

On maintenance models in severely and long-term limited membrane bioreactor cultivations

Anja Drews and Matthias Kraume

corresponding author: Anja Drews

TU Berlin, Verfahrenstechnik, Sekr. MA 5-7, Straße des 17. Juni 136, 10623 Berlin, Germany.

anja.drews@tu-berlin.de, Phone: +49-30-31424852, Fax: +49-30-31421134

Summary

Membrane bioreactors (MBR) are combinations of common bioreactors and membrane separation units for biomass retention. Through increased biomass concentration, they allow increased productivity (or smaller reactor volume, respectively). Besides high biomass concentrations, operation at very low growth rates is typical for MBRs. In this regime, maintenance metabolism where substrate uptake only yields energy for cell survival becomes of higher importance than in processes run at higher growth rates. While thermodynamically based correlations for the prediction of maintenance coefficients are available for chemostat or other medium growth rate processes, some authors have mentioned a change in energy demand in MBRs and a dependence of maintenance parameters on operating conditions. Due to the fact that often mixed cultures are used and resulting from the different evaluation methods used by different authors, views on the possible influences on maintenance parameters differ. However, it is accepted that common models describing microbial growth and production of metabolites or degradation of pollutants do not consider the effects caused by severe limitations and therefore cannot sufficiently be applied to MBRs. In this study, maintenance parameters were determined for a model organism (*Ustilago maydis*) and results from different evaluation methods were compared. A continuous fit of respiration data gave more consistent results than the traditional method of plotting specific uptake vs. growth rate. They suggest that below $\mu = 10\% \mu_{\max}$ the maintenance coefficient drops to a third of the value in short-term limited cultures.

Keywords: maintenance energy, membrane bioreactor, substrate limitations, carbon dioxide evolution rate, online estimation, *Ustilago maydis*

1 Introduction

Due to the retention of biomass, membrane bioreactors (MBR) allow increased volumetric productivity. In comparison to batch, a 40-fold increase in ethanol production by *Saccharomyces cerevisiae* has been reported (Arnot and Howell, 2001). With the organism used in this study, ferrichrome productivity was increased by a factor of 12 (Drews and Kraume, 2005). By decoupling of hydraulic and biomass retention times, MBRs offer an additional degree of freedom for process control in comparison to chemostats.

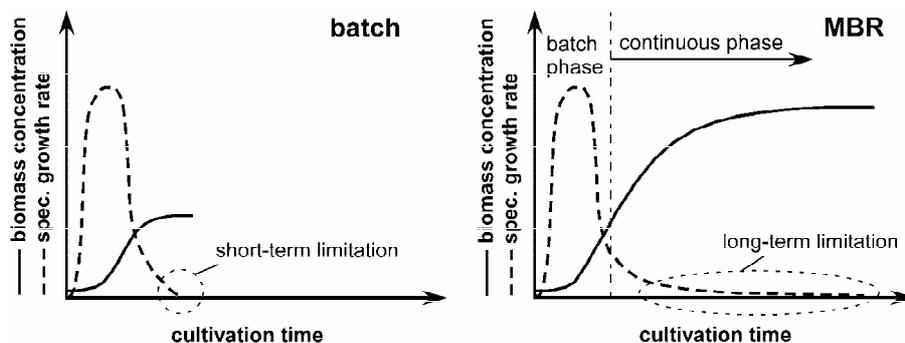


Fig. 1: Development of biomass concentration and specific growth rate in a batch and in an MBR cultivation.

In a typical MBR process as sketched in Fig. 1, due to the rising biomass concentration each cell is subjected to an increasing substrate limitation which leads to a decrease in growth rate down to zero growth. In this region, maintenance metabolism which always takes place in parallel to growth metabolism gains higher importance. While very low growth rates are often encountered in nature, the physiological consequences have not been explored as exhaustively as those at higher growth rates or at starvation conditions (Konopka, 2000). In biological wastewater treatment, where MBRs are already widely established, this effect is observed as decreased sludge production at higher sludge ages. High cell densities or progressing limitations can also lead to a change in metabolism. This means that kinetic parameters determined in common experiments like fed-batch and chemostat do not sufficiently describe conditions in MBRs. If a change in growth behaviour occurs at low growth rates, a change in production behaviour is of course conceivable (Drews and Kraume, 2005), too, and must be borne in mind when designing a biological production process.

Resulting from the interest in decreased excess sludge production in wastewater treatment, literature on maintenance in MBRs traditionally deals with mixed cultures. To eliminate the effects of varying populations and microbial communities, and that of non-hydrolysable substrates, however, kinetic measurements should be carried out in pure cultures. In this study, *Ustilago maydis* is used as a model organism to determine maintenance parameters. *Ustilago maydis* is a phytopathogenic fungus growing yeast-like in oval shaped single cells (length approx. 10µm). It can be used for production of ferrichrome, a siderophore with a variety of medical and agricultural applications (Neilands, 1995).

2 Theory

For design, monitoring, and control of a process, reliable models are required. Balance equations for the individual components (biomass, substrates, nutrients, and metabolites) are coupled via yield coefficients. Biomass yields from substrate uptake can be considered constant over wide ranges of growth rates. However, especially at very low growth rates, other phenomena must be taken into account. To describe such phenomena, Pirt (1965) introduced the maintenance concept whereby part of the substrate is always used for cell survival and not for reproduction, the corresponding substrate uptake rate (expressed as specific rate m_s) therefore only yielding energy for maintenance processes like renewal of the cell membrane and maintenance of transmembrane ion gradients (Bulthuis et al., 1989). This rate depends on the type of the limiting substrate and on temperature (Tijhuis et al., 1993). $Y_{B/s}^g$ represents the true yield which relates the formed biomass to the substrate mass used for growth as opposed to maintenance purposes.

$$Y_{B/s}^g = \frac{\Delta c_B}{\Delta c_s^g} \quad (1)$$

According to Pirt (1965), the substrate uptake rate can be expressed as:

$$-\dot{r}_s = \frac{\dot{r}_B}{Y_{B/s}^g} + m_s \cdot c_B \quad \Leftrightarrow \quad \sigma = \frac{\mu}{Y_{B/s}^g} + m_s \quad (2)$$

$$\text{or: } \frac{1}{Y_{B/s}} = \frac{1}{Y_{B/s}^g} + \frac{m_s}{\mu} \quad (3)$$

$$\text{with: } Y_{B/s} = \frac{\Delta c_B}{\Delta c_s^g + \Delta c_s^m} \quad (4)$$

From eq. (3) it becomes obvious that $Y_{B/s} \neq \text{const}$, or in other words $Y_{B/s} = f(\mu)$. Constant values can only be assumed for $\mu \gg m_s$, i.e., at large growth rates, substrate uptake is dominated by growth purposes and m_s can be neglected.

Another perception underlies the model of endogenous metabolism. Here, substrate uptake at net zero growth is explained by lysis of some of the already produced cells (Herbert, 1958). The lysis products serve as substrates for other cells (cryptic growth). Like maintenance, this endogenous metabolism always takes place in parallel to anabolism and can be neglected during sufficient substrate supply.

$$\dot{r}_B = \mu_s \cdot c_B - \mu_e \cdot c_B = \mu \cdot c_B \quad (5)$$

If lysis products are not completely reused for growth, the lysis rate μ_e is often called death rate or death coefficient k_D . In terms of their mathematical model description both maintenance and the endogenous metabolism are equivalent: Since

$$\mu_s = \sigma \cdot Y_{B/s}^g \quad (6)$$

eq. (5) can be transformed into eq. (2):

$$m_s = \frac{\mu_e}{Y_{B/s}^g} \quad (7)$$

Current concepts assume that organisms do not die just like that (only predation, adverse conditions, toxic substances or viruses lead to death) but under limitations fall into dormancy during which they do not require any maintenance energy, and from which they can be awoken again months later at fresh substrate supply (Van Loosdrecht and Henze, 1999). Some authors mention the occurrence of subpopulations which grow at a lower rate than the culture on average (Müller and Babel, 1996) or even „sleep“ (Pirt 1987, quoted in Bulthuis et al., 1989). According to Konopka (2000), however, these mechanisms are not dominant in MBRs. Another explanation is that of a critical biomass concentration above which growth is strongly inhibited (quorum sensing). Menshutina et al. (2001) give 115 g L^{-1} for *Lactobacillus casei*.

Whichever phenomenon prevails, a large number of processes can be satisfactorily described by the Pirt concept. It has often been observed and described that m_s is not constant (Pirt, 1965; Tjihuis et al., 1993). Deviations from the linear relationship between \dot{r}_s and \dot{r}_B (or σ and μ , respectively) seem to occur when growth is limited by a substrate or nutrient other than the energy substrate (Neijssel and Tempest 1976, quoted in Bulthuis et al., 1989; Pirt, 1982). To account for this, Pirt (1982) introduced a growth dependent term (see Fig. 2):

$$\sigma = \frac{\mu}{Y_{B/s}^g} + m_s + m'_s \cdot \left(1 - \frac{\mu}{\mu_{max}}\right) \quad (8)$$

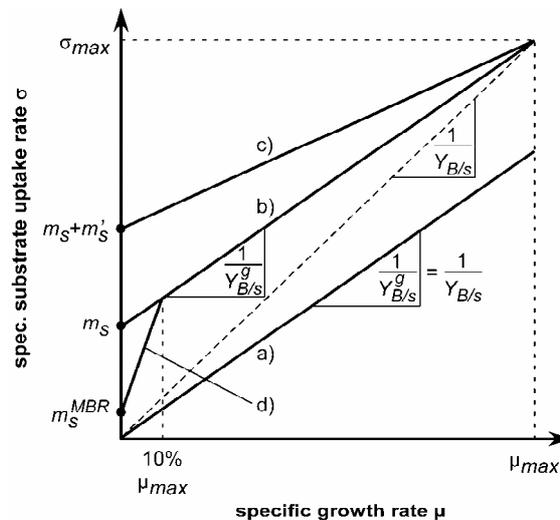


Fig. 2: Illustration of the maintenance concept according to Pirt: a) without maintenance coefficient, b) with constant maintenance coefficient (eq. (2), with --- apparent yield, eq. (4)) and c) with growth dependent maintenance coefficient (eq. (8)), d) at severe limitations, e.g. in an MBR

The formation of energy storage substances such as glycogen is assumed to represent the growth dependent part of the maintenance metabolism under sufficient energy substrate supply (Van Loosdrecht and Henze, 1999; Pirt, 1982). In practice, however, this growth dependent term is seldom considered.

Ihssen and Egli (2004), however, state that the specific growth rate and neither the nature of the limiting nutrient nor cell density is responsible for expression of the stress response sigma factor RpoS, an RNA polymerase subunit which enables *E. coli* to rapidly adapt to various stress conditions and thereby to survive under severe limitations. A common response to nutrient limitation is increased synthesis of membrane-based permeases for the limiting nutrient (Konopka, 2000).

Knowledge of the parameters maintenance coefficient m_s and true yield $Y_{B/s}^g$ permits modelling of the process including final biomass concentration at steady state and substrate removal rate $-\dot{r}_s$. In a comprehensive literature study, Tjihuis et al. (1993) derived a thermodynamically based correlation for maintenance requirements in chemostat cultivations valid for different autotrophic, heterotrophic, aerobic, and anaerobic organisms and a temperature range of 5 - 75 °C. The authors found an Arrhenius-type relationship which gives the energy equivalent of the maintenance coefficient per mole C with a relative deviation of ± 41 %:

$$e_{m,C} = 5.7 \cdot \exp \left[\frac{-6.94 \cdot 10^4}{R} \text{ J mol}^{-1} \cdot \left(\frac{1}{T} - \frac{1}{298K} \right) \right] \text{ kJ (mol h)}^{-1} \quad (9)$$

1 mol C is contained in approx. 25 g dry matter (Bulthuis et al., 1989; Tjihuis et al., 1993; van Verseveld et al., 1984), thus, $e_{m,C}$ can be transferred into e_m , the energy equivalent per biomass in kJ (g h)^{-1} . This in turn can be converted into m_s using the Gibbs free energy for oxidation of each substrate. Kinetic measurements are frequently carried out in chemostats as they can be operated at steady state and at the desired specific growth rate. Thus, in comparison to batch experiments kinetics determination is more precise and reliable (Sipkema et al., 1998). For investigating the phenomena at very low or even zero growth rates, however, chemostats are not suited since due to $\mu = D$ zero growth is impossible by definition. Different authors (Bulthuis et al., 1989; Tappe et al., 1996; Van Loosdrecht and Henze, 1999; van Verseveld et al., 1984) give approx. 0.01 h^{-1} as the order of magnitude for the smallest technically possible dilution or growth rate in chemostats. Extrapolation of data towards zero growth is problematic because below $\mu_{crit} \approx 10$ % μ_{max} microorganisms undergo severe changes in metabolism (Van Loosdrecht and Henze, 1999; van Verseveld et al., 1984; Konopka, 2000). Applying the Pirt concept, several authors have reported a significant reduction of maintenance demand at very low growth rates in comparison to chemostat data (Pirt, 1987; Bulthuis et al., 1989; van Verseveld et al., 1984; Low and Chase, 1999; Müller and Babel, 1996; Tros et al., 1996). For an MBR cultivation of *Pseudomonas*, Tros et al. (1996) found m_s -values one-third that obtained in chemostats. Contrary to that, using *Paracoccus denitrificans* in the same study, van Verseveld et al. (1984) observed a slight increase in maintenance energy in comparison with a chemostat.

While quite a few variations – mainly decreases – of m_s have been observed in MBRs, opinions on its dependence on operating parameters differ. No generally valid correlation for the influence of hydraulic residence time, specific growth rate or sludge age has been reported yet. For wastewater treatment MBRs, Bouillot et al. (1990) and Wisniewski et al. (1999) suggest $m_s \approx 0.04 \text{ mgCOD (mgVSS h)}^{-1}$ and $Y_{B/s}^g \approx 0.36 \text{ mgVSS (mgCOD)}^{-1}$. By plotting $\sigma = f(\mu)$, Bulthuis et al. (1989) found a decrease of both m_s and $Y_{B/s}^g$ at lower hydraulic residence times. In a study cultivating *P. fluorescens* on synthetic wastewater, however, Bouillot et al. (1990) found that neither $Y_{B/s}^g$ nor m_s were influenced by dilution or breeding rate (even at < 5 % of μ_{max}). In chemostat cultures, recombinant *Zymomonas mobilis* showed lower values for both maintenance coefficient and yield $Y_{B/ATP}$ as D dropped below 0.1 h^{-1} (Lawford and Rousseau, 2000). According to Wisniewski et al. (1999), hydraulic residence time in MBRs does not influence the maintenance coefficient.

Several authors found three to four linear growth phases in MBRs in which concentrations of regulatory nucleotides differ strongly (Chesbro et al., 1990; Müller and Babel, 1996; Tros et al., 1996). During the first phase, the nucleotide concentration rises until it causes an abrupt inhibition of ribosomal protein synthesis (stringent response), which in turn results in a lower growth rate and eventually a lower maintenance coefficient (Van Loosdrecht and Henze, 1999). Van Verseveld et al. (1984) argue that biosynthesis rates drop abruptly at a specific growth rate characteristic for each strain. This can be observed as a sudden increase in maintenance coefficient and a drop in biomass yield (Tros et al., 1996; Van Verseveld et al., 1984) and is in contrast to the Pirt concept which assumes continuous progression of yields. In continuous fits of concentration data to the Pirt model, Van Verseveld et al. (1984) found that the fitted true yield $Y_{B/s}^g$ was smaller than the measured yield $Y_{B/s}$, which defies any biological explanation (and eq. (3)). Thus, continuous parameter fits might be practical and technically useful but are mechanistically incorrect. Instead, the maintenance coefficient compensates for various effects (Bulthuis et al., 1989) and is a mere model artefact. However, as already mentioned, a large number of processes can still be satisfactorily described by the Pirt concept as long as the parameters are determined in the right range of operation, i.e. of specific growth rate (see Fig. 3).

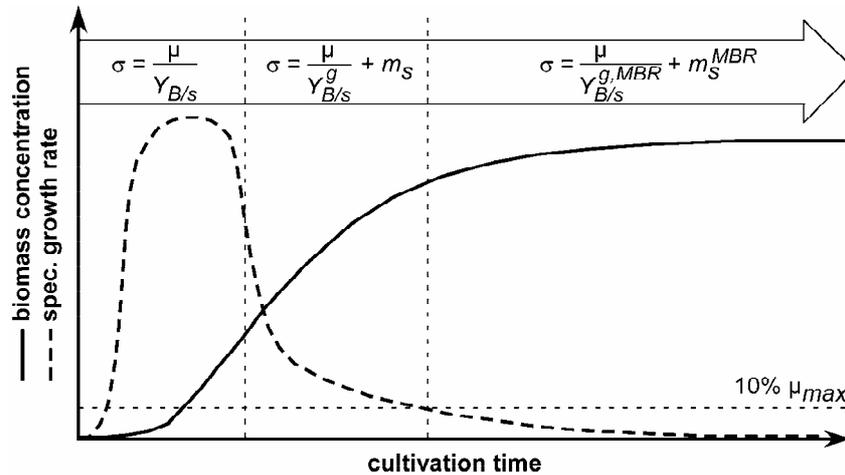


Fig. 3: Progressing need for different maintenance models

Besides selecting the right type of process, the way measured data are plotted and evaluated plays an important role in determining kinetics. In addition to plotting $\sigma = f(\mu)$, continuous operation allows other – and according to Bouillot et al. (1990) more precise – evaluation methods. According to eq. (3), plotting the inverse of yield over the inversed specific growth rate – or breeding rate D_B , respectively – yields a straight line with the slope m_s and $(Y_{B/s}^g)^{-1}$ as the ordinate intercept (see Fig. 4 a)). Assuming steady state, the substrate mass balance yields:

$$\frac{\dot{V}}{V_R} \cdot (c_{s,in} - c_s) = \frac{\mu \cdot c_B}{Y_{B/s}^g} + m_s \cdot c_B \quad (10)$$

or using the dilution rate D :

$$D \cdot (c_{s,in} - c_s) = \frac{\mu \cdot c_B}{Y_{B/s}^g} + m_s \cdot c_B \quad (11)$$

Plotting this for zero growth yields a straight line as shown in Fig. 4 b) (Beyeler et al., 1984).

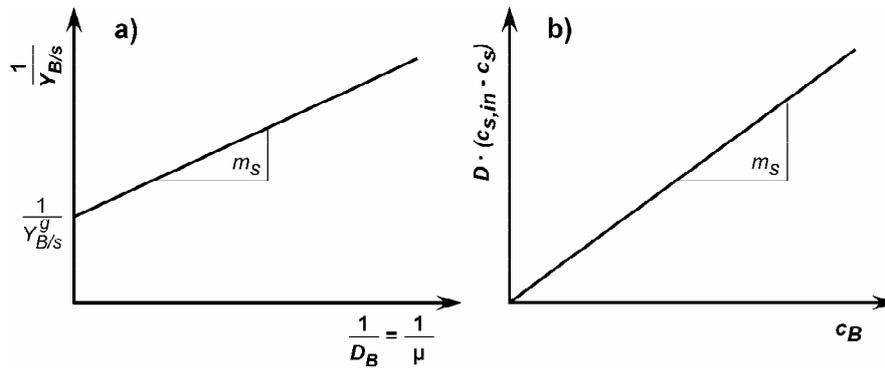


Fig. 4: Data evaluation for determination of maintenance parameters in continuous culture: a) acc. to eq. (3), b) acc. to eq. (11) at $\mu = 0 \text{ h}^{-1}$ (e.g. in MBRs).

All these methods involve measuring biomass and substrate concentration which often can only be carried out offline after sampling, and which therefore is a tedious and time-consuming process. Instead, when the required kinetic parameters are known the carbon dioxide evolution rate can be used for a model-based online estimation of biomass concentration (Lubenova et al., 2003; Drews, 2004) or, vice versa, for a fit of kinetic parameters. Herbert (1958) already observed a linear relationship between specific carbon dioxide production rate and specific growth rate which has successfully been used for estimation of contaminant depletion in soils (Schoefs et al., 2004):

$$\frac{\dot{r}_{CO_2}}{c_B} = Y_{CO_2/B}^g \cdot \mu + m_{CO_2} \quad (12)$$

Similar to eq. (2), this equation contains a yield coefficient $Y_{CO_2/B}^g$ and a maintenance coefficient m_{CO_2} . The substrate taken up for maintenance purposes is solely used for energy production, i.e. it is completely respired and not integrated into biomass or other metabolites. During catabolism, stoichiometric oxidation of 1 mol glucose, e.g., yields 6 mol CO_2 . Using the molecular weights of glucose and CO_2 , the maintenance coefficients can thus be converted into one another:

$$m_{CO_2} = m_s \cdot \frac{6 \cdot \tilde{M}_{CO_2}}{\tilde{M}_{Gl}} \quad (13)$$

From a fit of parameters in eq. (12) to measured biomass concentrations, maintenance parameters can thus be determined, too.

The minimum substrate concentration at which growth can be initiated also differs from that found by extrapolation of chemostat data (Tros et al., 1996). Assuming a Michaelis-Menten-type substrate uptake,

$$\sigma = \sigma_{max} \cdot \frac{c_s}{K_S + c_s} \quad (14)$$

yields the minimum substrate concentration at which $\sigma = m_s$:

$$c_{s,min} = \frac{K_S \cdot m_s}{\sigma_{max} - m_s} \quad (15)$$

From eq. (15) it is apparent that $c_{s,min}$ decreases at lower values of m_s as long as K_S does not increase which it will not do at decreasing growth rates. Tros et al. (1996) determined a minimum acetate concentration of $26 \mu\text{mol L}^{-1}$ in an MBR in contrast to $109 \mu\text{mol L}^{-1}$ in a chemostat. The authors also found a decrease of the half-saturation constant K_S down to a quarter of the value measured in the stationary phase of a batch run. The findings are in agreement with Wick et al. (2001) who for *E. coli* determined a 10-fold increase in glucose affinity under long-term glucose limited conditions which shows that the above mentioned constraint that K_S should not increase is complied with.

3 Materials and Methods

3.1 Organism and media

Ustilago maydis (strains *urbs*- and wild type FB1, both kindly donated by S. Leong) was stored in glycerol stocks (25%) at $-80\text{ }^{\circ}\text{C}$. After a 3-day inoculation on potato-dextrose-agar cells were transferred into shaking flasks containing a modified Grimm-Allen-medium (see Table I) with glucose as the main carbon source. Flasks were shaken for approx. 24h at 100 min^{-1} and at a temperature of $27\text{ }^{\circ}\text{C}$ until they were transferred into the fermentor.

Table I: Media compositions

component	concentration [g L^{-1}]		
	shaking flasks, starting medium batch and continuous runs	starting medium fed-batch runs	feed solutions continuous runs
Glucose $\cdot\text{H}_2\text{O}$	20	20 - 30	20 - 200
$\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$	3	-	3 - 21
$(\text{NH}_4)_2\text{SO}_4$	-	2.83	-
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	$8 \cdot 10^{-2}$	$8 \cdot 10^{-2}$	$8 \cdot 10^{-2}$
K_2HPO_4	3	-	3 - 6
KH_2PO_4	-	6.8	-
NaSO_4	0.88	-	0.88
$\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$	1	-	1 - 2
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	$1.5 \cdot 10^{-4}$	-	-
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	$6.25 \cdot 10^{-6}$	$6.25 \cdot 10^{-6}$	$1.25 \cdot 10^{-5}$
$\text{MnCl}_2 \cdot 5\text{H}_2\text{O}$	$3.05 \cdot 10^{-5}$	$3.05 \cdot 10^{-5}$	$6.1 \cdot 10^{-5}$
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	$2.67 \cdot 10^{-3}$	$2.67 \cdot 10^{-3}$	$2.67 - 5.34 \cdot 10^{-3}$
	+ NH_4OH until pH 6.8	+ NaOH until pH 6.8	+ NH_4OH until pH 6.8

3.2 Apparatus and fermentation procedures

For cultivation, a 5 L glass fermentor (B.Braun Int., Germany) was used. In MBR runs, this was equipped with an external ceramic tubular membrane module for biomass retention (Pall Schumacher, Germany). Temperature was controlled at $27\text{ }^{\circ}\text{C}$, pH at 7.2, and pO_2 at 40 %. Fig. 5 shows the experimental set-up.

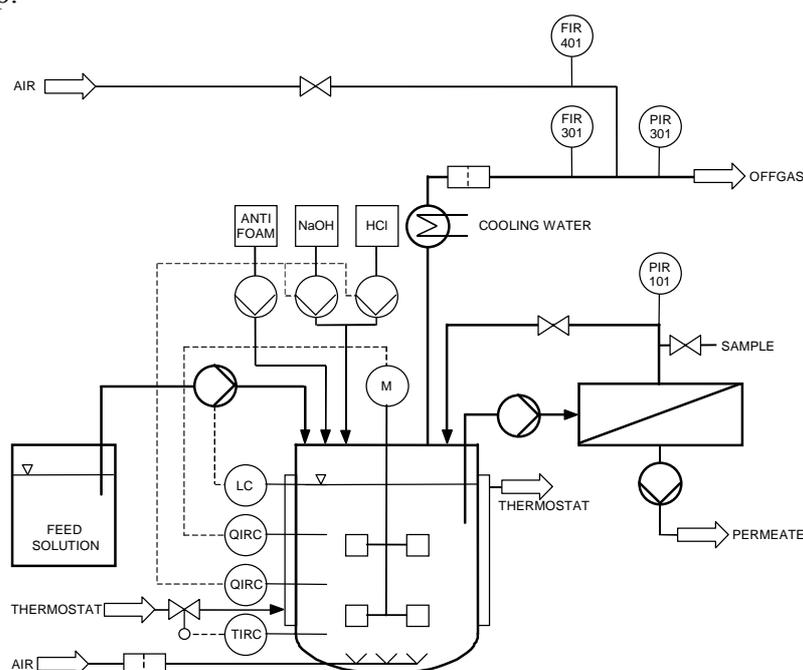


Fig. 5: Experimental set-up

To study the effects of glucose and ammonia limitation, respectively, and the effect of short- and long-term limitation, the following batch, fed-batch, chemostat and MBR cultivations were carried out (see Table II). In continuous runs, the feed concentrations and glucose to ammonia ratios were varied to give carbon or ammonia limitations successively. Previously determined growth kinetics (Drews, 2004) and frequent concentration measurements allowed judgement of the currently limiting substrate or nutrient.

Table II: List of cultivations

Name	Aim of trial / type of limitation
B1	batch data; ammonium limitation
F1	constant glucose and other nutrients feed; ammonium limited for 5 hours, then ammonium spike (twice)
F2	constant ammonia and other nutrients feed; glucose-limited
F3	repetition of F2 (reproducibility)
C1	with $\mu > 0.01 \text{ h}^{-1}$, limitations less severe than in MBR runs
MBR1	ammonia-limited
MBR2	by change of nutrient and substrate feed rates, glucose- and ammonia-limited, successively
MBR3	by change of nutrient and substrate feed rates, glucose- and ammonia-limited, successively
MBR4	glucose-limited

3.3 Analyses

Biomass concentration was determined by turbidity measurements at 600 nm (UV-120-01, Shimadzu) calibrated against dry weight measurements of washed cells. For calibration, 16 samples from different growth phases were used to avoid the influence of cell size, morphology and age. Across the extinction range, some of these samples were used directly while others were diluted to cover a broad range of matrix concentrations. The relationship between extinction and concentration was found to be linear in the range of 0.2 – 0.8 corresponding to 0.1 – 0.5 g L⁻¹. Samples containing higher concentrations were diluted accordingly. Relative deviation between results for the same sample at different dilutions was < 3% even at high concentrations, i.e. high dilution factors. For each measurement, the average of duplicate samples with a relative deviation < 5% from each other was used. Cross-checks, i.e. both turbidity and dry weight measurements, were performed for random samples. These yielded relative deviations < 10% even at biomass concentrations > 100 g L⁻¹.

For determination of substrates and nutrients concentrations, commercial test kits were used (glucose: enzymatic test kit liquicolor by Human GmbH, Germany; ammonium: LCK 303 and 304 by Dr. Lange GmbH, Germany). All samples were membrane filtered before analyses.

CO₂ in the offgas was measured using a gas analyser S 710 (Maihak, Germany). The measuring range of the detection module FINOR was 0-5000 ppm. When necessary, an exactly dosed air stream was added for dilution.

4 Results

4.1 Prediction of maintenance coefficient for medium limitation

In order to be able to evaluate the validity of maintenance coefficients derived from cultivation data, the maintenance coefficient for medium limitation is predicted using eq. (9). Using the cell carbon content at medium growth rates which was found to be approx. 1 mol C per 26 g biomass and is thus close to the value reported in (Tijhuis et al., 1993), eq. (9) yields $e_m = 264 \pm 108 \text{ J (g h)}^{-1}$ or $m_s = 0.0167 \pm 0.0068 \text{ h}^{-1}$, respectively, at a cultivation temperature of 27 °C. For *U. maydis*, μ_{max} was found to be 0.28 h⁻¹ and $K_S = 0.7 \text{ g L}^{-1}$ in batch and fed-batch cultures (Drews 2004). Using these values and assuming a typical value of $Y_{B/S} = 0.5$, the minimum substrate concentration for growth is $0.0217 \pm 0.009 \text{ g L}^{-1}$ according to eq. (15).

4.2 Common evaluation methods

4.2.1 Short-term limited cultivations

Fig. 6 shows different evaluation plots for the fed-batch run F2 and the chemostat run C1. For the glucose limited cultivation the glucose uptake rate can be expressed as (see Fig. 6 a)):

$$\sigma = 1.847 \cdot \mu + 0.049 \text{ h}^{-1} \quad (16)$$

According to eq. (2), this means that true yield $Y_{B/S}^g = 0.54$. At 0.05 h^{-1} , the value determined for m_s is more than twice that predicted using eq. (9). For an ammonia limited batch cultivation (data not shown), $m_s = 0.016 \text{ h}^{-1}$ was determined from the slope of the glucose concentration over time during zero growth in the stationary phase. For the same batch, $m_s = 0.017 \text{ h}^{-1}$ was determined from the CO_2 evolution rate at zero growth and using eq. (13). Both values match the prediction exactly and are lower than those derived from the $\sigma = f(\mu)$ plot for F2. According to Fig. 2 and eq. (8), the maintenance coefficient for the ammonia limited batch culture should be higher than that of the glucose limited fed-batch which is not found here. With $m_s = 0.026 \text{ h}^{-1}$, evaluation of chemostat data according to Fig. 4 a) also yields a lower value than the $\sigma = f(\mu)$ plot for F2 (see Fig. 6 b)). From a cumulative plot of mass of glucose used over mass of biomass formed in F2 (Fig. 7), a biomass yield of $Y_{B/S} = 0.32$ can be found while Fig. 6 a) yields $Y_{B/S} = 0.46$. This deviation also shows the plotting specific rates is a rather insensitive method.

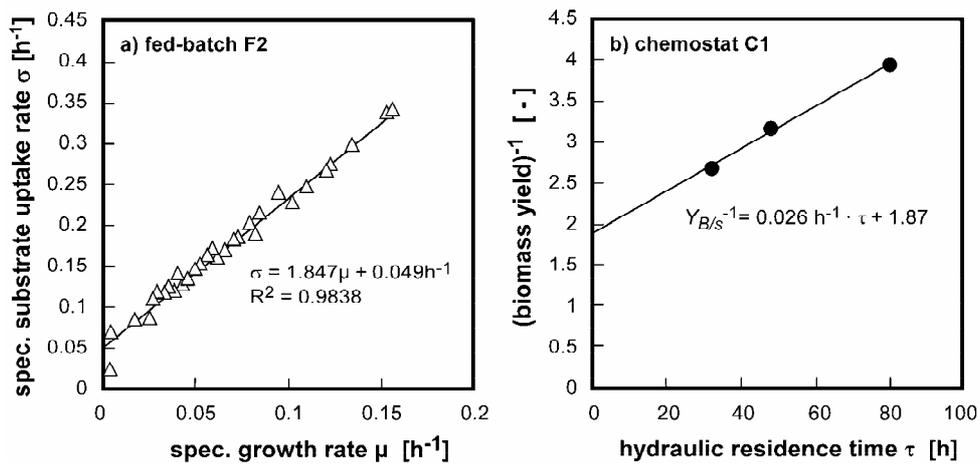


Fig. 6: Determination of substrate uptake kinetics: a) acc. to Fig. 2 for F2, b) acc. to Fig. 4 a) for C1

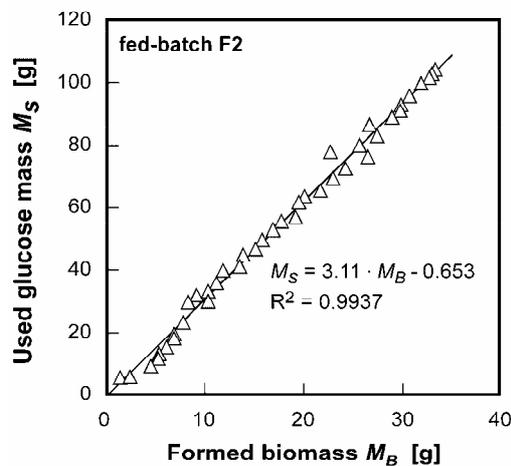


Fig. 7: Used glucose over formed biomass for F2

4.2.2 Long-term limited cultivations

In MBR cultivations, a decrease in maintenance demand is expected. At zero growth, this can be determined according to eq. (11) and Fig. 4 b). Since MBR cultivations in this study were mainly carried out to increase productivity, only few data points at $\mu = 0 \text{ h}^{-1}$ are available. Plotting these for MBR4 (see Fig. 8) results in $m_s = 0.015 \text{ h}^{-1}$, again a value within the predicted range despite the low amount of data points.

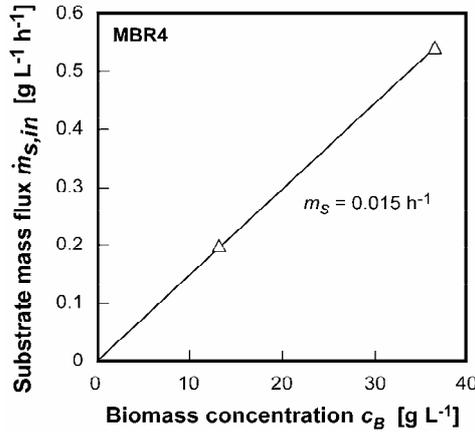


Fig. 8: Determination of maintenance coefficient at zero growth (MBR4) acc. to Fig. 11 b)

4.3 Evaluation using the CO₂ production rate

Using online data for \dot{r}_{CO_2} , parameters in eq. (12) were fitted to biomass concentrations for each cultivation. From these individual optimum fits, extreme values of m_{CO_2} and $Y_{CO_2}^g/B$ were chosen and combined according to Table III for the plots in Fig. 9 and 10.

Table III: Parameter sets for eq. (12)

Parameter set	1	2	3	4
m_{CO_2} [10^{-2} h^{-1}]	3.9	1.2	1.2	3.9
$Y_{CO_2}^g/B$	0.6	1.5	0.6	1.5

Fig. 9 clearly shows that growth in F1 and F2 can be correctly modelled using parameter set 1 which results in curves close to the respective optimum fit (maximum deviation at the end of F1 is -4%). F3 and B1 are also well represented until the beginning of the stationary phase (max. deviation $+20\%$). Set 2 underestimates biomass concentrations during growth by up to 30% while sets 3 and 4 give values that are always too high or too low, respectively. From this, $m_{CO_2} = 0.039 \text{ h}^{-1}$ and $Y_{CO_2}^g/B = 0.6$ can be deduced as the optimum parameter set for short-term limited cultivation of *U. maydis* at 27°C . The corresponding substrate value $m_s = 0.027 \text{ h}^{-1}$ is very close to that obtained from Fig. 6 b) for the chemostat cultivation, proving the applicability of the CO₂ method. Fig. 10 shows model results for the MBR cultivations. In MBR1 and MBR3, a larger fraction of the reactor content was withdrawn at a certain point during the cultivation. Hence, a new starting value was set for biomass modelling which is why all curves “merge” at one point. Set 2 proves to be best suited for all MBR cultivations. It correctly predicts growth over more than 400h. Larger deviations can only be observed in MBR2 during which cells were subject to less severe limitations. The parameter sets 1 and 4 which as a result of large maintenance coefficients both underestimate growth, result in similar values towards the end albeit largely different true yields. This highlights that in MBR cultivations the correct value of the maintenance coefficient is more important than that of the true yield.

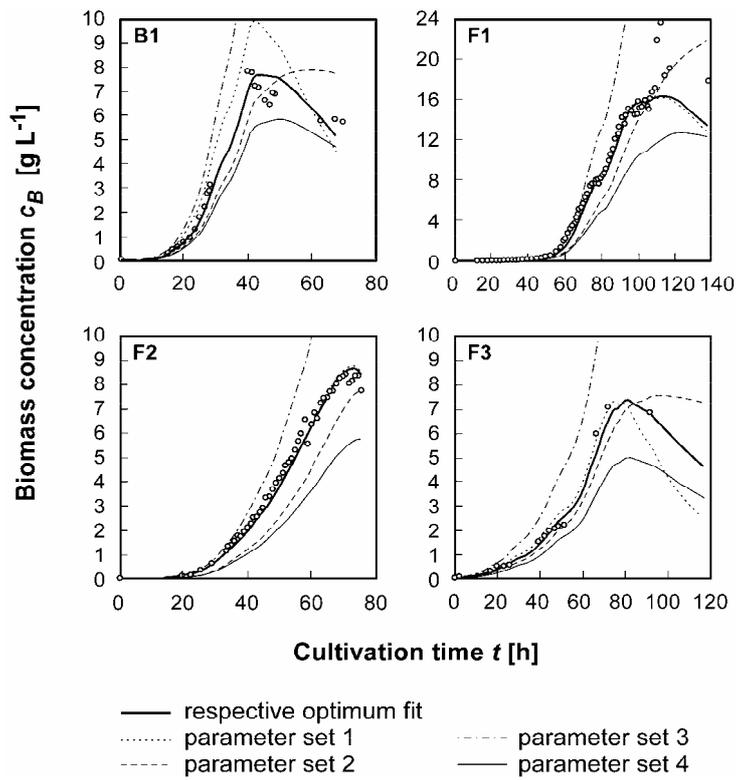


Fig. 9: Modelling of biomass concentration using the CO₂ production rate for different MBR runs using parameter sets acc. to Table III.

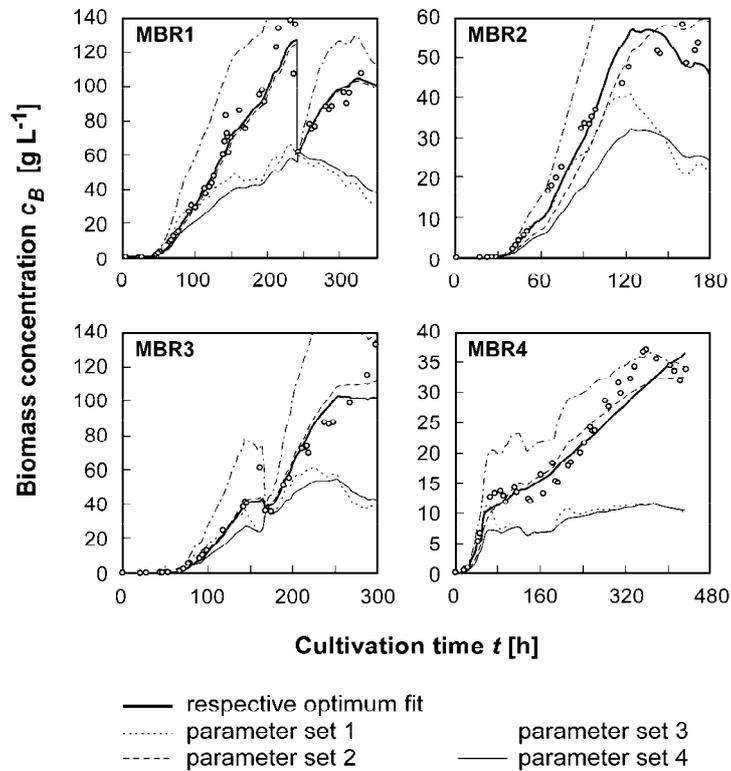


Fig. 10: Modelling of biomass concentration using the CO₂ production rate for different MBR runs using parameter sets acc. to Table III.

As can be seen, using an inaccurate value of m_{CO_2} can lead to dramatic miscalculation of process data. Using $m_{CO_2} = 0.039 \text{ h}^{-1}$, the optimum for short-term limited cultures, the model clearly underestimates biomass formation in MBR cultivations. In the long run, this is well represented by using $m_{CO_2}^{MBR} = 0.012 \text{ h}^{-1}$, i.e. approx. 1/3 of the aforementioned value. Since these results were obtained at largely different biomass concentrations, quorum sensing as the cause for a shift in kinetics can be ruled out. To summarise results, eq. (12) is plotted in Fig. 11 with values determined for short- and long-term limited cultures. The maintenance coefficient m_{CO_2} drops to around a third of the value determined for short-term limitation, while CO_2 yield rises to approx. twice the initial value. According to eq. (12), the respective values for m_{CO_2} correspond to $m_s = 0.027 \text{ h}^{-1}$ for short- and $m_s^{MBR} = 0.0082 \text{ h}^{-1}$ for long-term limitation. These values are just below or just above the range predicted by eq. (9) when using the cell carbon content suggested by (Bulthuis et al., 1989; Tijhuis et al., 1993; van Verseveld et al., 1984). In this study, the cell carbon content was found to be approx. 1 mol C per 26 g dry matter with a decreasing tendency (to approx. 1 mol per 29 g) for lower growth rates. This decrease is too small to explain the drop in carbon uptake at small growth rates. To discuss the effect on the minimum substrate concentration, we initially let $K_S = \text{const}$. The maintenance coefficients then correspond to $c_{s,min} = 0.035 \text{ g L}^{-1}$ and $c_{s,min}^{MBR} = 0.01 \text{ g L}^{-1}$ according to eq. (15), i.e., here, too, a drop to a third can be observed which is in accordance to Tros et al. (1996). Assuming a decrease in K_S as can be expected under long-term cultivation, this trend would be even more pronounced, i.e., $c_{s,min}$ would drop even further.

A shift in maintenance requirements can be explained by changes in metabolism caused by progressing limitations. These changes occur abruptly at growth rates typical for each strain. Anomalities of the Pirt concept have been observed for growth rates less than approx. 10% μ_{max} . Measured specific growth rates in MBR cultivations carried out in this study were $10^{-3} - 10^{-2} \text{ h}^{-1}$. The intersection of lines obtained from independent parameter fits in Fig. 11 gives $\mu_{crit} = 0.03 \text{ h}^{-1}$ which indeed represents 11% of μ_{max} .

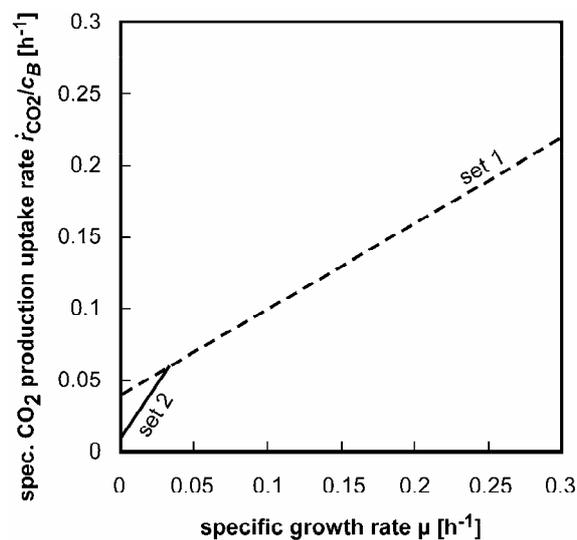


Fig. 11: Dependence of specific CO_2 production rate on specific growth rate during short- (set 1) and long-term limited culture (set 2)

5 Conclusions

The aim of this study was to determine maintenance parameters according to Pirt for growth and substrate uptake of *U. maydis* in medium or short-term limited and in severely or long-term limited cultures. To obtain those maintenance parameters, different evaluation methods were used: the traditional method of plotting specific rates, plotting the inverse of biomass yield over residence time in continuous culture, and a continuous fit of CO₂ production parameters to biomass concentrations.

Table IV: Maintenance parameters determined via different methods

	m_s [h ⁻¹]	$Y_{B/s}^g$	m_{CO_2} [h ⁻¹]	$Y_{CO_2/B}^g$
prediction (eq. (9)) with 1 mol C (26 g) ⁻¹	0.0167 ± 0.0068	-	0.0245 ± 0.01	-
fed-batch (F2):				
uptake/evolution at zero growth	0.016	-	0.026	-
specific rates	0.049	0.54	0.051	0.43
fit to eq. (12) and using eq. (13)	0.027	-	0.039	0.60
chemostat (C1):				
$Y_{B/s}^{-1} = f(\tau)$	0.026	0.53	-	-
membrane bioreactor (MBR4):				
uptake/evolution at zero growth	0.015	-	0.011...0.014	-
fit to eq. (12) and using eq. (13)	0.008	-	0.011	1.4

Table IV lists some maintenance parameters determined in this study. It was found that plotting specific rates yields much higher maintenance coefficients and true yields than other methods. Apart from those, a reasonable agreement of parameters for either short- or long-term limitation can be seen. Maintenance coefficients for short-term limitation are also in accordance with those predicted by a thermodynamically based correlation (eq. (9)). For a fed-batch cultivation (F2), a continuous fit of CO₂ production rate to measured biomass concentration gave higher results for m_s and m_{CO_2} than glucose uptake or CO₂ production rate at zero growth. For MBR4, m_{CO_2} values from both methods are comparable while m_s determined via substrate uptake rate at zero growth was higher than that calculated from m_{CO_2} . Using eq. (13), m_s and m_{CO_2} determined at zero growth do not correspond like they should. Using just one respective set of parameters for short- and long-term limited cultures, biomass concentrations can be predicted from CO₂ production over a period of up to 400 hours (relative deviation approx. 10 %) for different runs of the respective cultivation type. It is concluded that a continuous fit of respiration kinetics to biomass concentration is more reliable than plotting a limited number of points from offline measurements. No significant differences between glucose or ammonia limitation were observed which is in contrast to Pirt (1982).

Due to the different possible mechanisms (continuous or discontinuous maintenance, endogenous metabolism, cryptic growth), a number of models exist to describe phenomena jointly termed maintenance. The Pirt concept might not be suited for understanding biochemical processes, but it is suited for technical application as long as model parameters are determined in the respective range of operation. While m_s can be predicted by a thermodynamically based correlation with a rather good accuracy, these values cannot be adopted to MBR or other low growth processes. It is stressed that a drop in maintenance demand must be borne in mind when modelling and designing an MBR process.

Nomenclature

c	concentration	$[\text{g L}^{-1}]$
D	dilution rate	$[\text{h}^{-1}]$
e	energy equivalent of the maintenance coefficient	$[\text{J g}^{-1} \text{h}^{-1}]$
K	half-saturation constant	$[\text{g L}^{-1}]$
m	maintenance coefficient	$[\text{h}^{-1}]$
M	mass	$[\text{g}]$
\dot{m}	mass flux	$[\text{g L}^{-1} \text{h}^{-1}]$
\tilde{M}	molar mass	$[\text{g mol}^{-1}]$
\dot{r}	rate of reaction	$[\text{g L}^{-1} \text{h}^{-1}]$
R	molar gas constant	$[\text{J mol}^{-1} \text{K}^{-1}]$
t	time	$[\text{h}]$
T	temperature	$[\text{°C}]$
V	volume	$[\text{m}^3]$
\dot{V}	volumetric flow rate	$[\text{L h}^{-1}]$
Y	yield coefficient	$[\text{g g}^{-1}]$
μ	specific growth rate	$[\text{h}^{-1}]$
σ	specific substrate uptake rate	$[\text{h}^{-1}]$
τ	residence time	$[\text{h}]$

Subscripts and superscripts

$crit$	critical growth rate
eq	equivalent
B	biomass
C	carbon source (glucose)
D	death
e	endogenous
in	in
g	growth
m	maintenance
max	maximal
min	minimal
S	saturation
s	substrate (carbon source)

abbreviations

COD	chemical oxygen demand
MBR	membrane bioreactor
RQ	respiratory coefficient
VSS	volatile suspended solids

References

- Arnot T, Howell J. 2001. Manipulation of cell physiology in membrane bioreactors. MBR 3, Cranfield University, 44-55.
- Beyeler W, Rogers PL, Fiechter A. 1984. A simple technique for the direct determination of maintenance energy coefficient: An example with *Zymomonas mobilis*. *Appl Microbiol Biotechnol* 19:277-280.
- Bouillot P, Canales A, Pareilleux AH, Goma, G. 1990. Membrane bioreactors for the evaluation of maintenance phenomena in waste water treatment. *J Ferment Bioeng* 69:178-183.
- Bulthuis BA, Koningstein GM, Stouthamer AH, van Verseveld HW. 1989. A comparison between aerobic growth of *Bacillus licheniformis* in continuous culture and partial-recycling fermentor, with contributions to the discussion on maintenance energy demand. *Arch Microbiol* 152:499-507.
- Chesbro W, Arbige M, Eifert R. 1990. When nutrient limitation places bacteria in the domains of slow growth: metabolic, morphologic and cell cycle behaviour. *FEMS Microbiol Ecol* 74:103-120.
- Drews A, Kraume M. 2005. Process Improvement by Application of Membrane Bioreactors. *Trans IChemE, Chem Eng Res Des* 83(A3):276-284.
- Drews A. 2004. Kontinuierliche Ferrichromproduktion mit *Ustilago maydis* im Membranbioreaktor. PhD thesis TU Berlin. Fortschr.-Ber. VDI Reihe 17 Nr. 246. Düsseldorf:VDI-Verlag.
- Herbert D. 1958. Some principles of continuous culture. *Rec Progr Microbiol* 381-396.
- Issen J, Egli T. 2004. Specific growth rate and not cell density controls the general stress response in *Escherichia coli*. *Microbiol* 150:1637-1648.
- Konopka A. 2000. Microbial physiological state at low growth rate in natural and engineered ecosystems. *Current Opinion in Microbiology* 3:244-247.
- Lawford HG, Rousseau JD. 2000. Comparative energetics of glucose and xylose metabolism in recombinant *Zymomonas mobilis*. *Appl Biochem Biotechnol* 84:277-293.
- Low, E.W. and Chase, H.A. 1999. The effect of maintenance energy requirements on biomass production during waste water treatment. *Wat. Res.* 33 (3), 847-853.
- Lubenova V, Rocha I, Ferreira EC. 2003. Estimation of multiple biomass growth rates and biomass concentration in a class of bioprocesses. *Bioproc Biosyst Eng* 25:395-406.
- Menshutina N, Skorohodov A, Guseva E, Fick M, Boudrant J. 2001. Lactic acid fermentation in a cell recycling fermentor: modelling of performances using mechanical and environmental stresses. *Récents Progrès en Génie des Procédés* 15 :107-114.
- Müller RH, Babel W. 1996. Measurement of Growth at Very Low Rates ($\mu \geq 0$), an Approach To Study the Energy Requirement for the Survival of *Alcaligenes eutrophus* JMP 134. *Appl Env Microbiol* 62:147-151.
- Neilands JB. 1995. Siderophores: Structure and Function of Microbial Transport Compounds. *J Biol Chem* 270:26723-26726.
- Pirt SJ. 1965. The maintenance energy of bacteria in growing cultures. *Proc. Of the Royal Soc. of London* 163 B:224-231.
- Pirt SJ. 1982. Maintenance Energy: a General Model for Energy-Limited and Energy-Sufficient Growth. *Arch Microbiol* 133:300-302.
- Schoefs O, Perrier M, Samson R. 2004. Estimation of contaminant depletion in unsaturated soils using a reduced-order biodegradation model and carbon dioxide measurement. *Appl Microbiol Biotechnol* 64:53-61.
- Sipkema EM, de Koning W, Ganzeveld KJ, Janssen DB, Beenackers AACM. 1998. Experimental pulse technique for the study of microbial kinetics in continuous culture. *J Biotechnol* 64:159-176.
- Tappe W, Tomaschewski C, Ritterhaus S, Groeneweg J. 1996. Cultivation of nitrifying bacteria in the retentostat, a simple fermenter with internal biomass retention. *FEMS Microbiol Ecol* 19:47-52.
- Tijhuis L, Van Loosdrecht MCM, Heijnen JJ. 1993. A thermodynamically based correlation for maintenance Gibbs energy requirements in aerobic and anaerobic chemotrophic growth. *Biotechnol Bioeng* 42:509-519.
- Tros ME, Bosma TNP, Schraa G, Zehnder AJB. 1996. Measurement of Minimum Substrate Concentration (S_{min}) in a Recycling Fermentor and Its Prediction from the Kinetic Parameters of *Pseudomonas sp.* Strain B13 from Batch and Chemostat Cultures. *Appl Env Microbiol* 62:3655-3661.
- Van Loosdrecht MCM, Henze M. 1999. Maintenance, endogeneous respiration, lysis, decay and predation. *Wat Sci Tech* 39:107-117.
- van Verseveld HW, Chesbro WR, Braster M, Stouthamer AH. 1984. Eubacteria have 3 growth modes keyed to nutrient flow. *Arch Microbiol* 137:176-184.
- Wick LM, Quadroni M, Egli T. 2001. Short- and long-term changes in proteome composition and kinetic properties in a culture of *Escherichia coli* during transition from glucose-excess to glucose-limited growth conditions in continuous culture and *vice versa*. *Env Microbiol* 3:588-599.
- Wisniewski C, Leon Cruz A, Grasmick A. 1999. Kinetics of organic carbon removal by a mixed culture in a membrane bioreactor. *Biochem Eng J* 3:61-69.