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Experimental pulse technique for the study of microbial kinetics in continuous culture

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Abstract

A novel technique was developed for studying the growth kinetics of microorganisms in continuous culture. The method is based on following small perturbations of a chemostat culture by on-line measurement of the dynamic response in oxygen consumption rates. A mathematical model, incorporating microbial kinetics and mass transfer between gas and liquid phases, was applied to interpret the data. Facilitating the use of very small disturbances, the technique is non-disruptive as well as fast and accurate. The technique was used to study the growth kinetics of two cultures, *Methylosinus trichosporium* OB3b growing on methane, both in the presence and in the absence of copper, and *Burkholderia (Pseudomonas) cepacia* G4 growing on phenol. Using headspace flushes, gas blocks and liquid substrate pulse experiments, estimates for limiting substrate concentrations, maximum conversion rates V_{max} and half saturation constants K_s could rapidly be obtained. For *M. trichosporium* OB3b it was found that it had a far higher affinity for methane when particulate methane monooxygenase (pMMO) was expressed than when the soluble form (sMMO) was expressed under copper limitation. While for *B. cepacia* G4 the oxygen consumption pattern during a phenol pulse in the chemostat indicated that phenol was transiently converted to an intermediate (4-hydroxy-2-oxo-valerate), so that initially less oxygen was used per mole of phenol.

Keywords: Transient; Dynamic; Oxygen consumption pattern; Chemostat; *Methylosinus trichosporium* OB3b; *Burkholderia (Pseudomonas) cepacia* G4

1. Introduction

The development and optimization of industrial fermentation processes ask for a clear picture of the relevant microbial kinetics. Traditionally,

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measurements to determine the kinetics are carried out with batch culture experiments. Often, good interpretation of the results is difficult due to the influence of culture history (Braha, 1987; Templeton and Grady, 1988) and changing conditions (Villadsen, 1990). Measurements in continuous cultures under steady-state operation, in which the environment is well-controlled, do not have these disadvantages, and the results obtained are generally more reliable and reproducible (Scragg, 1991). However, this technique is time consuming, because several steady-states have to be analysed to obtain sufficient information. Furthermore, microorganisms are known to change their physiology when the growth conditions are altered.

In order to simplify this process, it is possible to study the effect of disturbances at a single steady-state. Until now, a number of groups used this technique. For example, Yang and Humphrey (1975) and Allsop et al. (1993) studied the effect of step changes in dilution rate or phenol feed concentration to acquire information on the substrate inhibition kinetics of *Pseudomonas putida* cultures. They found different patterns in the dynamic response depending on the size of the step change. Chi and Howell (1976) used the same step change techniques but also studied short term effects by injecting pulses of the inhibitory substrate phenol in a *Pseudomonas* culture. Because of the relatively insensitive parameters measured (biomass and phenol concentration with a detection limit of 0.1 mM) large disturbances (above 1 mM of initial phenol concentration) had to be applied in order to obtain informative responses. Another example of pulse studies is found in the work of Al-Awadhi et al. (1990). They studied the transient behavior of the methylotroph *Bacillus* sp. NCIB 12522 by injecting pulses of the limiting substrates methanol and ammonia.

In the work mentioned thus far, only extracellular concentrations were measured. Others, whose main objective was metabolic regulation, applied the same kind of disturbances but determined intracellular concentrations as well (RNA, DNA, ATP, etc.) (Chu and Papoutsakis, 1987a,b; Baloo and Ramkrishna, 1991a,b; Rizzi et al., 1993). However, in none of these studies the information provided by the oxygen consumption pattern of the cells was used as a means of determining the reaction kinetics.

Here we present a technique involving substrate pulses, in which oxygen is used to probe the metabolic behavior of the cells. Because of the sensitivity of oxygen as an indicator, far smaller disturbances can be studied than in the work presented thus far. So the technique is non-disruptive and allows for a fast and accurate analysis of kinetic processes while hardly affecting the physiology of the cells.

Two approaches to obtain information from the dissolved oxygen data are discussed. Firstly a very rapid and more qualitative method, using a combination of the most prominent characteristics of the curve. This method provides estimations of for example the residual substrate concentration. Secondly an interpretation of the data via a model. In the model microbial kinetics as well as mass transfer between gas and liquid phases are incorporated. With the help of numerical fitting techniques, this second method yields values for standard biokinetic parameters such as the maximum conversion rate V_{max} and the half saturation constant K_s. But it may also yield information about the transient accumulation of intermediates.

2. Modeling and theoretical aspects

2.1. Model assumptions

The chemostat system studied is schematically described in Fig. 1. Three phases are considered: two gas phases, a bubble gas phase GB and a headspace gas phase GH, and one liquid phase L. Gas enters at the bottom, bubbles through the liquid into the headspace and out of the reactor. Mass transfer takes place between both gas phases and the liquid phase and is described by the volumetric mass transfer coefficients k_La_B (bubble) and k_La_H (headspace).

The k_La_H is considered because for small vessels (up to 250 l), such as used in this study, the contribution of the headspace mass transfer cannot be neglected (Fuchs et al., 1971; Miller, 1974;

Nishikawa et al., 1981). The headspace mass transfer contribution for our 1 l reactor turned out to be around 25% of the total mass transfer, that for our 3 l reactor around 10% (gas flow 30 ml min^{-1}; data not shown). However, changes in gas flow rate due to headspace mass transfer were neglected ($\phi_{GH,out} = \phi_{GB,out}$, error < 5%).

The gas-liquid mass transfer resistance is entirely in the liquid film at the gas-liquid interface. The interfacial liquid concentration C_L^* is in equilibrium with the gas phase and follows Henry's law ($C_L^* = C_G/He$, with C_G the gas concentration and He the Henry coefficient). The microbial reaction rates are assumed not to influence the gas-liquid mass transfer mechanism. In bioreactors of the type studied here, only under extreme conditions (very high cell densities, vigorous mixing), significant deviations of this assumption can be expected (Yagi and Yoshida, 1975; Merchuk, 1977; Ju and Sundararajan, 1992).

All three phases considered are assumed to be ideally mixed. Although this is a simplification of the complicated flow pattern of the bubbles in the stirred tank, in well stirred small reactors for the liquid phase and the headspace gas phase it is easily approached (Westerterp et al., 1963; Hanhart et al., 1963). For the bubble gas phase the situation is more complicated. Because in the systems described here only a limited difference exists between the inlet and outlet gas concentrations (< 50%), ideal mixing and plug flow type approaches lead to similar results (within 5%), and ideal mixing was chosen for simplicity. For systems in which the gas phase is (almost) depleted more of a plug flow type approach needs to be followed in order to give an accurate description of the driving force for mass transfer.

Finally, the pressure in the reactor is assumed to be constant.

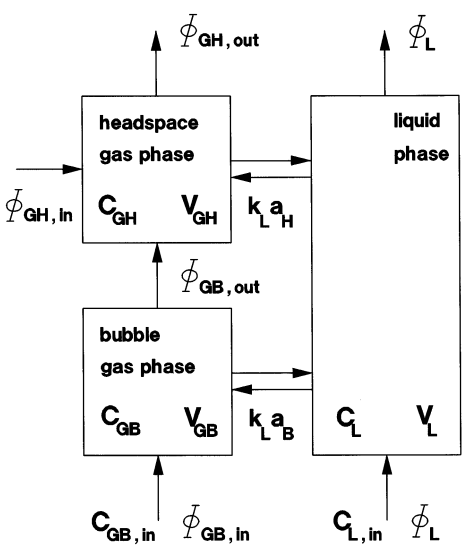

Fig. 1. Schematic representation of the chemostat system studied.

2.2. Microbial kinetics

Two aerobic organisms are considered: *Methylosinus trichosporium* OB3b and *Burkholderia cepacia* G4, growing on methane and phenol, respectively. For both organisms substrate conversion (R_S, S = methane or phenol) is described with Monod type kinetics. Because the first step of the metabolism involves in both cases the consumption of molecular oxygen, a second Monod term is included to make the conversion oxygen dependent. This also prevents substrate conversion in the absence of oxygen:

$$R_S = -V_{max,S}\frac{C_{L,S}}{C_{L,S}+K_{s,S}}\frac{C_{L,O_2}}{C_{L,O_2}+K_{s,O_2}}C_X \qquad (1)$$

with C_{L,O_2} = dissolved oxygen concentration [M]; $C_{L,S}$ = liquid substrate concentration [M]; C_X = cell concentration [kg l^{-1}]; $K_{s,i}$ = half saturation constant of substrate (i = S) and oxygen (i = O_2) [M]; R_S = volumetric substrate conversion rate, negative in case of compound consumption [mol s^{-1} l^{-1}]; $V_{max,S}$ = maximum specific conversion rate substrate S [mol s^{-1} kg^{-1}].

Phenol degradation by *B. cepacia* G4 is documented to follow Haldane type kinetics (Folsom et al., 1990). Although this can easily be included in Eq. (1), this was not done here because in none of the experiments discussed substrate inhibition played a role.

Growth is described by a constant yield term Y_{XS} [kg mol^{-1}] and a term for maintenance based on Beeftink et al. (1990):

$$R_X = -Y_{XS}(R_s - R_m) \qquad (2)$$

with $-R_m$ = volumetric maintenance substrate consumption function [mol s^{-1} l^{-1}]; R_X = volumetric cell production rate [kg s^{-1} l^{-1}].

The exact formulation of the maintenance function is still controversial (Pirt, 1982, 1987; Roels, 1983; Stouthamer et al., 1989). Because the model is not intended to describe very low growth rates, where maintenance plays an important role, the maintenance function was not included in the model ($R_m = 0$). With this assumption the maximum growth rate μ_{max} can easily be derived from $V_{max,S}$: $\mu_{max} = V_{max,S}\ Y_{XS}$.

Oxygen is treated as a second essential substrate, with its own yield Y_{XO} [kg mol^{-1}], so that oxygen and substrate consumption are strictly coupled. With one type of experiments, in which the substrate supply is blocked, this concept is not adequate. It is well known that cells have endogenous respiration when they are incubated without substrate. This is due to the conversion of internal pools of storage materials. So in the substrate block experiments, endogenous respiration was included as a constant rate (e_{O_2} [mol s^{-1} kg^{-1}]) starting to operate at the onset of the experiment. This e_{O_2} can be compared with normally measured biomass decay rates.

2.3. Reactor kinetics

In order to describe the growth of the organisms in a continuous culture, the microbial rate expressions R_X, R_S and R_{O_2} are combined with reactor specific expressions in mass balances. The changes in the cell and liquid substrate concentrations in time are described as:

$$\frac{dC_X}{dt} = D(-C_X) + R_X \tag{3}$$

$$\frac{dC_{L,S}}{dt} = D(C_{L,in,S} - C_{L,S}) + k_L a_{B,S}\left(\frac{C_{GB,S}}{He_S} - C_{L,S}\right) + k_L a_{H,S}\left(\frac{C_{GH,S}}{He_S} - C_{L,S}\right) + R_S \tag{4}$$

with $C_{GB,S}$ = substrate concentration in bubble gas phase [M]; $C_{GH,S}$ = substrate concentration in headspace gas phase [M]; $C_{L,in,S}$ = substrate concentration in the liquid feed [M]; D = dilution rate ($= \phi_L/V_L$) [s^{-1}]; He_S = Henry coefficient of substrate [−]; $k_L a_{B,S}$ = volumetric mass transfer coefficient of the bubble phase for substrate [s^{-1}]; $k_L a_{H,S}$ = volumetric mass transfer coefficient of the headspace for substrate [s^{-1}]; t = time [s].

The substrate balance applies both to volatile substrates supplied through the gas phase ($C_{L,in,S} = 0$), and to non-volatile substrates (both $k_L a$ terms omitted).

The time dependent gas phase mass balances for a volatile substrate are, using the assumption $\phi_{GB,out} = \phi_{GH,out}$:

$$\frac{dC_{GB,S}}{dt} = \frac{1}{V_{GB}}\left(\phi_{GB,in}C_{GB,in,S} - \phi_{GH,out}C_{GB,S} - k_L a_{B,S}\left(\frac{C_{GB,S}}{He_S} - C_{L,S}\right)V_L\right) \tag{5}$$

and

$$\frac{dC_{GH,S}}{dt} = \frac{1}{V_{GH}}\left(\phi_{GH,out}(C_{GB,S} - C_{GH,S}) - k_L a_{H,S}\left(\frac{C_{GH,S}}{He_S} - C_{L,S}\right)V_L\right) \tag{6}$$

for the bubble phase and the headspace, respectively, with $C_{GB,in,S}$ = substrate concentration in the gas flow entering the reactor [M]; V_{GB} = bubble gas volume [l]; V_{GH} = headspace volume [l]; V_L = liquid volume [l]; $\phi_{GB,in}$ = ingoing gas flow rate [l s^{-1}]; $\phi_{GH,out}$ = gas flow rate leaving headspace [l s^{-1}].

2.4. Estimation of system parameters

The model contains a considerable number of constants, many of which can easily be determined (flows, volumes etc.). The less obvious parameter estimations are discussed briefly.

The outgoing gas flow rate $\phi_{GH,out}$ was calculated from the nitrogen concentrations in ingoing and outgoing gas flows in steady-state (C_{GB,in,N_2} and C_{GH,N_2}):

$$\phi_{GH,out} = \frac{C_{GB,in,N_2}}{C_{GH,N_2}}\phi_{GB,in} \tag{7}$$

This supposes that N_2 is not used by the organism, which is generally the case when another nitrogen source is present in excess. By using this indirect technique, possible errors in the direct

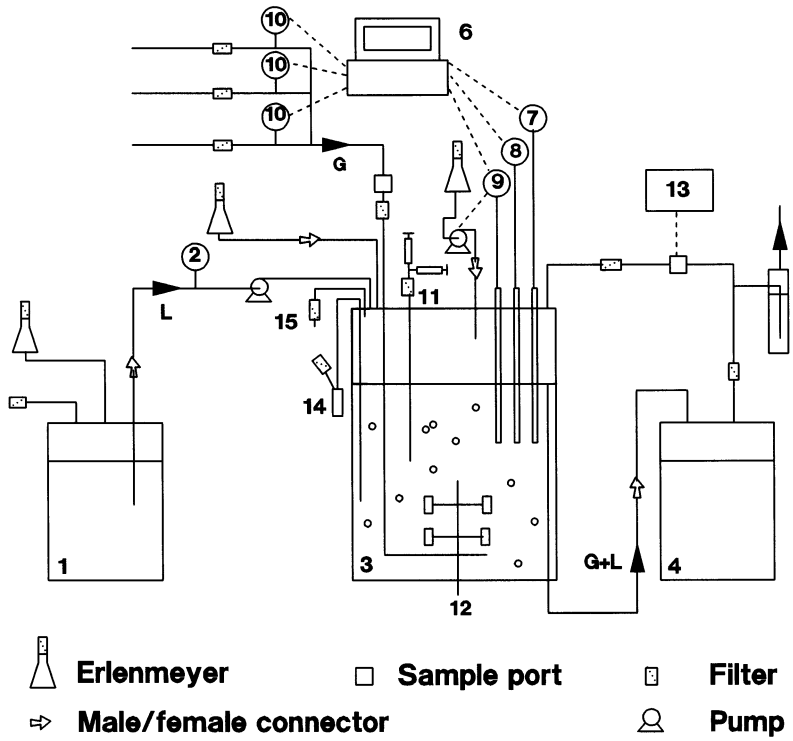

Fig. 2. Experimental set-up for the transient chemostat experiments. G: Gas flow; L: Liquid flow; 1: Nutrient tank; 2: Burette; 3: Bioreactor; 4: Waste tank; 5: Water column; 6: Computer; 7: Thermo-element; 8: Oxygen electrode; 9: pH controller; 10: Mass flow controller; 11: Syringe system (pulses); 12: Motor; 13: Gas chromatograph; 14: Outlet sample tube; 15: Headspace flush inlet.

measurement due to the large volume of the waste tank that strongly reacts to pressure and temperature changes are avoided.

Values for the mass transfer coefficients for oxygen (k_La_{B,O_2},k_La_{H,O_2}) were derived as follows. Steady-state measurements of dissolved oxygen and the concentrations of oxygen in the ingoing and outgoing gas yielded an overall mass transfer coefficient (k_La_{ov}). Subsequently, data from an experiment in which the headspace was briefly flushed with pure oxygen were used to determine the contribution of the headspace (k_La_{H,O_2}) to the overall mass transfer, thus defining k_La_{B,O_2} as well. Mathematically, the steady-state oxygen gas phase balances given in Eqs. (5) and (6), combined with Eq. (7) and steady-state measurements for C_{L,O_2},C_{GB,in,O_2},C_{GB,in,N_2},C_{GH,O_2} and C_{GH,N_2}, yielded one equation with two unknowns (k_La_{B,O_2} and k_La_{H,O_2}). Estimates for k_La_{H,O_2} were then obtained by fitting headspace flush data.

Similarly, for the mass transfer coefficients for methane (k_La_{B,CH_4},k_La_{H,CH_4}) one equation with two unknowns was obtained. Values for both unknowns were subsequently determined by using the k_La values for oxygen and the equation:

$$\frac{k_La_{H,O_2}}{k_La_{B,O_2}} = \frac{k_La_{H,CH_4}}{k_La_{B,CH_4}} \quad (8)$$

Henry coefficients were obtained from literature (Fogg and Gerrard, 1991).

3. Experimental

3.1. Experimental set-up

The experimental set-up (Fig. 2) consists of a continuously operated 3 l bioreactor, equipped with two submerged turbine impellors and four baffles. It was operated at constant T and pH.

Ingoing gases were supplied via mass flow controllers (Bronkhorst, NL). Liquid was fed by a peristaltic pump. Both gas and liquid outflows were led through a constant level overflow. The reactor was maintained at an overpressure of 40 cm H_2O.

Dissolved oxygen (C_{L,O_2}) was measured with a sterilizable amperometric electrode (Ingold, Urdorf, CH). Off-gas concentrations (O_2, CH_4, N_2) were measured on-line by gas chromatography (GC) (Chrompack CP9001; TCD, carboplot P7, 0.53 mm i.d., carrier gas helium, 200 kPa, 30°C). In the subsequent calculation of the gas concentrations in the reactor the overpressure was accounted for.

Both C_{L,O_2} and GC data were collected in a computer. Sampling frequencies were based on the expected experimental response times.

Liquid samples were taken from the reactor and analysed for biomass, phenol and intermediate concentrations using spectroscopic and HPLC techniques.

To allow for the sensitive measurement of small effects in oxygen consumption rate, flow rates were kept very low (10–50 ml min^{-1}) compared to those applied in general. Analysis of the system beforehand concerning this aspect, including the cell density which should not be too high, is needed to obtain good results.

3.2. Experimental procedures

Dynamic experiments were performed starting from steady-state. The types of experiments performed include:

1. *Headspace flush* experiments: the concentration of the volatile compounds in the headspace was changed 'instantaneously' by flushing the headspace during 10 s at a gas flow rate of 1 l min^{-1}.
2. *Substrate pulse* experiments: with a syringe (Fig. 2) a 1–5 ml liquid compound was added through a filter (0.22 μm pore diameter) into the reactor, followed by an injection of a similar volume of demineralized water from a second syringe to clean the injection system. The exact volume injected was determined by weighing the syringe before and after the injection.
3. *Gas block* and *liquid block* experiments: the gas flow and/or liquid flow were stopped during a limited period of time.

Liquid samples taken from the reactor were quenched in phosphoric acid (0°C, 0.1 mM final concentration), which is assumed to suffice for the determination of the relevant extracellular metabolites that are converted relatively slowly. After centrifuging (20 min, 6000 rpm, Sorvall RC-5B) the supernatant was analysed by HPLC.

Calibration of the 100% air saturation of the oxygen electrode was done regularly by turning off the limiting carbon substrate supply and saturating the liquid with air. The 100% level was assumed to be reached as soon as the C_{L,O_2} no longer increased, usually after a few hours. At this time maintenance was assumed to be negligibly small. During the saturation, gas flow rate and impeller speed were maintained at steady-state values.

The oxygen electrode response time, which was of the same order of magnitude as the rate of change in C_{L,O_2} expected in the chemostat culture (5–15 s), was measured. The electrode appeared to have a first order response. The response time, which changed within the time scale of the chemostat culture experiments (months), was measured in-situ for each set of transient experiments separately by injecting a pulse of freshly dissolved sodium dithionite (about 2 times molar excess), thus instantaneously reducing C_{L,O_2} to zero. Although severely disturbing the steady-state, this treatment proved to be not lethal to the cells.

3.3. Analysis

Cell dry weight was determined from filtering culture medium and drying the filter overnight at 105°C. Routinely, cell densities were derived from optical density measurements (OD_{450}, Hitachi 100-60) using experimentally determined conversion factors (at $OD_{450} = 1$: OB3b 0.143 g l^{-1}; G4 0.167 g l^{-1}).

Phenol and 4-hydroxy-2-oxovalerate (HOV) concentrations were determined using isocratic high-pressure liquid chromatography (Pharmacia LKB BioTechnology AB) with a Chrompack nu-

cleosil RP-18 column (25 cm × 4.6 mm i.d.) using a flow rate of 1.25 ml min^{-1}. The eluent composition was 30% acetonitrile and 70% milli-Q; a 0.4 ml injection volume was used. The detection frequency was 80 Hz, and spectral data were collected and analysed with the WaveScan EG HPLC Analysis Software Program (LKB 2146) (phenol 210 nm, HOV 240 nm).

3.4. Materials

The experimental set-up for *M. trichosporium* OB3b was similar to the continuous culture described by Oldenhuis and Janssen (1993). The culture was grown under copper limitation, unless stated otherwise.

B. cepacia G4 (Nelson et al., 1986) was a gift from M.S. Shields, U.S. Environmental Protection Agency, Gulf Breeze, FL. The feed medium for the continuous culture of *B. cepacia* G4 consisted of phenol (5 mM), KNO_3 (1 g l^{-1}), $Na_2HPO_4.12H_2O$ (5.3 g l^{-1}), KH_2PO_4 (1.4 g l^{-1}), $MgSO_4.7H_2O$ (0.2 g l^{-1}), trace elements (5 ml l^{-1}) (Janssen et al., 1984) and vitamins (1 ml l^{-1}) (Heijthuizen and Hansen, 1986). The pH was maintained at 7.0 (regulated with NaOH) and the temperature at 30°C.

The culture was assumed to be in steady-state when five residence times had passed without significant changes in cell concentration.

4-Hydroxy-2-oxovalerate (HOV) was prepared by complete hydroxylation of 4-methyl-2-oxobutyrolactone (Dagley and Gibson, 1965). The lactone was synthesized according to the method described by Rossi and Schinz (1948).

3.5. Computational methods

Simulation and fitting were carried out using the software program Scientist™ (MicroMath, Salt Lake City, Utah). Integration was performed using the Episode routine (Byrne and Hindmarsh, 1976), which contains a numerical method especially developed to deal with stiff differential equations. Least squares minimization procedures were used for fitting. When fitting dynamic experiments, the steady-state description was always included, thus assuring that the solutions describe the experimental data of both the steady-state and the unsteady-state processes. The experiments (pulse or step changes in gas flow etc.) were simulated as events starting at a certain time t. This starting time t, the experimental time-equals-zero, was often not known exactly due to system delays. In the fitting procedure, it was then treated as a variable with well defined boundaries and optimized along with the kinetic parameters. In headspace flush experiments, also the flush time period, which cannot be adjusted accurately, was treated as a variable and optimized.

Sensitivity analyses were carried out with a linearization technique described by Blau and Kuenker (1990). For each experiment, a normalized sensitivity function N was calculated. $N_{i;j}$, with i state variables and j parameters, can be interpreted as the percentage change in the state variable x_i for a 1% increase in the parameter p_j. Corrections were made for changes in steady-state values. For each experiment, the sensitivity of all of the parameters was calculated. In the experiments discussed only the sensitive parameters are mentioned.

4. Results and discussion

4.1. Study of the growth kinetics of *M. trichosporium OB3b*

In order to be able to calculate from transient experiments kinetic parameters of microorganisms that grow on volatile compounds, we formulated a model and tested it with *M. trichosporium* OB3b growing on methane. The model consisted of a biomass balance, and of liquid phase and gas phase balances for methane and oxygen. The value of the oxygen half saturation constant K_{s,O_2}, which was not critical for the simulations presented, was estimated as 1 μM. Steady-state, headspace flush and gas block experiments were carried out, the effects on the oxygen consumption and off-gas concentrations were measured and the model was fitted to the data.

Values for the yields and the overall k_La were calculated from steady-state measurements (Table 1). These values were then used in the transient experiments from which the other kinetic parameters were derived.

4.1.1. Estimation of yields

In steady-state, the left hand accumulation terms in the model equations (Eqs. (3)–(6)) are zero and the system description reduces to three time independent balances for biomass, dissolved oxygen and methane.

The yield of biomass on methane (Y_{XS}) was obtained by combining the biomass and methane balances and assuming the dissolved methane concentration to be negligible. As a check, since it was not determined experimentally, the liquid contribution to the total flow of methane leaving the system was calculated to be less than 0.2% using literature values for $V_{max,S}$ (μ_{max}) and $K_{s,S}$ (Table 1). The Y_{XS} value calculated (0.39 g g^{-1}) falls well within the large range of yields (0.17–0.70 g g^{-1}) pulished for growth on methane (Leak and Dalton, 1986).

The yield value Y_{XO} could subsequently be obtained from the oxygen balance, because all parameters were known.

Table 1
Parameter values used in the model describing the *M. trichosporium* OB3b system (S = methane)

Parameter	Value	Unit	Type of experiment
e_{O_2}	9.5	nmol min^{-1} mg^{-1}	Transient
e_{O_2} (+Cu)	2.1	nmol min^{-1} mg^{-1}	Transient
k_La_{B,O_2}	0.35	min^{-1}	Transient
$k_La_{B,S}$	0.38	min^{-1}	Transient
k_La_{H,O_2}	0.024	min^{-1}	Transient
$k_La_{H,S}$	0.026	min^{-1}	Transient
k_La_{ov}	0.40	min^{-1}	Steady-state
K_{s,O_2}	1	μM	
$K_{s,S}$	37	μM	Transient
$K_{s,S}$ (+Cu)	3	μM	Transient
V_{GB}	10	ml	
V_{GH}	0.53	l	
V_L	2.5	l	
$V_{max,S}$	290	nmol min^{-1} mg^{-1}	Transient
Y_{XO}	4.4	g mol^{-1}	Steady-state
Y_{XS}	6.3	g mol^{-1}	Steady-state
$\phi_{GB,in}$	42	ml min^{-1}	
$\phi_{GH,out}$	34	ml min^{-1}	Steady-state
ϕ_L	2.1	ml min^{-1}	
Literature values			
He_{O_2}	35	–	Fogg and Gerrard (1991)
He_S	33	–	Fogg and Gerrard (1991)
$K_{s,S}$	92	μM	Oldenhuis et al. (1991)
$V_{max,S}$	210	nmol min^{-1} mg^{-1}	Oldenhuis et al. (1991)
$V_{max,S}$ (+Cu)	290	nmol min^{-1} mg^{-1}	Park et al. (1992)

All values apply to the copper limited culture; $K_{s,S}$, e_{O_2} and $V_{max,S}$ values are shown also for the copper supplemented culture (+Cu). The type of experiment the value was derived from is included, and literature values are given separately.

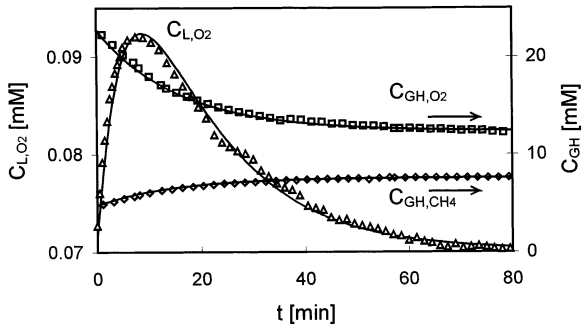

Fig. 3. Headspace flush experiment for *M. trichosporium* OB3b system. Starting from steady-state ($D = 0.05$ h^{-1}; $C_X = 0.52$ g l^{-1}), at $t = 0$, a 10-s lasting flush of oxygen (1 l min^{-1}) was led over the headspace. Shown are experimental data of C_{L,O_2} (△), C_{GH,O_2} (□) and C_{GH,CH_4} (◇) as a function of time together with the model fit (-) from which the headspace aeration contribution was derived.

4.1.2. Estimation of mass transfer coefficients

In order to determine the contribution of the headspace aeration to the total mass transfer, a headspace flush experiment was performed (Fig. 3), as described above. The contribution of the headspace aeration to the total mass transfer was found to be 6.4% (Table 1). The model accurately described the observed C_{L,O_2}, C_{GH,O_2} and C_{GH,CH_4} with time (Fig. 3).

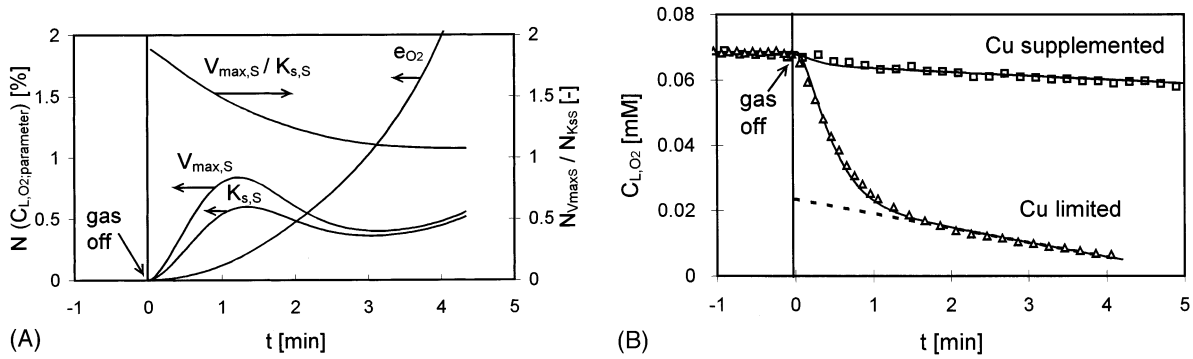

Fig. 4. Gas block experiment with copper limited and copper supplemented cultures of *M. trichosporium* OB3b growing on methane. Starting from steady-state (Cu limited: $D = 0.05\ h^{-1}$; $C_X = 0.52\ g\ l^{-1}$. Cu supplemented: $D = 0.03\ h^{-1}$; $C_X = 0.60\ g\ l^{-1}$) at $t = 0$ the gas flow was turned off. (A) Sensitivity of C_{L,O_2} for changes in the parameters $K_{s,S}$ ($N_{C_{L,O_2};K_{s,S}}$), $V_{max,S}$ ($N_{C_{L,O_2};V_{max,S}}$), e_{O_2} ($N_{C_{L,O_2};e_{O_2}}$); and the ratio $N_{C_{L,O_2};V_{max,S}}/N_{C_{L,O_2};K_{s,S}}$ (right axis) as a function of time. (B) Experimental C_{L,O_2} data for the Cu limited (△) and Cu supplemented (□) cultures and best fits obtained (-) as a function of time. The tangent that was used for the quick estimate for the Cu limited culture is also shown (..).

4.1.3. Estimation of $V_{max,S}$, $K_{s,S}$ and e_{O_2} from a gas block experiment

The growth parameters $V_{max,S}$ and $K_{s,S}$, as well as e_{O_2}, the term describing the depletion of internal pools occurring in the absence of growth substrate, were estimated from the C_{L,O_2} data of a gas block experiment. In this experiment, the C- and O-substrate supplies were cut off simultaneously.

The gas block was expected to lead to a decrease in C_{L,O_2} with time, with a rate governed by several factors:

1. Methane consumption, influenced by $V_{max,S}$ and $K_{s,S}$ and linked directly to oxygen consumption via Y_{XS}/Y_{XO};
2. Oxygen consumption for endogenous metabolism, resulting from depletion of internal pools. This is taken to be constant during the gas block experiment and described by e_{O_2}; and
3. Mass transfer between gas and liquid phases.

To determine whether the unknown kinetic parameters ($V_{max,S}$, $K_{s,S}$, e_{O_2}) could accurately be obtained from such a gas block experiment, a sensitivity analysis was performed using literature values for the kinetic parameters. $V_{max,S}$ and $K_{s,S}$ appeared to be sensitive during the first 2 min of the gas block, e_{O_2} only after this time (Fig. 4A). This is explained by the rapid residual dissolved methane consumption during the first 2 min, a process which is influenced by the growth parameters $V_{max,S}$ and $K_{s,S}$, and the slow oxygen consumption for endogenous metabolism, which is influenced by e_{O_2} and becomes important only after the residual methane is depleted. During the period of dissolved methane consumption both parameters $V_{max,S}$ and $K_{s,S}$ can be obtained independently, as implied by the changing ratio of sensitivities $N_{C_{L,O_2};V_{max,S}}/N_{C_{L,O_2};K_{s,S}}$ (Fig. 4A).

In the decline in the liquid phase oxygen concentration (Fig. 4B, Cu limited), two phases could be distinguished. There was an initial rapid decline, followed by a more gradual decrease after 1.5 min. The first phase was due to consumption of dissolved methane, the second to endogenous metabolism.

A rough estimate of the dissolved methane concentration C_{L,CH_4}, which was used as a starting value for the fitting procedure, could be obtained from the experimental data by extrapolating the tangent of the second, slowly decreasing, part of the curve to $t = 0$ (Fig. 4B). Using the steady-state ratio between oxygen and methane consumption (Y_{XS}/Y_{XO}, Table 1), C_{L,CH_4} was calculated to be 33 μM. This estimate was in close agreement with the value obtained from fitting the model to the data (37 μM).

The discrepancy between rough estimate (33 μM) and fitted value (37 μM) is due to the mass transfer between gas and liquid phases which was

neglected in determining the estimate. In the fitting procedure it was accounted for, implying that the estimate becomes less accurate with increasing influence of the mass transfer (large $k_L a_H$ values).

The values for $V_{max,S}$ (290 nmol min^{-1} mg^{-1}, or $\mu_{max} = 0.11$ h^{-1}) and $K_{s,S}$ (37 μM) obtained from the fitting procedure were similar to literature values (210 nmol min^{-1} mg^{-1} and 92 μM, respectively) (Oldenhuis et al., 1991).

From the value for the endogenous respiration obtained in the fitting procedure ($e_{O_2} = 9.5$ nmol min^{-1} mg^{-1}), a biomass decay rate was calculated using a conversion factor of 1.4 mg of O_2 per mg of biomass. This rate (0.31 d^{-1}) compared well with literature values for other organisms (Bilbo et al., 1992; Chang et al., 1993).

4.1.4. pMMO has a higher affinity for methane than sMMO

M. trichosporium OB3b expresses different methane monooxygenases (MMOs), depending on the amount of copper available (Park et al., 1992). In the absence of copper, a soluble form (sMMO) is expressed. When copper is available in excess, a membrane bound MMO (pMMO) is present. A gas block for a continuous culture grown with excess copper showed only a slow and gradual decrease in liquid phase oxygen concentration (Fig. 4B). In contrast to the gas block for the culture grown under copper limitation, no initial rapid decline in the C_{L,O_2} data was visible. Since the V_{max} values of sMMO and pMMO are similar (Table 1), it was concluded that very little methane was dissolved, implying a small $K_{s,S}$ value resulting in a very short methane consumption period (seconds). A simulation using a literature value for the $V_{max,S}$ of pMMO (290 nmol min^{-1} mg^{-1}, Park et al., 1992) and a $K_{s,S}$ value of 3 μM appeared to give a good representation of the data (Fig. 4B). This indicates that pMMO has approximately a ten-fold higher affinity for methane than sMMO.

4.1.5. Discussion on gas block experiment

The two examples given (Fig. 4B) illustrate that accurate values for the growth parameters $V_{max,S}$ and $K_{s,S}$ can only be obtained from a gas block experiment if the $K_{s,S}$ value is relatively high (above 5 μM). If the organism has a higher affinity for the limiting substrate, the decrease in dissolved oxygen concentration is very small and the estimation of both – strongly related – growth parameters is virtually impossible. However, if a literature value for either of the two parameters is available, an estimate of the other parameter can be obtained, as shown for the copper supplemented case.

Another factor hindering accurate estimation of $K_{s,S}$, particularly in the case of low $K_{s,S}$ values, is the depletion of internal pools. Obviously taking e_{O_2} as a constant is a simplification of reality as with time internal pools will be depleted and e_{O_2}will decrease. The e_{O_2} directly after the gas supply is switched off will therefore usually be underestimated, implying an overestimation of the residual dissolved limiting substrate concentration and $K_{s,S}$ value. The related uncertainty, a few micromolars of oxygen, is independent of the actual $K_{s,S}$ value, implying a decreasing relative uncertainty with an increasing $K_{s,S}$ value.

4.2. Study of the growth kinetics of *B. cepacia* G4

In order to obtain information on the microbial kinetics of microorganisms that grow on non-volatile substrates, a model was formulated and tested with *B. cepacia* G4 growing on phenol using transient chemostat experiments. Steady-state, headspace flush, gas and/or liquid block, and substrate pulse experiments with phenol were carried out and the effect on the oxygen consumption rate was measured. The model consisted of a biomass balance, a phenol balance and gas and liquid oxygen balances.

Maintenance, which could become important at the relatively low growth rate used (15% of μ_{max}), was determined to be negligible (Fig. 5, $R_m < 5\%$ of R_S, Eq. (2)). The value obtained (50 nmol mg^{-1} h^{-1}) was between values published for growth of *B. cepacia* G4 on phenol (Solomon et al., 1994) and on toluene (Mars et al., 1996).

4.2.1. Estimation of yields and mass transfer coefficients

Similar to the procedure followed for *M. trichosporium* OB3b, values for the yields (Y_{XS}, Y_{XO}) of *B. cepacia* G4 and the overall k_La were obtained from steady-state measuments (Table 2). The dissolved phenol concentration, which appeared to be below the detection limit of 2 μM, was neglected.

For this culture, in which the limiting substrate is supplied via the liquid phase, the headspace aeration contribution was derived from two types of dynamic experiments: a headspace flush experiment such as described for *M. trichosporium* OB3b (data not shown), and a gas block, in which the gas flow was switched off during a restricted period of time (min), keeping C_{L,O_2} well above limiting values (>5 μM) (Fig. 6). From both experiments similar results were obtained, implying a headspace aeration contribution of 14% (Table 2).

4.2.2. Estimation of $V_{max,S}$ and $K_{s,S}$ by phenol pulses

In order to obtain information on the phenol conversion kinetics, small pulses of phenol were injected into the chemostat (initial concentrations of 10–50 μM). Right after the injection, the cells started converting the additional phenol as shown by an instantaneous and rapid decrease in C_{L,O_2} with time (Fig. 7A). After all additional phenol had been converted, the disturbed oxygen balance was slowly restored.

As expected, in a sensitivity analysis of this experiment the kinetic parameters for phenol conversion ($V_{max,S}$ and $K_{s,S}$) were found to be sensitive during the few minutes of rapid decrease only (Fig. 7B). The subsequent restoration of the oxygen balance was governed mostly by the mass transfer parameters (data not shown).

Using literature values for $V_{max,S}$ and $K_{s,S}$ (Folsom et al., 1990) (Table 2), the model predicted

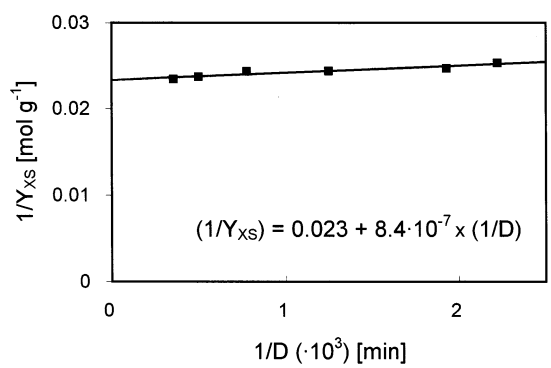

Fig. 5. Observed yield as a function of dilution rate for the *B. cepacia* G4 chemostat culture growing on phenol. Via $1/Y_{XS} = 1/Y_G + m/D$ values for the true growth yield Y_G and the maintenance coefficient m of 43 g mol^{-1} and 50 nmol mg^{-1} h^{-1}, respectively, were derived. Experimental data (■) and regression curve (-).

Table 2
Parameter values used in the model describing the *B. cepacia* G4 system (S = phenol)

Parameter	Value	Unit	Type of experiment
e_{O_2}	16	nmol min^{-1} mg^{-1}	Transient
$I_{dyn,OS}$	2.4	mol mol^{-1}	Transient
$I_{ini,OS}$	2.2	mol mol^{-1}	Transient
k_La_{B,O_2}	0.26	min^{-1}	Transient
k_La_{H,O_2}	0.042	min^{-1}	Transient
k_La_{ov}	0.37	min^{-1}	Steady-state
K_{s,O_2}	1.0	μM	Transient
$K_{s,S}$	5.1	μM	Transient
V_{GB}	5	l	
V_{GH}	0.5	l	
V_L	2.5	l	
$V_{max,S}$	200	nmol min^{-1} mg^{-1}	Transient
Y_{XO}	11	g mol^{-1}	Steady-state
Y_{XS}	41	g mol^{-1}	Steady-state
$\phi_{GB,in}$	12	ml min^{-1}	
$\phi_{GH,out}$	12	ml min^{-1}	Steady-state
ϕ_L	1.3	ml min^{-1}	
Literature values			
He_{O_2}	35	–	Fogg and Gerrard (1991)
$K_{s,S}$	8.5	μM	Folsom et al. (1990)
$V_{max,S}$	230	nmol min^{-1} mg^{-1}	Folsom et al. (1990)

The type of experiment the value was derived from is included. Literature values are given separately.

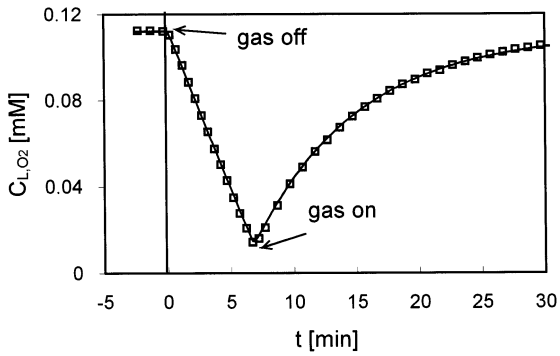

Fig. 6. Gas block experiment for *B. cepacia* G4 system. Starting from steady-state ($D = 0.03\ h^{-1}$; $C_X = 0.20\ g\ l^{-1}$), at $t = 0$ the ingoing gas flow was turned off, at $t = 6.5$ min it was turned on again. Experimental C_{L,O_2} data (□) and best model fit (-) are shown as a function of time.

that for a pulse injection of 45 μM of phenol, oxygen limitation should occur (Fig. 7A). This also followed from a rapid estimation based on the yield values (Y_{XS}/Y_{XO}) (Table 2): for the conversion of 45 μM of phenol around 0.17 mM of oxygen is needed, and only around 0.11 mM of oxygen was present. No such limitation was observed, however (Fig. 7A). Because all additional phenol had disappeared as soon as C_{L,O_2} started to increase again after the dip (data not shown), the conclusion had to be drawn that the amount of oxygen consumed per mole of phenol during the pulse differed from steady-state.

4.2.3. Intermediate formation

An explanation for the discrepancy between expected and measured oxygen consumption after the phenol injection could be the transient accumulation of some intermediate product. Both Li and Humphrey (1989) and Allsop et al. (1993) found similar behavior for another phenol converting organism (*Pseudomonas putida*) under comparable unsteady-state conditions. In order to check this hypothesis, the model was adjusted by taking the ratio of oxygen over phenol ($I_{dyn,OS} = Y_{XS}/Y_{XO}$) during the decrease in C_{L,O_2} as a (constant) fit parameter.

A sensitivity analysis with the adjusted model showed that in a phenol pulse experiment the parameters $V_{max,S}$, $K_{s,S}$ and $I_{dyn,OS}$ are sensitive. The depth of the dip in C_{L,O_2} is governed mostly by $I_{dyn,OS}$ and the initial slope mostly by $V_{max,S}$ (data not shown). The value of $K_{s,S}$ has little influence on the C_{L,O_2} data. Although $I_{dyn,OS}$ also affected the initial slope and $V_{max,S}$ the depth of the dip (they are related), their maximum effect occurred at different time points, allowing for reliable values for $I_{dyn,OS}$ and $V_{max,S}$ to be calculated.

With a value for $I_{dyn,OS}$ of 2.4, instead of the steady-state value of 3.7 (Table 2), both the depth in and the initial slope of the dissolved oxygen levels could satisfactorily be described (Fig. 7A). The values for the phenol conversion parameters obtained in this fit, $V_{max,S} = 200$ nmol min^{-1}

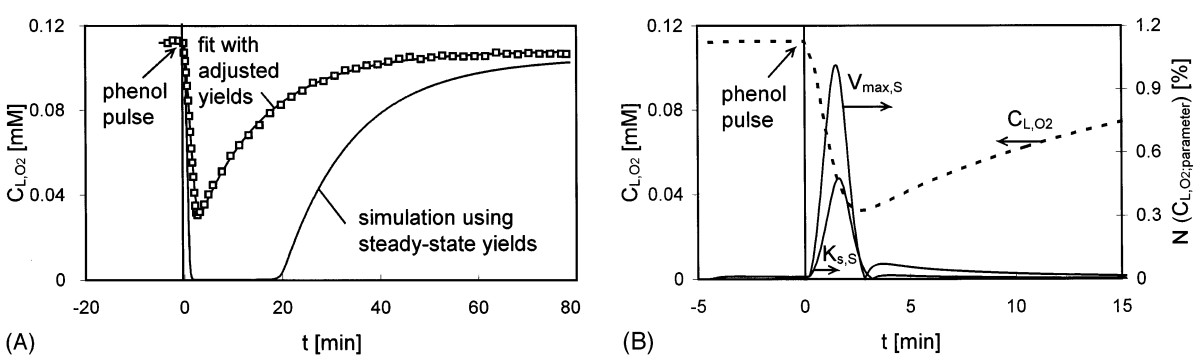

Fig. 7. Phenol pulse experiment with a continuous culture of *B. cepacia* G4 growing on phenol. Starting from steady-state ($D = 0.03\ h^{-1}$; $C_X = 0.20\ g\ l^{-1}$), at $t = 0$ a pulse of phenol was injected into the chemostat. (A) Experimental C_{L,O_2} data (□) for a phenol pulse of 45 μM. Shown are simulations (-) using steady-state yield values for Y_{XO} and Y_{XS} (Table 2); and an adjusted yield ratio $I_{dyn,OS}$ of 2.4 during the decrease in C_{L,O_2}. (B) C_{L,O_2} data of simulation for a phenol pulse of 21 μM (- -) and sensitivity of C_{L,O_2} (-) for changes in the parameters $K_{s,S}$ ($N_{C_{L,O_2};K_{s,S}}$) and $V_{max,S}$ ($N_{C_{L,O_2};V_{max,S}}$) (right axis) as a function of time.

mg^{-1} and $K_{s,S} = 5.1$ μM, are close to the values found by Folsom et al. (1990) (230 nmol min^{-1} mg^{-1} and 8.5 μM, respectively).

By correcting for the steady-state phenol conversion during the pulse, a yield ratio $I_{ini,OS}$ was obtained: 2.2 moles of O_2 per mole of phenol. $I_{ini,OS}$ represents the conversion of the additional phenol and gives an indication as to the nature of the intermediates that transiently accumulate.

If all additional phenol is initially converted to α-hydroxymuconic-semialdehyde, the intermediate detected by Li and Humphrey (1989), an $I_{ini,OS}$ value of 1.5 is expected (one monooxygenase and one dioxygenase step implying the consumption of two moles of O_2, and the consumption of one mole of NADH) (Shingler et al., 1992). Since this compound has a strong yellow color and no color change was observed, significant accumulation is not likely.

If the additional phenol is initially converted to one of the intermediates 4-oxalocrotonate, 2-oxopent-4-enoate, 4-hydroxy-2-oxovalerate, or aceetaldehyde and pyruvate, a value for $I_{ini,OS}$ of 2 is expected (O_2 and NADH consumption as described above, with an additional production of one mole of NADH) (Shingler et al., 1992).

However, if during the conversion of phenol to intermediate part of the intermediate is already processed further, this would result in higher values for $I_{ini,OS}$ than 1.5 and 2, respectively, depending on the relative rates.

To analyse the presence of the intermediates, they were accumulated by repeatedly injecting small amounts of phenol into the culture while maintaining aerobic conditions ($\phi_{GB,in}$ adjusted). A single compound was found and identified as 4-hydroxy-2-oxovalerate (HOV) using mass spectrometry. Subsequently, HOV was synthesized and used for quantification in a standard pulse experiment. The 45 μM phenol pulse response (Fig. 8) was qualitatively in agreement with the conclusion drawn based on the oxygen data ($I_{ini,OS}$ above 2) since around 70% of the phenol accumulated as HOV, whereas the remainder was processed further.

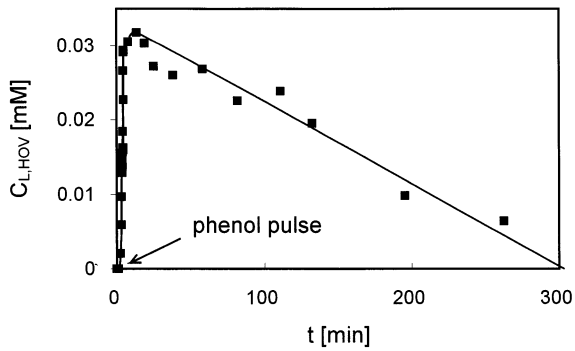

Fig. 8. Accumulation of 4-hydroxy-2-oxovalerate during a phenol pulse experiment with a continuous culture of *B. cepacia* G4 growing on phenol. Starting from steady-state ($D = 0.03$ h^{-1}; $C_X = 0.20$ g l^{-1}), a 45 μM pulse of phenol was injected into the chemostat. Experimental data (■) and trend line.

4.2.4. Estimation of kinetic parameters from liquid sampling data

Due to the disruption of the culture and the relatively low sampling frequencies (≈ 1 min), liquid sampling data (phenol, HOV) is far less accurate than dissolved oxygen data. In the phenol pulse experiment for example, phenol is depleted within 3 min, so obtaining reliable estimates for kinetic parameters from the dissolved phenol data is virtually impossible. On the other hand, for very slow processes such as consumption of accumulated HOV (Fig. 8), it is possible to obtain reliable estimates from the liquid sampling data. In this case, dissolved oxygen data is not very accurate as a result of the limited oxygen consumption involved and the slight drift of the oxygen probe over long periods of time (h).

4.2.5. Estimation of residual phenol concentration and $V_{max,S}$ and $K_{s,S}$ from a gas-liquid block

The concentration of the residual phenol was measured in a combined gas and liquid block experiment. Here, both the phenol and oxygen supplies were switched off simultaneously. This experiment is similar to the gas block experiment for the *M. trichosporium* OB3b system. As discussed for *M. trichosporium* OB3b, two distinct regions were expected: one of fast decrease in which the dissolved and readily convertible substrates (phenol and intermediates if present) are

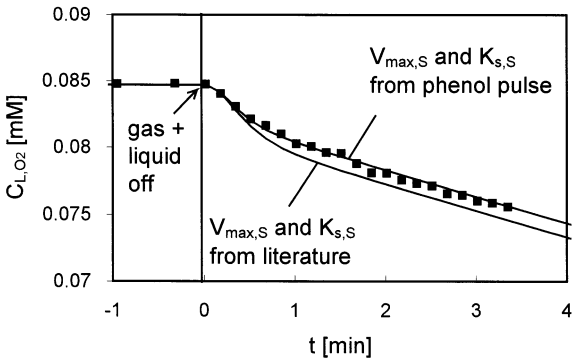

Fig. 9. Combined liquid and gas block experiment with a continuous culture of *B. cepacia* G4 growing on phenol. Starting from steady-state ($D = 0.03\ h^{-1}$; $C_X = 0.20\ g\ l^{-1}$), at $t = 0$ simultaneously the liquid and gas flows were stopped. Experimental data (■) and simulations (-) using the $V_{max,S}$ and $K_{s,S}$ values obtained from (1) the phenol pulse experiment and (2) literature (Folsom et al., 1990) (Table 2). In both simulations $e_{O_2} = 16$ nmol min^{-1} mg^{-1}.

rapidly converted; and one of very slow decrease in which internal pools are depleted and only endogenous respiration occurs.

In the continuous culture of *B. cepacia* G4 only very little limiting substrate (phenol, HOV) appeared to be present (Fig. 9, mind the C_{L,O_2} scale).

As was discussed for the gas block experiment with the culture of *M. trichosporium* OB3b grown with excess copper, the small amount of dissolved phenol (and intermediates) does not allow for accurate estimates for the phenol conversion parameters to be derived here. However, comparison of different sets of parameters obtained from other and independent experiments or from literature is possible. In this case, a simulation with values for $V_{max,S}$ and $K_{s,S}$ obtained from the phenol pulse experiment (using an e_{O_2} value of 16 nmol min^{-1} mg^{-1}) gives a slightly better representation of the C_{L,O_2} data than with literature values (Folsom et al., 1990) (Fig. 9). This is not surprising, because growth conditions in the phenol pulse experiment were identical to those in the gas-liquid block experiment, whereas the literature values were obtained using cells grown differently. The use of the different sets of parameters resulted in residual phenol concentrations of 0.8 μM (phenol pulse parameters) and 1.1 μM (literature parameters). Both these values are below the detection limit of our HPLC analysis method. This is in accordance with experimental results (data not shown) and implies that a more accurate estimate cannot be obtained by analyzing liquid samples.

Note that particularly in this type of experiment the mass transfer from the headspace to the liquid played an important role, because its contribution was of the same order of magnitude as the endogenous metabolism ($e_{O_2}C_X$ is around 55% of the headspace mass transfer rate $k_L a_{H,O_2}(C_{GH,O_2}/He_{O_2} - C_{L,O_2})$). By accurately incorporating this phenomenon, a realistic value for e_{O_2} (16 nmol min^{-1} mg^{-1}, or a biomass decay rate of 0.5 d^{-1}) was obtained that is similar to values described for comparable organisms (Chang et al., 1993).

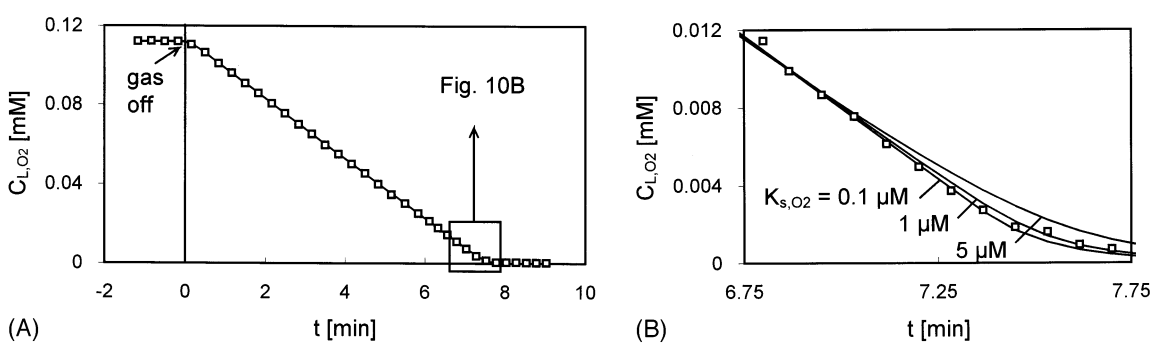

Fig. 10. Gas block experiment with a continuous culture of *B. cepacia* G4 growing on phenol. Starting from steady-state ($D = 0.03\ h^{-1}$; $C_X = 0.20\ g\ l^{-1}$), at $t = 0$ the gas flow was switched off. Experimental C_{L,O_2} data (□) and fit using $K_{s,O_2} = 1$ μM (-) as a function of time (A), and a detail showing simulations for various K_{s,O_2} values (0.1, 1 and 5 μM) (B).

4.2.6. Estimation of K_{s,O_2} from a gas block

The oxygen half saturation constant K_{s,O_2} was estimated from a gas block experiment (Fig. 10). Here, right after the gas flow was switched off, C_{L,O_2} started to decrease at a rate governed by the rate of phenol addition (via Y_{XS}/Y_{XO}). Oxygen consumption did not slow down until oxygen became limiting at around 2 μM ($C_{L,O_2} \approx K_{s,O_2}$).

The model appears to describe the data realistically using the $V_{max,S}$ and $K_{s,S}$ values derived from the phenol pulse experiment, and steady-state values for Y_{XS}/Y_{XO} (Fig. 10A).

The exact position of the bend in the curve, appearing at oxygen limitation, is determined by the K_{s,O_2} value. Three simulations show that the actual K_{s,O_2} is somewhere close to 1 μM (Fig. 10B). A more accurate estimate could not be obtained due to the inaccuracy of the oxygen probe in this region.

4.2.7. A single set of parameters accurately describes three different types of dynamic experiments

Except for the adjusted initial stoichiometry in the phenol pulse experiment, all three different types of dynamic experiments discussed were realistically described with a simple Monod type model using a single set of parameters (Table 2): a phenol pulse experiment (Fig. 7A), an experiment in which the oxygen supply was switched off (Fig. 10) and an experiment in which both the phenol and the oxygen supplies were switched off (Fig. 9). By deriving from each type of experiment only one parameter (e_{O_2}, K_{s,O_2}), or two parameters ($V_{max,S}$ and $K_{s,S}$) combined with sensitivity analyses keeping all other parameters equal, mutual independency of the parameters was assured. Obviously, the simple model suffices in describing the dynamic situations studied, and the parameters determined give a realistic description of the organisms' behavior under a range of different steady-state and dynamic situations.

5. Evaluation technique and conclusion

The examples show that as soon as a chemostat is in steady-state, rapidly qualitative information on the microbial kinetics is obtained from simple transient experiments such as gas blocks and substrate pulse injections. Thus, the transient chemostat technique can, for example, be used to routinely characterise the type of enzyme system (sMMO or pMMO) expressed by a methanotrophic culture.

Also, after the different k_La values (k_La_B, k_La_H) have been derived from a headspace flush experiment, the dissolved oxygen data can be interpreted via a model that incorporates the microbial kinetics, yielding estimates for parameters such as $V_{max,S}$ and $K_{s,S}$. The accuracy of the estimates obtained depends on the type of system (dissolved or gaseous limiting substrate) and the type of kinetics. For organisms with a high $K_{s,S}$ value – here above 5 μM, the value depends on the amount of O_2 consumed per mole of substrate – accurate values are obtained (error $< 20\%$). For organisms with a low $K_{s,S}$ value, the procedure yields an estimate (order of magnitude).

Because mass transfer between gas and liquid phases is accounted for, our pulse technique is especially applicable for the study of cultures in which volatile substrates play a role (toluene, styrene etc.).

For a particular microorganism, the analysis may lead to the conclusion that in certain dynamic experiments the initial stoichiometry deviates from steady-state stoichiometry as demonstrated by the phenol pulse experiments with the culture of *B. cepacia* G4. A hypothesis as to the actual kinetics can be formulated based on theoretical studies using a slightly adjusted model. Because of the obvious deficiencies of the simple model used to describe these more complicated situations, the parameters obtained from these dynamic experiments only give an indication. Additional experiments with liquid sampling are then needed to gain more insight.

As the oxygen data are collected on-line, with this transient chemostat technique the system is not disturbed. For this reason, and because oxygen is a very sensitive indicator, relatively small disturbances can be used. With traditional methods the effect of such small disturbances could not be studied because of the complexity involved in sampling and analysis. Thus, this technique allows

for a larger choice in experimental set-up: type, size and length of disturbance. It provides therefore an opportunity to study both situations in which the growing cells have not had time to adapt (very small disturbances), and situations in which they have (larger disturbances).

5.1. Extensions

Our transient chemostat technique can easily be extended to fit other applications, for example based on techniques known from others. The following are examples.

In headspace flush experiments a very small change in liquid concentration of a volatile component can be obtained. Gas step experiments can be conducted by changing the ingoing concentration of the gases (in $\phi_{GB,in}$), keeping the total gas flow and thus the k_La values constant. Also, the headspace aeration flow, as shown by Oeggerli et al. (1995), and the dilution rate can be shifted up and down from steady-state values. Compared to previous work, in which the latter type of experiment was frequently used, our technique yields additional information in the form of the oxygen consumption pattern.

The technique can be extended by using other on-line or additional off-line measurements. Care should be taken that the sampling does not affect the experiment; selective electrodes or on-line off-gas analyzing techniques can be used.

Finally, when extended with experimental design such as described by Posten and Munack (1990, 1993), the technique will further gain in strength and versatility. Experiment 'creation' will have to be based on biological insight though. Subsequently, experimental design procedures can be used to optimize the experiments.

Acknowledgements

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Appendix. Nomenclature

C_G	gas concentration [M]
C_{GB}	bubble gas concentration [M]
C_{GH}	headspace gas concentration [M]
C_L	liquid concentration [M]
C_L^*	interfacial liquid concentration [M]
C_X	cell concentration [kg l^{-1}]
D	dilution rate ($=\phi_L/V_L$) [s^{-1}]
e_{O_2}	endogenous oxygen respiration coefficient [mol s^{-1} kg^{-1}]
He	Henry coefficient ($=C_G/C_L^*$) [−]
k_La	volumetic mass transfer coefficient [s^{-1}]
k_La_B	volumetric mass transfer coefficient bubble gas phase [s^{-1}]
k_La_H	volumetric mass transfer coefficient headspace gas phase [s^{-1}]
k_La_{ov}	overall volumetric mass transfer coefficient [s^{-1}]
K_s	half saturation constant [M]
$N_{i;j}$	normalized sensitivity function, with i = state variable and j = parameter [−]
R	volumetric conversion rate [mol s^{-1} l^{-1}]
$I_{dyn,OS}$ ($=Y_{XS}/Y_{XO}$)	ratio oxygen over phenol during phenol pulse [mol mol^{-1}]
$I_{ini,OS}$	ratio oxygen over phenol during phenol pulse due to

	conversion of additional phenol [mol mol^{-1}]
$-R_m$	volumetric maintenance substrate consumption function [mol s^{-1} l^{-1}]
R_X	volumetric cell production rate [kg s^{-1} l^{-1}]
t	time [s]
V_{GB}	bubble gas volume [l]
V_{GH}	headspace volume [l]
V_L	liquid volume [l]
V_{max}	maximum specific conversion rate [mol s^{-1} kg^{-1}]
Y_G	true growth yield [kg mol^{-1}]
Y_{XO}	yield of biomass on oxygen [kg mol^{-1}]
Y_{XS}	yield of biomass on substrate [kg mol^{-1}]
ϕ_{GB}	bubble gas flow rate [l s^{-1}]
ϕ_{GH}	headspace flow rate [l s^{-1}]
ϕ_L	liquid flow rate [l s^{-1}]
μ_{max}	maximum specific growth rate [s^{-1}]

Subscripts and abbreviations

GC	gas chromatograph
HOV	4-hydroxy-2-oxo-valerate
HPLC	high performance liquid chromatography
i.d.	internal diameter
in	in ingoing flow
out	in outgoing flow
S	substrate
T	temperature

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