Growth Dynamics of a Methylotroph (*Methylomonas* L3) in Continuous Cultures. I. Fast Transients Induced by Methanol Pulses and Methanol Accumulation

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The dynamic behavior of the Ribulose Monophosphate-type *Methylomonas* L3 in continuous cultures was studied, using methanol pulses to induce fast transients in steady-state cultures of single (methanol) and mixed (methanol plus formaldehyde) substrates. In several experiments, the methanol-uptake rate (MUR) profiles displayed negative MUR values for a time period following the methanol pulse, and significant amounts of methanol disappeared immediately following the pulse. These phenomena suggested the accumulation of methanol in the cells upon pulsing, apparently due to an active transport system. Accordingly, and in order to estimate the potential of the transport system for methanol accumulation, accumulation profiles were calculated for several pulse experiments. The calculations are based on a methanol balance and experimentally determined values of the cell volume and the true transient biomass yields. It is calculated that methanol accumulates up to 200-fold to very high intracellular concentrations. The accumulation is calculated to be much higher in single- (methanol) substrate cultures of low dilution rate than in cultures of high dilution rate or of mixed substrates. The specific growth rate immediately following the methanol pulse decreased in single-substrate cultures and increased in mixed-substrate ones. The biomass yield decreased after the methanol addition in all experiments; however, the drop was less severe in the mixed-substrate experiments. It is also suggested that formaldehyde as a methanol cosubstrate may be an effective means of providing more stable biomass yields and growth rates in reactors with imperfect mixing, and of protecting the reactor against accidentally induced methanol accumulation.

INTRODUCTION

The usefulness of the transient behavior as a tool in the understanding of regulatory mechanisms of microbial metabolism has been long recognized. Microbial growth dynamics induced by shifts in nutrient concentrations and the dilution rate have been employed to elucidate how microorganisms regulate their protein-synthesis machinery according to environmental changes.[1,2] Many new concepts and discoveries have evolved from those studies. Temperature, pH, and oxygen tension have also been employed as perturbed variables[3,4] to study the mechanisms of enzyme regulation, among others. Moreover, pulses or periodical feeding of a limiting nutrient have been used to enhance the production of catabolites, such as ethanol from glucose.[5,6] Pulse feedings of small reactors can also serve as simulators of large fermentors with imperfect mixing, whereby cells experience fluctuations in nutrient concentrations.[3,5–7] Studies on the transient behavior of growth can also help build better process models for control purposes.[8]

Transient phenomena are especially important in methylotrophic fermentations. Since the biomass yield and the specific growth rate are adversely affected by high concentrations of methanol (MeOH),[9–11] there is a risk of process instability that can lead to washout if the reactor MeOH level is increased as a result of some disturbance in a process parameter. The risk is higher if the feed MeOH concentration is high, which is typical of industrial processes. Thus, more information on the growth dynamics and the implementation of effective control strategies would be desirable.[12–14] Also, because of imcomplete mixing in industrial fermentors, especially in fermentors without mechanical mixing devices, such as loop fermentors, the biomass yield is lower due to the inhomogeneity of the reactor MeOH concentration.[12] This problem has been studied using pulse-feeding transients in small-scale reactors.[3,5–7] Currently, this problem is solved mainly by more expensive reactor design for improved mixing characteristics of the fermentor (such as em-

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ploying multiple feeding ports).[6,15] A better understanding of what happens when the cells experience fluctuations in the MeOH concentration can give us some hints on how to solve this problem at the microbial physiology level, e.g., by mutant selection or genetic engineering.

The extracellular MeOH profiles of the MeOH pulse experiments reported here display two unusual features: 1) negative values of the MeOH-uptake rate (MUR) during a certain period of time following the MeOH pulse in the continuous reactor, and 2) the disappearance of significant amounts of MeOH immediately following the pulse. These two features indicate accumulation of MeOH in the cells and suggest the existence of an active-transport system for MeOH. An estimation of the intracellular MeOH concentration following the pulse would be of both fundamental and practical interest. Because of its high membrane permeability, MeOH diffuses back very fast when accumulated by cells.[16] Also, the oxidation of MeOH cannot be effectively decoupled from its transport.[16] As a result, the usual substrate transport studies employing whole cells and radioactive tracers cannot be employed in these methylotrophs, and one must therefore resort to anaerobic conditions for such whole cell studies, as is detailed by Diwan.[16] Anaerobic conditions proved useful in showing the energy dependence of this MeOH transport system, but because of the energy limitations imposed by anaerobic conditions, the true *in vivo* potential of the transport system could not be demonstrated. The calculations presented here, although meant as mere estimates of the true MeOH accumulation gradients, provide a measure of the true *in vivo* potential of this system for MeOH accumulation. Since MeOH accumulation is energy dependent,[16] the accumulation gradient is a measure of the energy spent by the cells for MeOH transport, especially because MeOH diffuses back upon accumulation, and thus, it appears to act as an uncoupler of biosynthetic energy.[16]

MATERIALS AND METHODS

Organism, Media, and Culture Conditions

The MeOH utilizing bacterium *Methylomonas* L3, originally isolated and described by Hirt et al.,[11] was obtained by reconstitution from freeze-dried cultures. A basal-salts medium plus carbon substrates, MeOH or CH_2O, was used to grow strain L3.[11] Batch cultures of *M.* L3 were established on the basal-salts medium plus 0.4% v/v MeOH in 250 mL flasks in an incubator (New Brunswick Scientific, model G-25) at 30°C and 250 rpm.

Continuous cultures were established in a Bioflo-30 (New Brunswick Scientific) 1 L fermentor with a working volume of 350 mL. Agitation was set at 450 rpm, aeration was ca. 0.2 L/min, and the temperature was kept at 30°C. The culture pH was measured by an Ingold pH probe and was kept at 7.0 ± 0.1 by a pH controller (Horizon, model 5994) that controlled the addition of 1*N* NaOH. Dissolved oxygen (DO) was measured by a galvanic-type probe with a DO monitor (New Brunswick Scientific, DO50). The medium was sterilized by passing through a 0.2 μm capsule filter (Gelman Sciences) and was kept at pH 3.5 by adding HCl to minimize the risk of contamination. MeOH (final concentration at 0.8 g/L) or CH_2O (100 mg/L) was added to the sterile medium aseptically.

M. L3 was first grown in batch cultures followed by several subcultures to ensure active growth and was then inoculated to the fermentor with 3 mL of suspension from a late-exponential phase batch culture. The above procedure was especially important for the mixed-substrate culture with MeOH and CH_2O as substrates. The fermentor was first run in batch mode until the MeOH was exhausted, and then the medium feed pump was turned on and adjusted to the desired dilution rate. To avoid the build-up of wall growth, continuous operation was maintained for at most three weeks.

Transient Experiments

A MeOH-pulse transient was induced by injecting a certain amount (usually 3 mL) of MeOH into the steady-state chemostat at various dilution rates. Samples were withdrawn directly from the fermentor. Part of the sample was used in the measurement of turbidity for biomass determination; the rest was filtered through a 0.22 μm membrane filter (Gelman Sciences), and the filtrate was analyzed for MeOH and CH_2O. The sampling interval was larger than 15 min to avoid the possibility of altering the transient behavior by excessive sampling.

For the calculation of the specific growth rates and the transient biomass yields from the transient-experiment data, the derivatives of the biomass and the residual-MeOH concentration profiles were obtained from smooth-curve functions (polynomials) fitted by least-squares regression to the observed data. This procedure was employed to eliminate the errors that might have been introduced by simply using differences of data points to calculate the derivatives.

The transient values of the residual MeOH, residual CH_2O, dissolved oxygen, and biomass concentration were measured directly. The specific growth rate, the biomass yield, and the MUR were then calculated from the above-mentioned values. The specific growth rate was calculated from the biomass concentration profile

$$\mu = (dX/dt)/X + D \tag{1}$$

The MUR was calculated according to the mass balance of external MeOH on the reactor:

$$\text{MUR} = [(S_f - S)\,D - dS/dt]/X \qquad (2)$$

where S_f is the MeOH feed concentration, S is the MeOH concentration in the fermentor, D is the dilution rate, t is time, and X is the biomass concentration expressed in dry mass per unit volume. As mentioned above, the derivatives, dX/dt and dS/dt, were obtained from fitted smooth-curve functions, while other quantities in eqs. (1) and (2) were obtained directly from the experimental data. The apparent biomass yield was calculated from the specific growth rate μ and MUR:

$$Y = \mu/\text{MUR} \qquad (3)$$

Cell Volume

The volume of the cells was measured as the mannitol-impermeable space of the cells using ^3H_2O and [U—^{14}C] mannitol as previously reported.[17,18]

True Transient Biomass Yields

The true transient biomass yield on MeOH (Y_t), defined as the grams of biomass produced per gram of MeOH oxidized to CH_2O, was measured over a period of 15 or 60 min following the MeOH pulse by employing a $^{14}CH_3OH$-traced pulse. Following the addition of the traced MeOH pulse, CO_2 was collected as described previously.[19,20] At the end of the 15- or 60-min period, the fermentor fluid was pumped out quickly, it was mixed with the fluid collected in the product tank over the specified time period, and the reaction was stopped by HCl addition. That fluid was treated further[19,20] in order to determine the distribution of label between the biomass and total CO_2.

Using the notation of Refs. 19 and 20, if r_7 is the rate of MeOH incorporation into biomass and r_1 is the rate of MeOH oxidation to CH_2O, then r_7/r_1 is the fraction of MeOH incorporated into biomass. r_7/r_1 is related to the true yield Y_t by the expression[19,20]

$$Y_t\, r_1/r_7 = \text{MW}_b/32 \qquad (4)$$

where 32 is the molecular weight of MeOH and MW_b is the "molecular weight" of $CH_pO_qN_r$, which represents the biomass composition. For the continuous experiments of interest, $\text{MW}_b/32$ was determined experimentally to be 0.6.[20] Once r_7/r_1 is determined from the label distribution between the biomass and CO_2, Y_t is determined from eq. (4). Note that the experimentally determined r_7/r_1 values represent average quantities over the 15- or 60-min period, and so does the Y_t.

Methanol Concentration Gradient Across the Cell Membrane

It is possible to calculate, approximately, the total methanol amount in the reactor after the methanol pulses from the methanol mass balance over the reactor,

$$dS_T/dt = D(S_f - S_T) - \mu X/Y_t \qquad (5)$$

where S_T is the total amount (extracellular plus intracellular) of methanol per unit reactor volume. With the experimentally obtained values of S_f, μ, and X, an assumed value for Y_t, and the initial value of S_T, we can calculate the profile for S_T. The values for μ and X used in this calculation were obtained from the fitted smooth-curve function of the biomass concentration profile, as described above. The difference between the calculated S_T and the observed extracellular methanol concentration(s) is the intracellular methanol amount. This amount is then converted to concentration based on the intracellular volume of the biomass according to the following equation:

$$v\, S_i + (1 - v)\, S = S_T \qquad (6)$$

where v is the fraction of total reactor volume occupied by the cells, while S_i represents the intracellular methanol concentration. The value of v is calculated to be (1.6) ($10^{-6}X$) based on the value of the intracellular volume of *M.* L3, which was measured to be 1.3 ± 0.3 μL/mg dry biomass (average of six determinations) as described above. Note that we use the value with the maximum measured error (1.6 μL/mg) in order not to overestimate the intracellular MeOH concentration.

Since $v \ll 1$ (X is always less than 0.85 g/L for all calculations performed), eq. (6) can be rearranged as

$$S_i/S = (S_T/S - 1)/v \qquad (7)$$

from which the methanol gradient across the cell membrane (S_i/S) can be calculated.

Chemicals

[^{14}C]methanol, [^{14}C]mannitol, and ^3H_2O were purchased from Amersham, Arlington Heights, IL. Inorganic salts and acids were obtained from Baker Chemical Co., Phillipsburg, NJ, or Mallingckrodt, Inc., Paris, KY. CH_2O was prepared by heating for 48 h at 110°C in a paraformaldehyde water solution. Paraformaldehyde was obtained from Aldrich Chemical Co., Milwaukee, WI. Bacto-agar was purchased from Difco Laboratories, Detroit, MI.

Analyses

MeOH was determined by gas chromatography as described previously.[11] CH_2O was determined by the chromotropic-acid method[11] with an accuracy of ±0.5 mg/L. The turbidity of the cell suspension was determined by a spectrophotometer (Gilford 250) at 600 nm. Dry biomass weight was determined by drying prewashed cells of known turbidity in a preweighed aluminium dish in an oven at 105°C for 40 h.[21] The correlation of dry weight to turbidity was found to be 0.42 ± 0.01 g per absorbance unit at 600 nm for a 1-

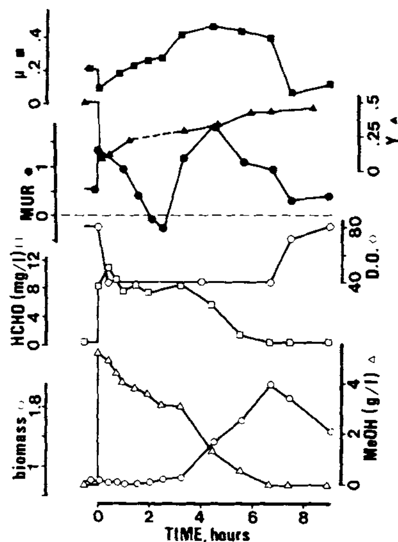

Figure 1. Response of a single-substrate continuous *M.* L3 culture to a methanol pulse (experiment #1: $D = 0.2\ h^{-1}$, pulse magnitude = 6.7 g/L). Biomass is expressed in OD_{600} units, MUR in g MeOH/h g dry biomass, Y is expressed in g dry biomass/g MeOH (the unrealistic high or negative values of Y caused by the negative MUR values were rejected in Figs. 1–3 and 6 as indicated by the dashed lines), DO is expressed in percent saturation, and μ is expressed in h^{-1}. Time zero corresponds to the addition of the methanol pulse.

cm-long light path through the spectrophotometric cuvette.

RESULTS

Methanol Pulse Experiments

Five experiments performed using MeOH as a substrate and another two experiments performed using MeOH plus CH_2O as substrates are presented here.

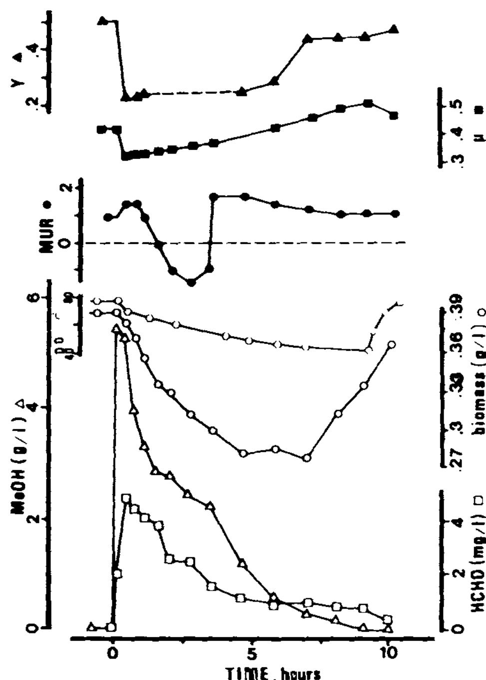

Figure 3. Response of a single-substrate continuous *M.* L3 culture to a methanol pulse (experiment #3: $D = 0.42\ h^{-1}$, pulse magnitude = 6.7 g/L). The units of the various quantities are given in the legend to Fig. 1.

The transient values of the residual MeOH, residual CH_2O, dissolved oxygen, and biomass concentration were measured directly. The specific growth rate, the biomass yield, and the specific MUR were then calculated from the above-mentioned values, as discussed in Materials and Methods. The results are plotted and presented in Figures 1–7.

Figures 1–4 show how the dilution rate affected the transient response of the cells to the MeOH pulses

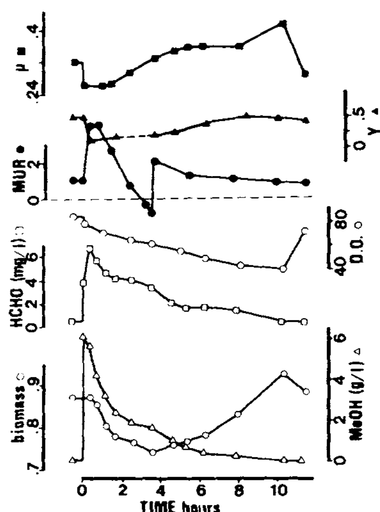

Figure 2. Response of a single-substrate continuous *M.* L3 culture to a methanol pulse (experiment #2: $D = 0.32\ h^{-1}$, pulse magnitude = 6.7 g/L). The units of the various quantities are given in the legend to Fig. 1.

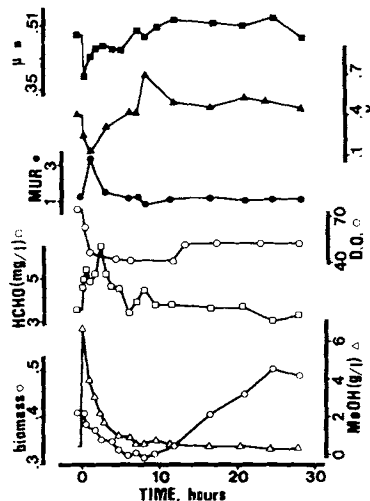

Figure 4. Response of a single-substrate continuous *M.* L3 culture to a methanol pulse (experiment #4: $D = 0.49\ h^{-1}$, pulse magnitude = 6.7 g/L). The units of the various quantities are given in the legend to Fig. 1.

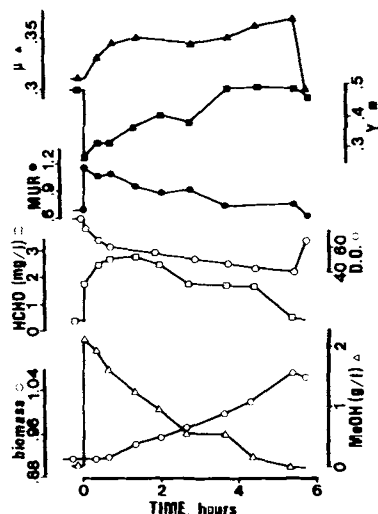

Figure 5. Response of a single-substrate continuous *M.* L3 culture to a methanol pulse (experiment #5: $D = 0.31\ h^{-1}$, pulse magnitude = 2.2 g/L). The units of the various quantities are given in the legend to Fig. 1.

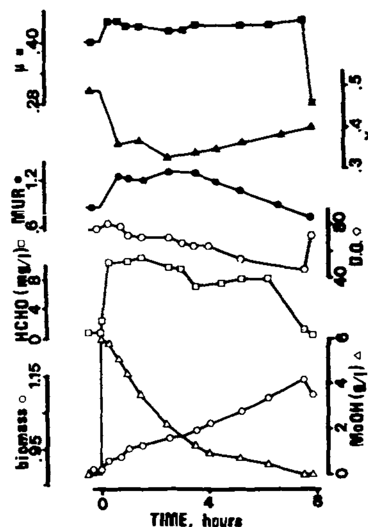

Figure 7. Response of a mixed-substrate continuous *M.* L3 culture to a methanol pulse (experiment #7: $D = 0.40\ h^{-1}$, pulse magnitude = 6.7 g/L). The units of the various quantities are given in the legend to Fig. 1.

(experiments #1–4). MeOH pulses were carried out by adding 3 mL of MeOH to the reactor. The dilution rates were 0.2, 0.32, 0.42, and 0.49 h^{-1}, respectively. A comparison of these four sets of experiments reveals some common features as well as some differences. Immediately after the MeOH pulse, the specific growth rate, the biomass yield, and the dissolved oxygen level dropped in all experiments. The magnitudes of the drop in the specific growth rate were about the same for all cases, i.e., 0.1 h^{-1} lower than before the MeOH pulse. The magnitude of the drop in the biomass yield was greater for the low dilution rate experiments, namely, when $D = 0.20$ and 0.32 h^{-1}. The DO profiles were similar for all runs in that they decreased until the residual MeOH reached its prepulse level; then the DO rose back rapidly.

The residual-MeOH and the MUR profiles were also similar, except for the 0.49 h^{-1} experiment. Because of the relatively high amounts of MeOH that disappeared between the time of MeOH pulsing and that of the first sample (ca. 1 min), we have checked if evaporation and ill mixing could be responsible for the MeOH loss, by carrying out blank experiments. While ill mixing was ruled out as a cause, we found that a substantial amount of MeOH, out of the normal 6.7 g/L intended for the pulse, was lost due to flash evaporation. Thus, the effective pulse concentration varied, apparently randomly, between 6.25 and 6.55 g/L. Further MeOH evaporation during the course of the experiment due to aeration and mixing was found to be negligible. Even after the correction for flash evaporation, large amounts of MeOH (0.3–1.05 g/L) disappeared immediately after the pulse injection in experiments #1–3, and the MUR descended to negative values within about 2–3 h. Negative MUR values were sustained for 0.5–1.5 h. These unusual phenomena can be explained if MeOH is transported into the cells by an active transport system.[22] This is because negative MUR values mean that MeOH is released by the cells, and this in turn means that the internal concentration of MeOH is higher than the external one. On the other hand, in experiment #4 a small amount, if any of MeOH disappeared, and there was no negative MUR observed. These unusual MeOH profiles and their relation to the transport system for MeOH will be discussed in more detail below. Because of the negative MUR values, unrealistically high (higher

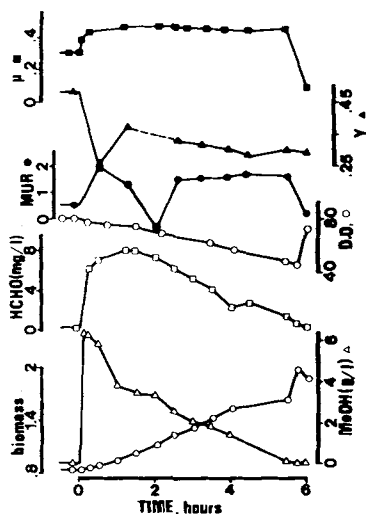

Figure 6. Response of a mixed-substrate continuous *M.* L3 culture to a methanol pulse (experiment #6: $D = 0.30\ h^{-1}$, pulse magnitude = 6.7 g/L). The units of the various quantities are given in the legend to Fig. 1.

than 0.7 [ref. 9]) and negative values of the apparent biomass yield (Y) are calculated (according to eq. [3]) right before, during, and right after the negative MUR-value period. These Y values are not plotted in Figures 1–3 and 6, but are indicated by the dashed lines of the Y profiles.

Residual CH_2O concentrations were measured in view of the important regulatory role CH_2O plays in these methylotrophs.[9–11] Experiment #1 displayed the highest residual-CH_2O concentration, while experiment #3 displayed the lowest one. It is possible that at lower growth rates, the capacity for CH_2O production, i.e., the MeOH dehydrogenase, is largely unsaturated compared with the capacity for CH_2O incorporation and oxidation.

To study the effect of the MeOH-pulse size on the dynamic behavior of growth, experiment #5 was carried out, and the results are presented in Figure 5. Experiment #5 was performed at a dilution rate of 0.31 h^{-1} with a 1 mL pulse, as compared with the 3 mL pulses used in experiments #1–4. In the experiment with the smaller MeOH pulse, the specific growth rate did not drop after the MeOH pulse, and there was no unusual MUR profile. The residual CH_2O concentration was less than 3 mg/L during the whole span of the experiment. However, the biomass yield dropped and fluctuated, in a fashion similar to that of experiments #1–4.

Pulse-transient experiments were also performed on mixed-substrate cultures (#6 and 7). 100 mg/L of CH_2O was used together with 790 mg/L MeOH as carbon substrates in the feed. At steady state, the residual levels of MeOH and CH_2O were below detection limits, which was typical of single-substrate cultures, as well. Furthermore, the biomass yield did not change when 100 mg/L of CH_2O was added to the feed tank of a steady-state culture grown on MeOH (data not shown). Therefore, the major effect of CH_2O in the feed was not to raise the residual CH_2O level, but rather to restructure the enzyme system responsible for its metabolism, in view of the increased CH_2O flux.

For experiments #6 and 7, the dilution rates were 0.3 and 0.4 h^{-1}, respectively. An MeOH pulse of 3 mL was used in both experiments. The results are presented in Figures 6 and 7. There were some major differences between the dynamic behavior of single- and mixed-substrate cultures. The specific growth rate profiles in experiments #6 and 7 displayed an upward trend right after the pulse perturbation and remained in the 0.42–0.48 h^{-1} range. Although the biomass yield dropped after the pulse perturbation, the drop was not as severe, and the biomass yield recovered to higher levels faster than the single-substrate experiments. The residual CH_2O level for experiments #6 and 7 was higher than in the single-substrate cases. To make these comparisons more clear, the profiles of the biomass yield and the specific growth rate for experiments #1, 3, 6, and 7 are replotted in Figures 8 and 9. The last two experiments seem to indicate that the mixed-substrate cultures adapt better to rapid additions of high MeOH amounts, that they display higher transient biomass yields, and that their growth is not quite as adversely affected by MeOH-pulse perturbations. Also, the high residual CH_2O level does not seem to have any negative effects on cellular metabolism. The MUR profile shows small negative values for a short time period in experiment #6 and no negative values in experiment #7. Finally, the amounts of MeOH disappearing immediately after the MeOH pulse were considerably smaller than those of experiments #1–3.

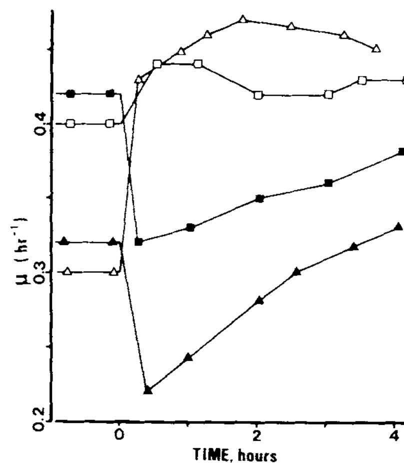

Figure 8. Comparison of the response to a methanol pulse between single- and mixed- substrate cultures: the specific growth rate profiles. Symbols: (△), mixed substrate, $D = 0.3\ h^{-1}$; (□), mixed substrate, $D = 0.4\ h^{-1}$; (▲), single substrate, $D = 0.33\ h^{-1}$; (■), single substrate, $D = 0.42\ h^{-1}$.

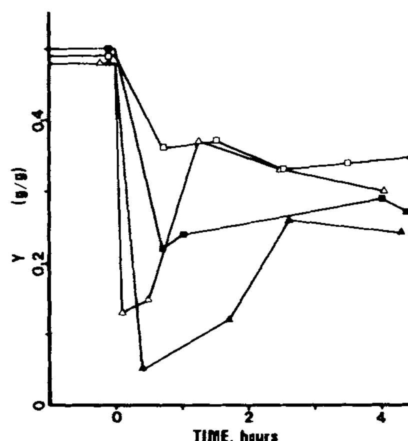

Figure 9. Comparison of the response to a methanol pulse between single- and mixed- substrate cultures: the biomass yield profiles. The unrealistic high or negative values of Y caused by the negative MUR values were rejected here. See Fig. 8 for symbols.

Table I. Measurement of transient biomass yield (Y_t) in continuous cultures of *M.* L3 upon pulsing a ^{14}C-labeled MeOH pulse of 6.7 g/L.

D h^{-1}	dpm × 10^{-4} Cells	CO_2	Broth	r_7/r_1	Steady State MOR[a]	Average transient MOR	Steady Y	State Y_t
0.2	1.89	3.31	239.6	0.364	0.53	0.68	0.38	0.22
0.32	14.5	11.84	1320	0.55	0.67	0.87	0.48	0.33

Y_t is the average transient true yield calculated at the end of a 15 min period after the pulse according to eq. (4). The ^{14}C recovery was almost 100% in both experiments.

[a] Specific rate of MeOH oxidation to CH_2O, g MeOH/h/g dry biomass.

True Transient Yields and MeOH Accumulation Profiles

The procedure described in Materials and Methods for calculating the MeOH accumulation gradient (S_i/S) requires the value of the true transient biomass yield (Y_t). For this yield we found significant changes with time following the MeOH pulse. By measuring the average Y_t over 15 and 60 min periods, we found that Y_t is at its lowest value immediately following the MeOH pulse and then increases with time to eventually attain its steady-state value (results not shown). Clearly, a precise determination of the S_i/S profile would require precise values of the instantaneous Y_t, which is not practically feasible. It would nevertheless be interesting to calculate S_i/S profiles based on very conservative constant (i.e., the lowest) Y_t values, in order to avoid grossly overestimating MeOH accumulation. Such S_i/S profiles are not meant as an accurate simulation of the true MeOH accumulation, but rather as a measure or indication of that accumulation. To this effect, we have measured the average true yields Y_t over a 15-min period following the MeOH pulse, in order to use these values as conservative estimates of the true instantaneous Y_t. Detailed results from two experiments for these Y_t measurments are presented in Table I where it is shown that the short-term transient yields drop by 30–40% from their steady-state values, while the MeOH oxidation to CH_2O rate increases by 30–55% as a result of the MeOH pulse.

The calculation of the S_i/S profiles is not only sensitive to the Y_t values used in the calculations, but also to (1) the value of the intracellular cell volume, (2) the initial amount of MeOH disappearing upon pulsing, as we have discussed, (3) the accuracy of the chromatographic MeOH analysis, and (4) the measurement of the biomass concentration. Regarding (1), we have used the maximal measured (see Materials and Methods) cell-volume value of 1.6 μL/mg. Regarding (2), as we have already discussed, the amount of MeOH disappearing upon pulsing is in itself a measure of the amount of MeOH accumulated by the cells. As we have discussed above, because of flash evaporation, the effective size of the intended 6.7 g/L MeOH pulse was found to vary randomly between 6.25 and 6.55 g/L. We thus use 6.25 g/L as the effective MeOH-pulse concentration in our calculations, in order to avoid overestimating the true amount of MeOH disappearing due to accumulation in the cells.

Figures 10 and 11 show the extracellular (S) MeOH profiles calculated for various assumed Y_t values, as well as the experimentally measured values, for the two experiments at D = 0.2 and 0.32 h^{-1} (Figs. 1 and 2, respectively). The corresponding S_i/S profiles are shown in Figures 12 and 13. Bars and/or points on the

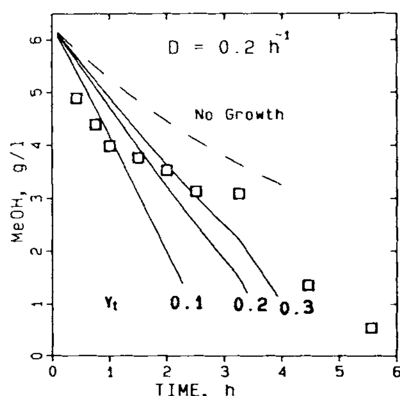

Figure 10. Extracellular methanol profiles calculated with various biomass-yield values, and experimental data (□) for the experiment of Fig. 1.

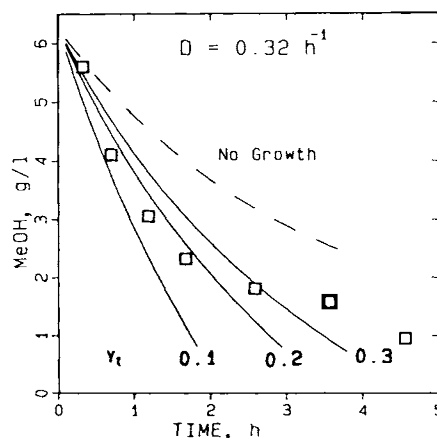

Figure 11. Extracellular methanol profiles calculated with various biomass-yield values, and experimental data (□) for the experiment of Fig. 2.

S_i/S profiles indicate the variations for a ±7% error in the chromatographic MeOH determination.

From Figures 10 and 12 we observe that for the $D = 0.2\ h^{-1}$ experiment the calculated MeOH accumulation is relatively insensitive to the value of Y_t used in the first 30–60 min after the pulse and that, unless the true Y_t is higher than 0.3 after 3 h, negative S_i/S values are calculated. There is an additional reason to suggest that the true yield Y_t is in fact higher than 0.2 after the first 30–60 min. In this experiment, as Figure 1 shows, MeOH is released by the cells between hours 2.0 and 2.8. This implies that, at least up to hour 2.8, S_i/S is positive. In contrast, the S_i/S profile calculated for $Y_t = 0.2$ shows negative values after approximately hour 1.6. The calculations of Figure 12 suggest MeOH concentration gradients up to ca. 200 and very high intracellular MeOH concentrations of 0.2 to above 0.6 g/mL.

For the $D = 0.32\ h^{-1}$ experiment (Figs. 11 and 13), the requirement of nonnegative S_i/S values suggests that the true Y_t is higher than 0.3 in the first 30 min or so, which is in line with the measured value of 0.33 (Table I). Since MeOH was found to be released by the cells between hours 3.0 and 3.8 (Fig. 2), it appears that the true Y_t is in fact higher than 0.3 after hour 2.5. Concentration gradients of up to 200 and S_i values of 0.2 to above 0.5 g/mL are suggested by the calculations of Figure 13.

We found that small, if any, MeOH-accumulation gradients are suggested by calculations similar to those of Figures 10–13 for those experiments of Figures 3–7 where small or no initial MeOH disappearance and no subsequent MeOH release were observed (calculations not shown).

DISCUSSION AND CONCLUSIONS

The disappearance of large amounts of MeOH (0.3–1.05 g/L) upon pulsing and the sustained negative MUR values are novel phenomena not only in methylotrophy, but also in microbial physiology and dynamics in general. Because of the novelty of these phenomena, and because of the practical and fundamental importance of their consequences, as we subsequently discuss, we carefully scrutinized the possibility of an artifact when we observed them for the first time. The experimental methodology and measurements were carefully analyzed, and the effect of experimental errors on the observation of these phenomena was evaluated. The possibility of an artifact was thus rejected. These phenomena were observed in a number of other experiments (not shown) which display the general characteristics and trends of experiments #1–5.

Since the disappearance of large amounts of MeOH upon pulsing and the negative MUR values were observed in experiments #1–3 and 6, but not in experiments #4, 5, and 7, these two phenomena are not a general nonspecific property of the *M.* L3 biomass. They are, rather, a unique property of particular physiological states (compare experiments #1–3 and 6 to experiments #4 and 7, in all of which a 6.7 g/L MeOH pulse was used), and particular experimental conditions (compare experiment #2 to experiment #5 where the physiological state of the cells was virtually the same, but the MeOH pulse was significantly different). In other words, the disappearance of large amounts MeOH immediately following the pulse, and the subsequent release of MeOH by the cells into the medium, cannot be due to a nonspecific binding of MeOH on

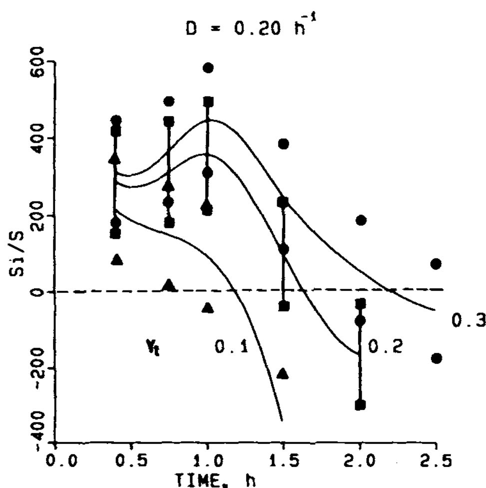

Figure 12. Methanol accumulation profiles calculated with various biomass-yield values for the experiment of Fig. 1. Points around the S_i/S profiles indicate variations for a ±7% error in the chromatographic MeOH analysis, as follows: (▲) for $Y_t = 0.1$; (■) for $Y_t = 0.2$; and (●) for $Y_t = 0.3$. For clarity, these points have been connected by bars only for the $Y_t = 0.2$ case.

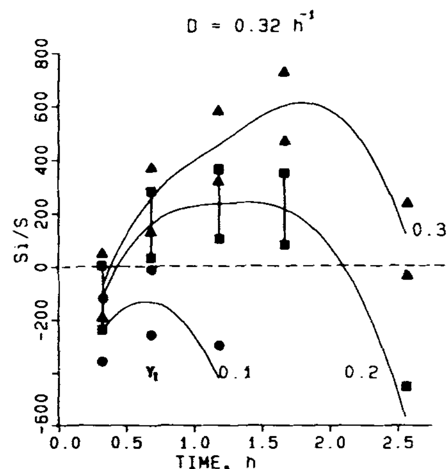

Figure 13. Methanol accumulation profiles calculated with various biomass-yield values for the experiment of Fig. 2. Points around the S_i/S profiles indicate variations for a ±7% error in the chromatographic MeOH analysis, as follows: (●) for $Y_t = 0.1$; (■) for $Y_t = 0.2$; and (▲) for $Y_t = 0.3$. Points are connected by bars only for the $Y_t = 0.2$ case.

the biomass, and are thus the result of an MeOH accumulation in the cells. Therefore, the negative values in the MUR profiles of the MeOH-pulse transients suggest the existence of an active transport system. MeOH is a small and uncharged molecule that has very high permeability through lipid-bilayer membranes[23] and may thus cross the cell membrane readily by simple diffusion. A common argument against the existence of an active transport system for MeOH is the fact that, the MeOH dehydrogenase being located on the outer side of the cell membrane (periplasmic space), MeOH does not need to be transported in the cell.[24–26] However, methylamine dehydrogenase is also located in the periplasmic space of methylotrophs,[27] and yet there is evidence that methylamine is actively transported by the cells.[23] Because of the negative MUR values observed during these transients and because of the apparent MeOH accumulation in the cells, values of the biomass yield Y calculated according to eq. (3) can be unrealistically high or negative, as we have already mentioned. Thus, transient values of the apparent Y calculated according to eq. (3) do not represent the true efficiency of biomass formation and should not be used for process monitoring and control since they may result in seriously erroneous decisions. Unfortunately, there exists no easy alternative method for calculating Y on line, as the method for measuring the true transient yields Y_t (see Materials and Methods) is both tedious and an off-line one. Unless the Y and MeOH concentration values are carefully screened, process monitoring and control must therefore rely on measurable process parameters other than Y and the reactor MeOH concentration under conditions of such severe reactor transients.

The drop in the specific growth rate observed upon methanol addition was checked in various ways to make sure it was not an artifact. The possibilities that high MeOH concentrations may kill the cells, or that the addition of MeOH may alter the light-scattering property of the cell suspension, were carefully examined and eliminated.[28] Viable cell counts and control dry weight determinations were used in these examinations.[28] In fact, the observed higher specific growth rates for the mixed-substrate experiments were enough to prove that the drop was not an artifact, but rather a property of the cell metabolism.

From the calculated values of methanol gradients across the cell membrane, it appears that in a number of experiments, methanol is accumulated inside the cells by up to 200-fold. However, the errors involved in the computation grow cumulatively with time and become very large when the residual methanol is low (see eq. [7]). Therefore, only the results calculated from the first 2 or 3 h of the transient experiments are meaningful. We cannot overstress that the S_i/S-profile calculations are only approximate calculations for suggestive purposes and subject to substantial error due to their extreme sensitivity to a number of parameters, as we have already discussed. While the calculations in Figures 10–13 have accounted for the sensitivity to the cell volume, the true yields Y_t, and the methanol concentration, by employing conservative values for those parameters, these calculations may still often predict unrealistically high accumulation gradients and intracellular concentrations for MeOH, due to error in the measurement of the biomass concentration. Indeed, while a 200- or even 300-fold accumulation gradient is not unrealistic *per se*, intracellular MeOH concentrations above 0.2–0.3 g/mL are at least suspect, if not totally unrealistic, because MeOH is a very potent solvent at such high concentrations. Based on an experimentally suggested[16] value of 0.1 mol ATP required for the transport of 1 mol MeOH, we have carried out conservative calculations, which, nevertheless, suggested that the biosynthetic energy necessary to transport such substantial MeOH amounts in the cells is likely available (results not shown).

Due to the high permeability of methanol through biological membranes, and the difficulty in finding methanol metabolic mutants in obligate methylotrophs, conventional studies on the methanol transport mechanism are quite difficult.[16] The procedures described in this study (experimental and computational) may lead to a new approach to the problem of MeOH transport. However, the current experimental procedures would need some modifications in order to produce more accurate and conclusive data. Such modification should include shorter sampling intervals for more accurate determinations of the MeOH and biomass concentrations and more measurements of the true transient Y_t values over more time intervals.

A possible scenario that seems to be suggested from the results of the MeOH-pulse transient experiments and the calculations of MeOH accumulation profiles is as follows. While MeOH is probably actively transported in all experiments at steady state, the MeOH pulse results in dramatically different accumulation profiles in different experiments, either because the transport system is saturated or because it is repressed at higher dilution rates and in mixed-substrate cultures. If the high intracellular MeOH concentrations suggested by the calculations of Figures 10 and 11 are realistic, the negative effect of MeOH on growth may be the result of nonspecific solvent action rather than specific growth inhibition. It is conceivable that such damaging accumulations of MeOH may be caused also by smaller MeOH fluctuations (such as these resulting from bioreactor ill mixing or nonsevere bioreactor transients[3,5–7,12]. This is because active transport systems follow a saturation-type behavior, whereby small changes in the extracellular substrate concentration result in significantly higher transport rates.

The better adaptation of mixed-substrate cultures to methanol pulses (Figs. 6 and 7) seems to arise from

the use of CH_2O as a cosubstrate. Since the residual CH_2O level is approximately the same for single- and mixed-substrate cultures, this better adaptation is most unlikely to be caused by the CH_2O concentration *per se*. The higher CH_2O flux to the cells in the mixed-substrate cultures relative to the MUR may play a role in this better adaptation. Significant changes of the metabolic pattern have been observed in continuous and batch cultures of strain L3 with CH_2O as a co-substrate.[19,20] Those changes may also make the cells less sensitive to MeOH pulses.

In view of the above and in view of the fact that small amounts of CH_2O as a MeOH cosubstrate do not adversely affect either the biomass yields or the cell growth, CH_2O in the feed may be an effective means to provide more stable biomass yields and growth rates in reactors with imperfect mixing. CH_2O in the feed may also provide better protection against accidentally induced transients that may result in MeOH accumulation and growth inhibition.

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NOMENCLATURE

D	dilution rate (h^{-1})
DO	dissolved oxygen level (percent saturation)
MUR	specific methanol uptake rate (g or mol MeOH/h/g dry biomass)
OD_{600}	optical density at 600 nm
r_1	rate of MeOH oxidation to CH_2O in eq. (4)
r_7	rate of MeOH incorporation into biomass in eq. (4)
S	residual methanol concentration (g/L)
S_f	feed methanol concentration (g/L)
S_i	intracellular methanol concentration (g/L)
S_T	total methanol concentration (g/L) based on reactor volume, in eq. (5)
v	ratio of intracellular to extracellular volume in the reactor
X	biomass concentration (g dry biomass/L)
Y	biomass yield (g dry biomass/g MeOH)
Y_t	true transient Y
μ	specific growth rate (h^{-1})

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