxic compounds such as formaldehyde or formic acid. If the specific yield coefficients are known, the calorespirometric control strategy can be used for all kinds of microbial cultivations. This way, biotechnological processes can be optimally controlled and, depending on the particular metabolic pathways of the applied microorganisms, a variety of bulk chemicals and fuels can be produced more sustainably.

Due to the many advantages of biocalorimetry, this method is especially suitable for industrial applications. Above all, because any up-scaling of the process will increase the precision of the signal. In the future, also other calorimetric measurements (e.g., megacalorimetry, photocalorimetry, calorimetry of bioelectrochemistry) could be used for process control and the integration of further online data such as oxygen uptake rate, FT-IR, or fluorescence spectroscopy could enlighten additional aspects of growth.

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Nomenclature

CO_2	carbon dioxide level in the exhaust gas [%]
F	substrate feeding rate [$L\,s^{-1}$]
K_S	half-saturation constant [$g\,L^{-1}$]
$m_{Q/CO}$	combined coefficient for heat production for biomass maintenance [$J\,g^{-1}\,s^{-1}$]
$m_{CO/S}$	combined coefficient for substrate consumption for biomass maintenance [$g\,g^{-1}\,s^{-1}$]
N	nitrogen concentration [$g\,L^{-1}$]
$\dot{Q}$	heat production rate [$W\,L^{-1}$]
$\Delta_R H_{CO_2}$	heat per CO_2 [$J\,mol^{-1}$]
r_X	biomass formation rate (PHB-free) [$g\,L^{-1}\,s^{-1}$]
X	biomass concentration (PHB-free) [$g\,L^{-1}$]
X^{tot}	total biomass concentration (including PHB) [$g\,L^{-1}$]
$Y_{Q/CO}$	combined heat yield coefficient for biomass and product formation [$J\,g^{-1}$]
$Y_{CO/S}$	combined yield coefficient for biomass and product formation per substrate [$g\,g^{-1}$]
ΔT	temperature difference [K]
μ	specific growth rate [h^{-1}]
μ_{max}	maximal specific growth rate [h^{-1}]

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