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GROWTH DYNAMICS AND CARBON-SUBSTRATE OXIDATION AND INCORPORATION PATTERNS OF METHYLOMONAS L3

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GROWTH DYNAMICS AND CARBON-SUBSTRATE OXIDATION AND INCORPORATION PATTERNS OF METHYLOMONAS L3

by

I-MING CHU

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

DOCTOR OF PHILOSOPHY

APPROVED, THESIS COMMITTEE

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May, 1985
ABSTRACT


The Ribulose Monophosphate-type (RuMP), obligate methanol-utilizing bacterium Methylomonas L3 has been used in the study of methylo trophic metabolism and fermentation dynamics.

The dynamic behavior of M. L3 in continuous cultures was studied in order to understand the regulatory mechanisms of cell growth under various transient situations. Transients generated by dilution-rate shifts and methanol-pulse additions were employed. The methanol-uptake rate (MUR) profiles of most experiments displayed the unusual phenomenon of showing negative MUR values for a time period following the methanol pulse. This phenomenon suggested the existence of a methanol active transport system. In single-substrate cultures, the specific growth rate decreased immediately after the methanol addition. In mixed-substrate cultures (with formaldehyde as a methanol co-substrate), the specific growth rate increased after the methanol addition. The biomass yield decreased after the methanol addition in all experiments; however, the drop was more severe in the single-substrate experiments. In dilution-rate shift experiments, overshoot and damped oscillatory behavior of
the specific growth rate was observed.

Radioactive labels were employed to elucidate the carbon substrate oxidation and incorporation patterns of strain L3. A thorough mathematical derivation of the proposed procedure was presented. $^{14}$C-labeled methanol, formate and $1-^{14}$C-glucose were used as tracers in a series of experiments with batch-grown, exponential-phase cells with different initial levels of substrate(s). From the distribution of the radioactivities in biomass and carbon dioxide, the rates of the carbon substrate oxidation reactions, the rates of the general decarboxylation and carboxylation reactions, as well as the rate of the direct biomass incorporation were obtained. The results showed that the two known oxidation pathways were operating in vivo, and that the extent of utilization of each pathway depended on the initial substrate(s) concentrations as well as on the state of the inoculum.

An unstructured growth model, incorporating the basic Monod formulation with substrate-inhibition and the maintenance energy concept, was constructed based on steady-state data. The ability of this model to predict the transient cell behavior was tested by comparison to the transient data obtained. The model performed better in predicting transient behavior due to dilution-rate shifts. For methanol-pulse transients, the model performed better at higher dilution rates.
TO MY PARENTS, TSUEY AND BENNY
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NOMENCLATURE

A  Q ratio for $^{14}$C-methanol; eq. 3-5.
B  Q ratio for ideally labeled $^{14}$C-glucose; eq. 3-6.
B' quantity defined by eq. 3-21.
C  Q ratio for $^{14}$C-formate; eq. 3-7.
D  dilution rate (l/hr).
D.O. dissolved oxygen level (% saturation).
E.C. energy charge, as defined in eq. 2-5.
E4P erythrose-4-phosphate.
FBP fructose biphosphate.
FCR formaldehyde consumption rate; eq. 2-4.
FDDH formaldehyde dehydrogenase.
FDH formate dehydrogenase.
F6P fructose-6-phosphate.
FUR formaldehyde uptake rate; eq. 2-4.
G3P glyceraldehyde-3-phosphate.
G6P glucose-6-phosphate.
(HCHO)$_0$ initial formaldehyde concentration.
Hu6P hexulose-6-phosphate.
KDPG 2-keto-3-deoxy-6-phosphogluconate.
m maintenance coefficient; eq.4-3.
MeOH methanol.
(MeOH)$_0$ initial methanol concentration (g/l).
MDH methanol dehydrogenase.
MUR methanol uptake rate (g MeOH/hr-g dry wt).
6PG  6-phosphogluconate.
O.D.  optical density.
PYR  pyruvate.
Q  ratio of radioactivity in biomass over the radioactivity in biomass and CO₂.
ri  rate i defined in Fig. 3.1.
R5P  ribose-5-phosphate.
Ru5P  ribulose-5-phosphate.
RuMP  ribulose monophosphate.
S  residual methanol concentration (g/l).
SCP  single-cell protein.
SDP  sedoheptulose diphosphatase.
SDS  sodium dodecyl sulfate.
Sf  feed methanol concentration (g/l).
Si  intracellular methanol concentration (g/l).
ST  overall methanol concentration (g/l).
Su7P  sedoheptulose-7-phosphate.
TA  transaldolase.
v  ratio of the intracellular volume over the extracellular volume in the bioreactor.
X  moles decarboxylated by general decarboxylation per 4 C-moles directly incorporated in biomass.
X  biomass concentration (g dry wt/l).
Xu5P  xylulose-5-phosphate.
Y  biomass yield (g dry wt/g MeOH).
Y  percentage of CO₂ produced by general decarboxylation which originates from the position 1 of 6PG.
Ymax  Maximum biomass yield; eq. 4-3.
\( \alpha \) fraction of carbon occupying the C-1 position on 6PG.

\( \beta \) Q ratio for 1-\(^{14}\)C-glucose; eq. 3-20.

\( \beta' \) quantity defined in eq. 3-22.

\( \gamma \) fraction of methanol oxidized to CO\(_2\) by cyclic scheme.

\( \delta_i \) fraction of carbon at the i-th position of the C\(_3\) skeleton lost to CO\(_2\) by general decarboxylation.

\( \varepsilon \) sum of \( \delta_1, \delta_2 \) and \( \delta_3 \).

\( \mu \) specific growth rate (1/hr).
Chapter 1

OVERVIEW OF METHYLOTROPHIC FERMENTATIONS

Fermentation processes based on methylochromic microorganisms have attracted extensive research and development efforts in the last twenty-five years. One reason for this enthusiasm for methylochromic fermentations is the low price and availability of the carbon substrates for these processes, methane and methanol. Methane is derived chiefly from natural gas, and may be produced from coal or from anaerobic fermentations of domestic and agricultural wastes. Methanol can be produced from methane, coal, wood and naphtha. Both substrates are produced in large quantities [1,2,3]. The diversity of the sources and the production scale of methane and methanol ensure the long term stability in supply and price of these one-carbon (C1) compounds, and make them suitable substrates for large-scale industrial fermentations. Beside the lower substrate cost, methylochromic cultures are less vulnerable to contamination than the traditional carbohydrate-based cultures, and thus, less stringent requirements for aseptic operation are necessary. Currently, methylochromic fermentations have found a number of industrial applications, including single-
cell protein (SCP) production, the production of enzymes (mainly dehydrogenases), coenzymes, amino acids, biopolymers and nucleotides [1]. In the future, recombinant DNA technology applied to methylotrophs may potentially lead to the production of a variety of bioproducts from C1 compounds [1]. Methylotrophs play an important role in the new field of biotechnology.

Methylotrophic microorganisms can be found in fungi, yeasts and bacteria, though yeasts and fungi can utilize only methanol but not methane [1,2]. Several strains of methanol-utilizing yeasts have been isolated, mainly for their potential for SCP production. Their biochemistry and physiology have been extensively reviewed [4].

Methylotrophic bacteria can be divided, according to their ability to utilize methane, into two main groups: those which can utilize methane and those which that cannot. Methane-utilizing bacteria are characterized by their complex inner membrane structure and the enzyme responsible for the oxidation of methane, methane monooxygenase [1]. Most of them are obligate methylotrophs, and can grow on methanol, as well. Their classification and biochemistry have been amply reviewed elsewhere [5,6]. It is sufficient to note here that there are two major carbon-assimilatory schemes used by these bacteria: the Ribulose Monophosphate (RuMP) cycle (type I) and the Serine Pathway (type II), although some species seem to have both [5].
The obligate methanol-utilizing bacteria are those that can grow on methanol but not on methane. These bacteria, including strain L3 employed in this research, use the 2-keto, 3-deoxy, 6-phosphogluconate aldolase/transaldolase (KDPG/TA) variant of the RuMP cycle (Fig. 1.1) for carbon assimilation and two distinct pathways (Fig. 1.2) for carbon dissimilation [1], which will be discussed in more detail in Chapter 3. Their inability to utilize carbon substrates other than methanol is believed to be a consequence of an incomplete Krebs, i.e. the tricarboxylic acid (TCA) cycle [1]. Facultative methanol-utilizing bacteria, on the other hand, are a much more diverse group of organisms. They can be found in many different genera of bacteria, e.g. Pseudomonas, Hyphomicrobium, and Arthrobacter [1,2]. Most of the facultative bacteria possess enzymes for the RuMP cycle fructose bisphosphate aldolase/sedoheptulose diphosphatase (FBP/SDP) variant or the serine pathway and have a complete Krebs cycle. The diversity of their metabolic activities can be applied to the production of many primary metabolites [1].

In the case of SCP production, the selection of the organism for the process is determined by many factors, including the reactor productivity, fixed and operational costs, availability of the raw materials and market conditions. Thus, various yeasts and bacterial strains have been selected according to different local situations. Yeasts can be marketed as human food since they have been
Figure 1.1. The ribulose monophosphate cycle, KDPG/TA variant [1].
Figure 1.2. The two substrate-oxidation schemes [1]. <a>: linear and <b> cyclic.
accepted in human diet for a long time. Yeasts also have many advantages with respect to process engineering of SCP production, such as easy harvesting due to their relatively large cell size, and their ability to grow at elevated temperatures and low pH values. However, SCP from bacteria has also been very attractive, because of higher protein content, higher biomass yields and because bacteria do not require any growth factors (e.g. vitamins) [1,2]. The problem of harvesting, due to the relatively smaller size of bacterial cells, can be overcome by flocculation techniques. The major drawback of bacterial SCP is the lower psychological acceptance for human consumption. Thus, bacterial SCP has been so far primarily marketed as an animal feed [2,5].

One of the most important factors which influence the economics of a SCP process is the biomass yield. At higher biomass yields, not only would the consumption of the substrates be more efficient, but oxygen-transfer and bioreactor-cooling requirements, two very expensive unit operations, would be also reduced [11]. This is because of the inverse relationships that exist between the oxygen requirement or the heat of fermentation and the biomass yield [11]. Obligate methanol-utilizing bacteria that use the RuMP cycle for carbon assimilation have the highest biomass yields among all methylo trophs [1,10-12] and are thus most desirable for industrial SCP processes [1,3,7,11].
There are two major topics that will be addressed in this thesis. The first one is the dynamic behavior of the cultures. This problem is very important for large-scale industrial processes, because in large fermentors the cells experience various disturbances caused by imperfect mixing and imperfect control [5,8,9], and, as a result, exhibit lower biomass yields than the yields observed in the laboratory. An understanding of the physiological events during those transient states would be very important to improve the performance of the cultures and the control strategies under such conditions. A series of experiments together with model simulations was employed to study this problem. Mixed-substrate cultures were also studied for the effects of the co-substrate, formaldehyde, on the dynamic behavior. The experimental part of this topic will be described in Chapter 2, and the simulation studies in Chapter 4.

The second topic that will be addressed here is the metabolic patterns of a RuMP-type obligate methanol-utilizing strain under different growth conditions. The theoretical biomass yields of methylotrophic bacteria have been studied by a number of researchers, using the energy-limited concept [10,11,12]. A major uncertainty in these predictions arises from the fact that it was not known which of the two possible substrate oxidation pathways was used in vivo by the cells, since one, the cyclic oxidation scheme, is energetically superior to the other one. Also, the
extent of CO₂ fixation by the cells was not known, and some had claimed [13] that CO₂ fixation would render the cells reduction-energy limited, which would imply that the biomass yield could not be calculated from energy-limited concepts. To clarify all such uncertainties, it is necessary to measure in vivo the metabolic patterns of the cells [9]. A radioactive-labeled tracer technique was developed and applied to batch cultures, as will be presented in Chapter 3.
Chapter 2

TRANSIENT BEHAVIOR OF CONTINUOUS CULTURES OF METHYLOMONAS L3

2.1 INTRODUCTION

The continuous culture technique has been widely used in the study of microbial physiology in the last 30 years [13,14,15], and has been also used in a number of industrial processes [16]. The continuous culture of microorganisms provides a time invariant system which allows one to manipulate various variables in a well-defined and an environmentally and physiologically reproducible system. Thus, earlier interest in the continuous culture of cells had focused on applications of the steady states created by this technique. Industrial applications have also concentrated on the steady-state continuous growth of cells for optimal reactor productivity. However, the importance of the transient behavior of cell growth in the study of microbial physiology and in industrial applications is recently gaining considerable recognition and interest. This is due not only to the fact that transient growth occurs much more often in the laboratory and in nature than steady state growth does, but also that transient phenomena can provide considerable information on the physiology and
metabolic regulation of microorganisms [17,18,19]. Furthermore, understanding of the dynamic behavior is essential to the development and implementation of advanced control strategies as well as in the better scale-up and operation of fermentation processes [20]. In view of the development of novel bioreactors (see e.g. [21]) or novel non-steady-state operation policies (such as the fed-batch operation) in recent years, information on the dynamic behavior is indeed of both practical and fundamental importance.

Transient phenomena in microbial growth are not new to scientists and engineers. In fact, the commonly employed microbial batch culture is a good example of a transient type of growth. Besides, transient phenomena are commonly observed in the natural environment as well as in industrial microbial processes. The difficulties of attaining reproducible and well-defined systems, however, have prevented a deeper understanding and application of dynamic phenomena in physiological and industrial processes. Nevertheless, the aforementioned difficulties can be overcome by carefully designed experiments, whereby artificial perturbations are introduced in well-defined steady state cultures, and the transient behavior induced by these perturbations is recorded and analyzed. Thus, the steady state and the transient growth can be carefully defined and correlated and are, most of all, reproducible. It is not surprising then that the dynamic behavior of
continuous cultures has been the focus of considerable research effort, and has also been extensively reviewed by a number of authors [22,23,24].

The usefulness of the transient behavior as a tool in the understanding of regulatory mechanisms of microbial growth and metabolism has been long recognized. The dynamic behavior of growth after shifts in nutrient concentrations and the dilution rate shift has been employed to elucidate how microorganisms regulate their protein-synthesis machinery according to environmental changes [17,19]. Many new concepts and discoveries have evolved from those studies. Temperature, pH and oxygen tension have been also employed as perturbed variables [25,26] 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 [18,27]. Pulse feeding of small reactors can also serve as simulators of large fermentors with imperfect mixing, whereby cells experience fluctuations in nutrient concentrations [18,25,28]. Studies on the transient behavior of growth can also help build better process models for control purposes [29], be they mechanistic models, or simple empirical models with various time constants, as will be discussed in Chapter 4.

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, there is a risk of process instability that can lead to washout if the reactor methanol level is increased as a result of some disturbance in a process parameter. The risk is higher if the feed methanol 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 [8,30,31]. Also, because of incomplete 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 methanol concentration [8]. This problem has been studied using pulse-feeding transients in small scale reactors [18,25,28]. Currently, this problem is solved mainly by more expensive reactor design for improved mixing characteristics of the fermentor (such as employing multiple feeding ports) [27,32]. A better understanding of what happens when the cells experience fluctuations in the methanol 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. Transient phenomena can also be helpful in revealing the kinetics or regulatory mechanisms of metabolism, which are of fundamental importance for understanding the microbial physiology and bioenergetics of methylotrophic growth. Dynamic data can also provide a basis for model
discrimination and elucidate the limitations of various models for methylotrophic growth.

Experiments of steady-state perturbations by methanol pulses and dilution-rate shifts were performed in order to study the dynamic response of the biological system. In particular, pulse perturbation experiments were expected to provide information on the methanol-transport mechanism, and the dynamic responses of the biomass yield, the specific growth rate, as well as the energy charge to methanol fluctuations. Dilution-rate shift experiments were performed to elucidate the system's biological response to milder disturbances, the so called slow transients.

2.2 MATERIALS AND METHODS

2.2.1 Organism

The methanol utilizing bacterium *Methylo monas* L3, originally isolated and described by Hirt et al. [33], was obtained by reconstitution from freeze-dried cultures. The culture was maintained on agar plates and subcultured weekly. A detailed description of strain L3 is presented in Appendix A.

2.2.2 Media and Culture Conditions

A basal salts medium plus carbon substrates, methanol or formaldehyde, was used to grow strain L3. For batch cultures, the medium contained the following ingredients in 1 liter distilled water: 0.2 g MgSO$_4$·7H$_2$O, 15 mg CaCl$_2$, 1 mg
FeSO\textsubscript{4}.7H\textsubscript{2}O, 40 mg KCl, 10 µg MnSO\textsubscript{4}.H\textsubscript{2}O, 10 µg Na\textsubscript{2}MoO\textsubscript{4}.2H\textsubscript{2}O, 70 µg ZnSO\textsubscript{4}.7H\textsubscript{2}O, 5 µg CuSO\textsubscript{4}.5H\textsubscript{2}O, 10 µg CoCl\textsubscript{2}.6H\textsubscript{2}O, 10 µg H\textsubscript{3}BO\textsubscript{3}, 0.5 g NH\textsubscript{4}Cl and 66 ml of pH 6.9, 0.6M phosphate buffer. The phosphate buffer was prepared by mixing equimolar amounts of Na\textsubscript{2}HPO\textsubscript{4} and KH\textsubscript{2}PO\textsubscript{4} in distilled water. Agar plates were prepared by adding 16 g of agar in 1 liter of basal salts medium. Methanol was added in the steam-sterilized medium. For continuous cultures, the basal medium was somewhat modified in that 4.5 mg FeSO\textsubscript{4}.7H\textsubscript{2}O was used in 1 liter medium, and the nitrogen source was changed to 1.2 g/l (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}. The phosphate buffer was also replaced by 0.66g/l NaH\textsubscript{2}PO\textsubscript{4}.H\textsubscript{2}O.

Batch cultures of M. L3 were established on the basal-salts medium plus 0.4% v/v methanol 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-liter fermentor with a working volume of 350 ml. Agitation was set at 450 rpm, aeration was ca. 0.2 lpm 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 (D.O.) was measured by a galvanic-type probe with a D.O. monitor (New Brunswick Scientific, D505). 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. Methanol (final concentration at 0.8 mg/l) or formaldehyde (100 mg/l) were 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 methanol and formaldehyde as substrates. The fermentor was first run in batch mode until the methanol was exhausted, as indicated by the rising dissolved-oxygen level; then, the medium feed pump was turned on and adjusted to the desired dilution rate. The reactor was assumed to have reached a steady state after at least three doubling times had passed without any observable fluctuations in the cell density, the dissolved-oxygen level and the residual methanol concentration. To avoid the build-up of wall growth, continuous operation was maintained for at most 3 weeks.

2.2.3 Transient Experiments

Pulse Transients. A methanol-pulse transient was induced by injecting a certain amount (usually 3.0 ml) of methanol 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 analyzed for methanol and formaldehyde levels. The sample size was about 4 ml and the sampling interval was larger than 15 minutes to avoid the possibility of altering the transient behavior by excessive sampling.

Dilution-Rate-Shift Transients. After the establishment of a steady-state in the chemostat, the dilution rate was shifted either up or down by changing the output rate of the medium feed pump. The sampling procedure was the same as in the pulse-transient case.

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-methanol concentration profiles were obtained from smooth-curve functions (polynomials) fitted by the least-squares regression method 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.

2.2.4 Energy-Charge Measurement

The ATP, ADP and AMP contents of M. L3 cells under a methanol-pulse transient were measured to determine the energy-charge profile of the cells. The procedures for the ATP, ADP and AMP determinations were similar to the ones
described earlier [34,35]. Samples were taken from the reactor before and after the pulse-addition of methanol at about 5 minute intervals with a sample size of about 1.5 ml. 1.0 ml of the sample was added to 1.0 ml of ice-cooled, 12% w/v perchloric acid in a 10x75 mm test tube, and was immediately mixed thoroughly. The time needed for this step, from sampling to mixing, was about 6–8 seconds. After incubation at 4 °C for 20 minutes, the acidified sample was spinned in a centrifuge (Sorvall RC5B) at 10,000×g for 8 minutes. The supernatant was carefully drained into another ice-cooled 10x75 mm test tube. To this test tube, 0.8 ml of 2N KOH and 0.5 ml of 1N KHCO₃ were added to bring the pH up to 7.4 ± 0.2. The precipitate, mainly KClO₄, was removed by centrifugation. The resultant supernatant, the, so called, cell extract, was kept on ice and analyzed for ATP within one hour.

To quantify ADP and AMP, it is necessary to convert ADP and AMP to ATP with specific phosphorylation enzymes so that the amounts of ADP and AMP can be determined by difference. To convert ADP to ATP, 0.5 ml of cell extract was added to a 10x75 mm test tube containing 0.5 ml of glycylglycine buffer. The buffer was made up of 50 mM of glycylglycine and 5 mM MgSO₄ at pH 7.4. Then, 0.1 mg of phosphoenolpyruvate (PEP) and 0.01 mg of pyruvate kinase were added to the test tube. After mixing and incubation at room temperature for 30 minutes, the content in the test tube was analyzed for ATP within 5 minutes. To convert both
AMP and ADP into ATP, 0.5 ml of cell extract was added to a 10x75 mm test tube containing 0.5 ml of the glycylglycine-MgSO₄ buffer described before. Then, 0.2 mg of PEP, 0.01 mg of pyruvate kinase and 0.01 mg of myokinase were added to the test tube. The sample was mixed and incubated at 37 °C for 1 hour and was analyzed for ATP immediately. Known quantities (usually 0.1 μmol) of ADP and AMP were also used in place of the cell extract for standardization purposes.

ATP was analyzed by the luminescence method in a whole-blood aggregometer equipped with a luminescence channel (Chrono-Log Co.). A small stirrer was placed inside the glass vial to provide constant mixing, and the temperature was controlled at 37 °C. Attenuation was set to 0.05 for best results. The luminescence signal was plotted on a recorder. Before any run began, 0.9 ml of glycylglycine-MgSO₄ buffer was added to the glass vial, which was then placed in the 37 °C holders of the aggregometer. After thermal equilibrium was attained, the vial was put into the measuring chamber of the instrument and the baseline output was observed. 0.02 ml of 40 mg/ml luciferase/luciferin was added to the vial and the baseline output was again checked. After that, 0.01 ml of the sample (cell extract or the samples prepared for ADP and AMP analysis) was added. The output signal was observed and recorded for 1 minute to allow the establishment of a traceable pattern, and then 10 μl of 1 μM ATP were added as internal standard.
2.2.5 Viable Counts

Culture fluid was diluted by sterile basal salts solution to appropriate concentrations. 0.1 ml of these diluted samples were spread, with a glass rod, on agar plates containing 2% v/v methanol [36]. Plates were incubated at 30 °C for 24 hours and the colonies formed were then counted. Colony counts were between 30 to 300 per plate for best results.

2.2.6 Chemicals

Inorganic salts and acids were obtained from Baker Chemical Co., Phillipsburg, NJ or Mallinckrodt, Inc., Paris, KY. Formaldehyde was prepared by heating at 110 °C for 48 hours a paraformaldehyde water solution. Paraformaldehyde was obtained from Aldrich Chemical Co., Milwaukee, WI. Methanol was obtained from EM Science, Gibbstown, NJ and Baker Chemical Co. Chromotropic acid, ATP (dipotassium salts), ADP (grade V), AMP (type III), pyruvate kinase (type III), myokinase (grade III), PEP, glycyglycine and luciferase-luciferin were obtained from Sigma Chemical Co. St. Louis, MO. Bacto-agar was purchased from Difco Laboratories, Detroit, Mich.

2.2.7 Analyses

Methanol was determined by gas chromatography. An Antek-300 G.C. equipped with a flame ionization detector was used. The 6-foot column used was packed with Porapak-Q,
80-100 mesh (Water Associates). Helium (Airco) was used as carrier gas and its flow rate was set at 30 ml/min. The column temperature was 120 °C, while the injector and detector temperatures were set at 180 °C and 195 °C, respectively. 1 μl syringe (Hamilton 7001) was used for sample injections. A recorder equipped with an integrator channel was used for the peak-area determinations. The limit of detection was 5 mg/l. Formaldehyde was determined by the chromotropic-acid method [33], 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 pre-washed cells of known turbidity in an pre-weighed aluminum dish in an oven at 105 °C for 40 hours and then measuring the total weight [36]. 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 cm long light path through the spectrophotometer cuvette.

2.3 RESULTS

2.3.1 Dilution Rate Shift Experiments

A dilution-rate shift-up experiment (run #1) was conducted by shifting the dilution rate from 0.32 l/hr to 0.48 l/hr. The shift-down experiment (run #2) was conducted by shifting the dilution rate from 0.32 l/hr to 0.15 l/hr. The profiles of turbidity, residual methanol, formaldehyde,
dissolved-oxygen level, specific growth rate and biomass yield are presented in Figs. 2.1 and 2.2. In the shift-up experiment, the residual methanol level increased gradually to 60 mg/l. The dissolved oxygen level behaved as expected in that it first went down because of the higher residual methanol concentrations that activated oxygen consumption, but after two hours it went back up due to the lower biomass concentration. The formaldehyde concentration went from an undetectable (less than 0.5 mg/l) level to about 1.0 mg/l immediately after the dilution rate shift and remained there. The profiles of turbidity and specific growth rate deserve more attention. The shift from 0.32 l/hr to 0.48 l/hr in dilution rate crosses through the region of the maximal biomass yield, which occurs approximately at a 0.43 l/hr dilution rate. The biomass yield is a convex function of the dilution rate (D), increasing with D until it reaches a maximum and then decreases as D approaches its washout value. The residual methanol and formaldehyde concentrations also increase from almost undetectable levels to relatively high values in the region between the maximal biomass yield and washout [33]. Therefore the specific growth rate is affected by both the biomass yield and the residual methanol concentration. The specific growth rate jumped to 0.46 l/hr after the shift because of the higher residual methanol level, and it increased gradually to 0.5 l/hr in one and half hour. Then the specific growth rate decreased due perhaps to the effect of the biomass yield.
Figure 2.1. Response of a single-substrate culture to a dilution-rate shift-up. The dilution rate was shifted from 0.32 to 0.38 l/hr. Biomass is expressed in O.D. 600 nm unit, MUR (methanol uptake rate) in g MeOH/hr-g dry wt, Y (biomass yield) in g dry wt/g MeOH.
Figure 2.2. Response of a single-substrate continuous culture to a dilution-rate shift-down. The dilution rate was shifted from 0.32 to 0.15 l/hr. Biomass is expressed in O.D. 600 nm unit, MUR (methanol uptake rate) in g MeOH/hr-g dry wt, Y (biomass yield) in g dry wt/g MeOH.
As mentioned before, the biomass yield is a convex function of the dilution rate at steady state, and, at the 0.48 - 0.50 l/hr range, the yield is lower than that at the 0.32 l/hr dilution rate. Thus, the culture could not maintain the biomass concentration (which is approximately proportional to the biomass yield) at its previous higher level, and both the biomass concentration and the specific growth rate had to decrease. As the biomass concentration decreased, the residual methanol level increased slowly due to the lower methanol consumption. Finally, a balance between methanol supply and demand was attained, and a new steady state at the 0.48 l/hr dilution rate was reached. It is thus seen that the profile of the specific growth rate follows a (damped) oscillatory behavior. Correspondingly, the biomass-yield profile showed first a decreasing phase followed by an increasing one, and then a decreasing phase again.

The specific-growth-rate profile of the shift-down experiment showed also a damped oscillatory behavior. The specific growth rate went down to 0.05 l/hr in less than 30 minutes after the dilution rate shift, and recovered after 2 hours from then to its new steady-state level. Residual methanol and formaldehyde were not detectable during the run. The dissolved-oxygen level rose to its new steady-state value immediately after shifting.

2.3.2 Methanol-Pulse Transient Experiments
Six experiments were performed using methanol as a substrate, and another two experiments were performed using methanol plus formaldehyde as substrates. The transient values of the residual methanol, residual formaldehyde, dissolved oxygen, and biomass concentration were measured directly. The specific growth rate, the biomass yield, and the methanol uptake rate were then calculated from the above mentioned values. The results are plotted and presented in Figs. 2.3 - 2.10. The specific growth rate was calculated from the biomass concentration profile,

$$\mu = \frac{dX/dt}{X} \quad (2-1)$$

The methanol uptake rate (MUR) was calculated according to the methanol mass balance on the reactor:

$$\text{MUR} = \frac{\left( S_f - S \right) D - dS/dt}{X} \quad (2-2)$$

where $S_f$ is the methanol feed concentration, $S$ is the methanol 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 in Materials and Methods, the derivatives, $dX/dt$ and $dS/dt$, were obtained from fitted smooth-curve functions while other quantities in eqs. 2-1 and 2-2 were obtained directly from the experimental data. The biomass yield was calculated from the specific growth rate $\mu$ and MUR,

$$Y = \frac{\mu}{\text{MUR}} \quad (2-3)$$
Figs. 2.3 to 2.6 show how the dilution rate affected the transient response of the cells to the methanol pulses (experiments #3 to #6). Methanol pulses were carried out by adding 3 ml of methanol to the reactor. The dilution rates were 0.2, 0.32, 0.42, and 0.49 l/hr, respectively. A comparison of these four sets of experiments reveals some common features as well as some differences. Immediately after the methanol 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 l/hr lower than before the methanol 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 l/hr. The dissolved oxygen (D.O.) profiles were similar for all runs in that they decreased until the residual methanol reached its pre-pulse level; then, the D.O. rose rapidly back. The residual-methanol and the methanol uptake rate profiles were also similar except for the 0.49 l/hr experiment. There were large amounts of methanol disappearing immediately following the pulse injection in experiments #3 to #5, and the methanol-uptake rate descended to negative values within about 2-3 hours. These unusual phenomena can be explained if methanol is transported into the cells via an active transport system [37]. This is because negative MUR values mean that methanol is released by the cells, and this in turn means that the internal concentration of methanol is
higher than the external one. On the other hand, experiment 
#6 did not show a large quantity of methanol disappearing 
or the negative MUR. These unusual methanol profiles and 
their relation to the transport system for methanol will be 
discussed in more detail in the next section. The residual-
formaldehyde profiles were also quite interesting. 
Experiment #3 displayed the highest residual-formaldehyde 
concentration, while experiment #5 the lowest one. It is 
possible that at lower growth rates, the capacity for 
formaldehyde production, i.e. the methanol dehydrogenase, is 
largely unsaturated as compared to the capacity for 
formaldehyde incorporation and oxidation. Therefore, when 
large amounts of methanol become available, a bottle-neck 
effect occurs as formaldehyde saturates its consumption 
pathways. At dilution rates higher than 0.4 l/hr, the high 
residual methanol (e.g. 350 mg/l in experiment #6), and 
formaldehyde levels may induce an extra-capacity for 
formaldehyde oxidation to the extent that the sudden 
increase in formaldehyde production does not cause the 
accumulation of formaldehyde, or, a more likely explanation, 
the capacity of formaldehyde production is saturated already 
at steady state due to the high residual methanol level, and 
that there is no sudden increase of formaldehyde production 
upon methanol pulse addition.

To study the effect of the methanol-pulse size on the 
dynamic behavior of growth, two additional sets of 
experiments (#7 and #8) were carried out, and the results
Figure 2.3. Response of a single-substrate continuous culture to a methanol pulse. (Dilution rate = 0.2 l/hr. Pulse magnitude = 6.7 g/l) Biomass is expressed in O.D. 600 nm unit, MUR (methanol uptake rate) in g MeOH/hr-g dry wt, Y (biomass yield) in g dry wt/g MeOH.
Figure 2.4. Response of a single-substrate continuous culture to a methanol pulse. (Dilution rate = 0.32 l/hr. Pulse magnitude = 6.7 g/l) Biomass is expressed in O.D. 600 nm unit, MUR (methanol uptake rate) in g MeOH/hr-g dry wt, Y (biomass yield) in g dry wt/g MeOH.
Figure 2.5. Response of a single-substrate continuous culture to a methanol pulse. (Dilution rate = 0.42 l/hr. Pulse magnitude = 6.7 g/l) Biomass is expressed in O.D. 600 nm unit, MUR (methanol uptake rate) in g MeOH/hr-g dry wt, Y (biomass yield) in g dry wt/g MeOH.
Figure 2.6. Response of a single-substrate continuous culture to a methanol pulse. (Dilution rate = 0.49 l/hr. Pulse magnitude = 6.7 g/l) Biomass is expressed in O.D. 600 nm unit, MUR (methanol uptake rate) in g MeOH/hr-g dry wt, Y (biomass yield) in g dry wt/g MeOH.
Figure 2.7. Response of a single-substrate continuous culture to a methanol pulse. (Dilution rate = 0.31 l/hr. Pulse magnitude = 2.2 g/l) Biomass is expressed in O.D. 600 nm unit, MUR (methanol uptake rate) in g MeOH/hr-g dry wt, Y (biomass yield) in g dry wt/g MeOH.
Figure 2.8. Response of a single-substrate continuous culture to a methanol pulse. (Dilution rate = 0.46 l/hr. Pulse magnitude = 13.4 g/l) Biomass is expressed in O.D. 600 nm unit, MUR (methanol uptake rate) in g MeOH/hr-g dry wt, Y (biomass yield) in g dry wt/g MeOH.
Figure 2.9. Response of a mixed-substrate continuous culture to a methanol pulse. (Dilution rate = 0.30 l/hr. Pulse magnitude = 6.7 g/l) Biomass is expressed in O.D. 600 nm unit, MUR (methanol uptake rate) in g MeOH/hr-g dry wt, Y (biomass yield) in g dry wt/g MeOH.
Figure 2.10. Response of a mixed-substrate continuous culture to a methanol pulse. (Dilution rate = 0.40 l/hr. Pulse magnitude = 6.7 g/l) Biomass is expressed in O.D. 600 nm unit, MUR (methanol uptake rate) in g MeOH/hr-g dry wt, Y (biomass yield) in g dry wt/g MeOH.
are presented in Figs 2.7 and 2.8. Experiment #7 was performed at a dilution rate of 0.31 l/hr with an 1 ml pulse, as compared to the 3 ml pulses used in experiments #3 to #6. A 6 ml pulse was used in Experiment #8 at a 0.46 l/hr dilution rate. In the experiment with the smaller methanol pulse (#7) the specific growth rate did not drop after the methanol pulse, and there was no unusual methanol-uptake profile. The residual formaldehyde 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 #3 to #6. For the larger methanol pulse, experiment #8 exhibited a rather severe drop in the specific growth rate as well as in the biomass yield at the beginning of the transient. However, both quantities recovered rapidly. The MUR did not go into a negative range, and the formaldehyde level was below 5 mg/l, a surprising low value in view of the amount of methanol added. There was also a large amount of methanol disappearing immediately after the pulse addition. These observations seem to confirm the notion that fast growing cultures are less likely to develop high residual formaldehyde levels and are less likely to have unusual MUR profiles. A unique phenomenon was also observed in experiment #8. The specific growth rate reached a high of 0.64 l/hr a value, which lasted for about three hours.

Pulse-transient experiments were also performed on mixed-substrate cultures. 100 mg/l of formaldehyde was used
together with 790 mg/l methanol as carbon substrates in the feed. At steady state, the residual levels of methanol and formaldehyde 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 formaldehyde were added to the feed tank of a steady-state culture grown on methanol (data not shown). Therefore, the major effect of formaldehyde in the feed was not to raise the residual formaldehyde level, but rather to restructure the enzyme system responsible for formaldehyde metabolism, in view of the increased formaldehyde flux. This is because the MUR is not equal to the consumption rate of formaldehyde (FCR), but rather:

\[ \text{MUR} + \text{FUR} = \text{FCR} \]  \hspace{1cm} (2-4)

where FUR is the formaldehyde uptake rate.

For experiments #9 and #10, the dilution rate was 0.3 and 0.4 l/hr, respectively. A methanol pulse of 3 ml was used in both experiments. The results are presented in Figs 2.9-2.10. There were some major differences between the dynamic behavior of single- and mixed-substrate cultures. To begin with, the specific growth rate profiles in experiments #9 and 10 displayed an upward trend right after the pulse perturbation, and remained at the 0.42 - 0.48 l/hr 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 formaldehyde level for experiments #9 and 10 was higher than in the single-substrate cases. To make these comparisons more clear, the profiles of the biomass yield, the specific growth rate and the residual formaldehyde level for experiments #4, 5, 9 and 10 are replotted in Figs. 2.11-2.13. These two experiments seem to indicate that the mixed-substrate cultures are adapting better to rapid additions of high methanol amounts, they display higher transient biomass yields, and that their growth is not quite adversely affected by methanol-pulse perturbations. Also, the high residual formaldehyde level does not seem to have any negative effects on cellular metabolism.

The energy charge, defined as

\[
\frac{[\text{ATP} + \frac{1}{2}\text{ADP}]}{[\text{ATP} + \text{ADP} + \text{AMP}]}
\]  \hspace{1cm} (2-5)

is an important parameter in microbial metabolic regulation [38]. It was thus felt that by measuring the energy charge and the ATP concentration before and after the methanol pulses, the transient behavior of methylotrophic growth to these pulses could be further elucidated. The results are presented in Table 2.1. At steady state, the energy charge of L3 was ca. 0.7, while after the methanol pulse, the energy charge as well as ATP concentration increased to much higher levels. The energy charge was above 0.9 immediately after the methanol perturbation and ascended gradually to even higher values. The steady-state energy charge obtained
Figure 2.11. Comparison of the response to methanol pulse between single- and mixed-substrate cultures: The specific growth rate profiles. Symbols: Δ mixed-substrate, D=0.3 l/hr, □ mixed-substrate, D=0.4 l/hr, ▲ single-substrate, D=0.33 l/hr, ■ single-substrate, D=0.42 l/hr.
Figure 2.12. Comparison of the response to methanol pulse between single- and mixed-substrate cultures: The biomass yield profiles. The unrealistic high or negative values caused by unusual MUR were rejected here. For symbols, see Fig. 2.11.
Figure 2.13. Comparison of the response to methanol pulse between single- and mixed-substrate cultures: The residual HCHO profiles. For symbols, see Fig. 2.11.
Table 2.1 Response of energy charge to methanol pulse. Pulse magnitude = 6.7 g/l. Dilution rate = 0.44 l/hr. E.C.: energy charge as defined in text. The levels of AMP, ADP and ATP were measured for cell samples taken at the indicated time relative to the methanol addition.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>AMP (nmol/mg dry wt)</th>
<th>ADP</th>
<th>ATP</th>
<th>E.C.</th>
</tr>
</thead>
<tbody>
<tr>
<td>-0.6</td>
<td>1.57</td>
<td>1.55</td>
<td>3.40</td>
<td>0.64</td>
</tr>
<tr>
<td>0.3</td>
<td>0.3</td>
<td>0.6</td>
<td>5.75</td>
<td>0.91</td>
</tr>
<tr>
<td>1.0</td>
<td>0.54</td>
<td>0.02</td>
<td>6.59</td>
<td>0.92</td>
</tr>
</tbody>
</table>

is comparable to that of Methylophilus methylotrophus [39].

2.3.3 Methanol Concentration Gradient Across the Cell Membrane

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

$$\frac{dS_T}{dt} = D(S_f - S_T) - \mu X/Y \quad (2-6)$$

where $S_T$ is the total amount (extracellular and intracellular) of methanol per unit reactor volume. With the experimentally obtained values of $S_f$, $\mu$ and $X$, an assumed value for $Y$ and the initial value of $S_T$, the profile of the calculated $S_T$ can be obtained. $\mu$ and $X$ values used in this calculation were obtained from the fitted smooth-curve function of the biomass concentration profile, as
described in Materials and Methods. The difference between the calculated and the observed extracellular methanol amounts 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:

\[ v \cdot S_i + (1-v) \cdot S = S_T \quad (2-7) \]

where \( v \) is the fraction of total reactor volume occupied by the cells, while \( S_i \) represents the intracellular methanol concentration. The value of \( v \) was calculated to be 0.005\( \cdot X \) based on the value of the intracellular volume of \( L3, 5 \mu l/mg \) dry biomass [37]. Since \( v \ll 1 \), eq. 2-7 can be rearranged as

\[ S_i/S = (S_T/S - 1)/v \quad (2-8) \]

and then the methanol gradient across the membrane can be calculated.

Examples of the resulting intracellular to extracellular methanol concentration ratios in four experiments (#3, 4, 7, 9) are presented in Figs. 2.14-2.17 with various assumed values of the biomass yield. It is clearly seen that the internal methanol concentrations are about one to two orders of magnitude higher than the external ones in these experiments.
Figure 2.14. The profile of methanol gradient across the cell membrane during transient experiment # 3.
Figure 2.15. The profile of methanol gradient across the cell membrane during transient experiment # 4
Figure 2.16. The profile of methanol gradient across the cell membrane during transient experiment # 7
Figure 2.17. The profile of methanol gradient across the cell membrane during transient experiment # 9
2.4 DISCUSSION

The transient experiments described above constitute an extensive study on the dynamic behavior of a methylothrophic bacterium. Most transient experiments that have been reported in the literature, have employed slow transient techniques, such as a dilution-rate shift, a substrate-concentration shift or substrate shift [40]. In those slow transients, cells experience gradual changes and, in most cases, can adjust promptly to the imposed changes [40]. Thus, the dynamic behavior of slow transients can often be predicted from the steady-state metabolic behavior of the cells. Fast transients, on the other hand, produce much more complex responses that may be difficult to analyze. For example, substrate transport would have little effect on transients resulting from a dilution-rate shift, but may affect dramatically the transient response to a substrate pulse as the present experiments demonstrated. However, a drastic perturbation, such as the one resulting from a substrate pulse, may reveal phenomena that could not be discerned otherwise. Besides, pulse transients can be used for selecting strains relatively insensitive to methanol fluctuations for large scale industrial applications.

The negative values in the MUR profiles of the methanol-pulse transients have suggested the existence of an active transport system. Methanol is a small and uncharged molecule which has very high permeability through lipid-
bilayer membranes [41]. Thus, methanol crosses the cell membrane readily by simple diffusion. A common argument against the existence of an active transport system for methanol is the fact that the methanol dehydrogenase being located on the outer side of the cell membrane (periplasmic space), methanol does not need to be transported in the cell [42,43,44]. However, methylamine dehydrogenase is also located in the periplasmic space of methylo trophs [45], and yet there is evidence that methylamine is actively transported by the cell [41]. From the calculated values of the methanol gradient across the cell membrane, it has been demonstrated that methanol is accumulated inside the cells. However, the errors involved in the computation grow cumulatively with time and in particular become very large when the residual methanol is low (see eq. 2-8). Therefore, only the results calculated from the first two or three hours of the transient experiments are meaningful. The assumed values of the biomass yield, which cover a reasonable range, do not affect the results very much. Due to the high permeability of methanol and the difficulty in finding methanol metabolic mutants in obligate methylo trophs, conventional studies on the methanol transport mechanism are quite difficult. The procedures described in this study (experimental and computational) may lead to a new approach to the problem of methanol transport. The current experimental procedures would need some modifications to be able to produce more accurate and
conclusive data, such as shorter sampling intervals and measuring the true biomass yield (as opposed to the observed apparent yield) by radioactive labels.

The drop in the specific growth rate observed upon methanol addition was checked in various ways to make sure it was not an artifact. Killing of the cells by the high methanol concentration may be the real cause of the apparent slow down in the growth rate. To check this possibility, viable cell counts were carried out for the cultures before and after methanol addition. The counts were not significantly different (Table 2.2), and this possibility was discarded. Another possibility to account for the drop in the optical density of the cells after the methanol addition is a different light-scattering property of the cell suspension due to the higher methanol concentration. This possibility was checked by performing dry-weight determinations for the cultures before and after methanol addition (Table 2.3). The results indicate that this was not the case either. In fact, the observed higher specific growth rates for the mixed-substrate experiments were enough to prove that the drop is not an artifact, but rather a property of the cell metabolism.

The observed energy-charge profile of the methanol-pulse transient does not support the notion that high methanol concentrations adversely affect the energy generating system, which would imply that substrate oxidation and ATP
Table 2.2 Viable cell counts before and after the methanol addition. Pulse magnitude = 6.7 g/l. Dilution rate = 0.40 l/hr. Viable cell count was performed for cell samples taken before and after the methanol addition. Averaged values are given in the last row together with the standard deviations in parentheses.

<table>
<thead>
<tr>
<th>$10^7$ cells/ml</th>
<th>before</th>
<th>after</th>
</tr>
</thead>
<tbody>
<tr>
<td>pulse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>145</td>
<td></td>
</tr>
<tr>
<td>135</td>
<td>113</td>
<td></td>
</tr>
<tr>
<td>121</td>
<td>124</td>
<td></td>
</tr>
<tr>
<td>114</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td><strong>117 (14)</strong></td>
<td><strong>124 (14)</strong></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3 Dry weight correlation of cells before and after the methanol addition. Pulse magnitude =6.7 g/l, dilution rate = 0.2 l/hr. Results were obtained from the average of two measurements.

<table>
<thead>
<tr>
<th></th>
<th>before</th>
<th>after</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$0.454 \pm 0.01$</td>
<td>$0.455 \pm 0.009$</td>
</tr>
</tbody>
</table>

phosphorylation could become uncoupled and result in lower biomass yields. It has been reported that, under excess methanol conditions, the electron transfer chain switches its terminal oxidase from cytochrome aa3 to cytochrome o,
and it was, thus, suspected (without direct evidence) that this switch might result in less efficient ATP production compared to methanol-limited conditions [44]. The present experimental data indicate that the cells synthesized much more ATP under high methanol concentrations, and thus the cells energy generating system did not appear to be greatly affected. In any case, the drop in the growth rate or the biomass yield was not the result of an insufficient ATP supply. However, the higher energy charge values found after the methanol pulse do point out to some possible explanations for the low biomass yield values obtained at high methanol concentrations. From the point of view of non-equilibrium thermodynamics, a high energy charge implies a lower efficiency in converting the protonmotive force into ATP [12,46,47]. Also, from the viewpoint of cellular metabolic regulation, cells would try to restore their energy charge to normal levels by consuming ATP for, perhaps, some "slip" reactions [48], since it has been postulated that most microbial cells maintain their energy charge at a constant level of ca. 0.8-0.9 in order to regulate catabolism and anabolism in a balanced and optimal way [38]. The latter explanation is especially plausible when growth is inhibited by high concentrations of methanol or formaldehyde, and the demand for ATP decreases. Thus, the lower biomass yields can be viewed as resulting from the effort of the cells to regulate their energy charge, and the inhibitory effect on growth exerted by methanol and
formaldehyde.

The toxic effect of methanol and formaldehyde on methylotrophic growth has been known for some time [33]. However, the mechanism of toxicity and its relationship to the cellular metabolism is still not known. For example, L3 can be grown in batch culture on formaldehyde alone as carbon substrate with an up to 250 mg/l initial concentration [33], if a series of batch cultures were carried out in a way that the formaldehyde level was gradually increased from batch to batch. On the other hand, 100 mg/l of formaldehyde injected directly in the fermentor will kill most of the cells (data not shown). This implies that the toxic effect of formaldehyde is not absolute, and that it is dependent on the physiological state of the culture. The nature of methanol toxicity is even more elusive. This is clearly shown in the pulse transient data presented here. Both the specific growth rate and the biomass yield recovered from their initial drops before the residual methanol concentration returned to undetectable levels (Figs. 2.3 to 2.10). However, small fluctuations (less than 20 mg/l) in the methanol concentration resulting from pulse feeding can sharply reduce the biomass yield [27]. Again, it appears that the toxic effect of methanol is dependent on the physiological state of the culture, as well.

The different dynamic behavior of the single- and mixed-
substrate cultures is very interesting in view of the above discussion on the toxicity of the two substrates. The better adaptation of mixed-substrate cultures to methanol pulses (Figs. 2.9 and 2.10) seems to arise from the use of formaldehyde as a co-substrate. Since the residual formaldehyde level is approximately the same for single- and mixed-substrate cultures, this better adaptation is most unlikely to be induced by the formaldehyde concentration per se. The higher formaldehyde flux to the cells of 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 the batch culture of strain L3 with formaldehyde as a co-substrate, as will be presented in Chapter 3. Those changes may also make the cells less sensitive to methanol pulses in continuous cultures.
Chapter 3

CARBON-SUBSTRATE OXIDATION AND INCORPORATION PATTERNS

3.1 INTRODUCTION

In obligate methanol utilizing bacteria, methanol oxidation to CO₂ can be accomplished via either a linear or cyclic oxidation scheme [1]. The reduced electron acceptors or cofactors in each enzymatic step of oxidation produce the necessary energy in the form of ATP for biosynthesis via oxidative phosphorylation. The amount of ATP produced from each oxidation step is determined by the nature of these electron acceptors, in that each electron acceptor is coupled with the respiratory electron transfer chain at different points, one, two or three phosphorylation sites away from oxygen, the final electron acceptor [1,11]. In the linear scheme (Fig. 1.2), methanol is converted to CO₂ by three dehydrogenases: methanol dehydrogenase (MDH), formaldehyde dehydrogenase (FDDH) and formate dehydrogenase (FDH). The electron acceptor associated with MDH has been identified as a novel pyrrolo-quinoline quinone [1,49]. Direct experimental measurements of the P/O ratio for methanol oxidation to formaldehyde have indicated that only one mole of ATP is formed per mole of methanol oxidized [1]. On the other hand, NAD-linked formaldehyde or formate
oxidation can produce up to 3 moles of ATP per mole of substrate oxidized [1]. The identity of the enzyme(s) responsible for formaldehyde oxidation to formate in vivo is still an open question [1], but even in the presence of an NAD-linked FDDH, formaldehyde can be oxidized to formate by a dehydrogenase similar to the MDH, thus yielding only one mole of ATP [1,11]. Thus, even if a methylo troph exhibits activities of an NAD-linked FDDH, YH$_2$ most probably yields less ATP than NADH$_2$ would. Cell-free extracts of batch grown cells of strain L3 exhibit no in vitro NAD-linked FDDH activity [33].

The cyclic oxidation scheme (Fig. 1.2) is the dissimilatory component of the RuMP cycle, and yields 2 moles of NAD(P)H$_2$. The requirement of NADPH$_2$ for biosynthesis purpose is fulfilled by the assimilatory part of the RuMP cycle [17]. Thus it may invariably produce 6 moles of ATP per mole of formaldehyde converted to CO$_2$. Therefore, methylo trophs employing this cyclic oxidation scheme would be the most energetically efficient ones and could, theoretically, exhibit biomass yields as high as 0.61-0.64 g dry-wt per g methanol [1,11]. The degree of utilization of each of the aforementioned oxidation scheme is thus both a practical and fundamental issue. From the viewpoint of fundamental microbial physiology, it is interesting to know if the degree of utilization of each scheme is affected by the culture conditions, if it correlates with the in vitro activities of the key enzymes
of the two schemes and how the cells regulate the extent of employment of each scheme. On the practical side, detailed knowledge of the substrate oxidation pattern can elucidate, firstly, the bioenergetics of methylo trophic growth and how culture conditions affect the coupling efficiency of energy metabolism, and secondly, the discrepancy that exists between theoretical predictions and experimentally observed biomass yields [1,11]. Such information can provide useful insights on process optimization and potentially suggest strategies for improving the microorganisms via mutant selection or recombinant DNA techniques.

The question of the extent of utilization of each oxidation scheme has been the subject of considerable effort and debate in the last few years [1,11,33,50-53]. The most accepted view [1], proposed by Zatman [52], states that obligate methylo trophs which can not utilize methane, employ predominantly the cyclic oxidation scheme. This conclusion is based on the low FDDH and FDH activities and high activities of the cyclic-oxidation enzymes that have been reported for these methylo trophs. However, in vitro enzyme activities constitute a rather weak basis for such a strong generalization, especially in view of some contradictory data that have appeared in the literature [53,54]. On the other hand, efforts to measure the extent of utilization of each oxidation scheme in vivo in Pseudomonas C [50,51] using several radioactive tracers have been seriously criticized [1]. The criticism arises from the use of uniformly-labeled
\( ^{14} \text{C}-\text{glucose as a tracer. This tracer overestimates the extent of utilization of the linear oxidation scheme at the expense of the cyclic one} [1], \) as will be discussed later.

Radioactive compounds have been used as tracers for the investigation of biochemical reaction mechanisms for many years. In most cases, experiments involving radioactive tracers are dealing with metabolic reactions in a qualitative manner, e.g. the identification of intermediates and pathways [55]. However, it is rather complicated to extract quantitative information from such tracer experiments [56]. Indeed, the difficulties encountered in such experiments may have prevented wider applications of radioactive tracer technique in quantitative studies of bacterial metabolism. The procedure proposed below overcomes those difficulties and can adequately solve the problem of measuring the \textit{in vivo} extent of utilization of each oxidation scheme in RuMP-type methylo trophs. Data obtained with this procedure from the continuous [57] and batch cultures of M. L3 presented below indicate that the cyclic oxidation scheme is not predominantly used, and that, in fact, the extent of utilization of each oxidation scheme varies with culture conditions.

3.2 MATERIALS AND METHODS

3.2.1 Organism and Culture Conditions
The growth media and conditions for the batch cultures of *Methylophaga* L3 on methanol at 30 °C have been described in Chapter 2.

### 3.2.2 Analyses

Methanol and formaldehyde analyses and biomass yield determinations were described in Chapter 2.

### 3.2.3 Batch Tracer Experiment

The microorganism was grown on various methanol and formaldehyde concentrations in 2-liter flasks with 400 ml media. When the culture reached mid-exponential phase, three portions of 50 ml culture suspension were withdrawn aseptically and put into three 250 ml flasks with magnetic stirrers inside the flasks. Each of the three labeled tracers (1-5 μCi) was then injected to one of the three 250 ml flasks. After two hours, growth was terminated by injecting 3 ml 100% w/v trichloroacetic acid (TCA). Acidification of the culture broth also released dissolved CO₂ from the broth. Sterile air was passed through each culture via ports in the stopper of the 250 ml flasks, during the entire run. 15 minutes after the injection of TCA, cells were harvested by centrifugation (Sorvall RC-5B) at 18000 x g for 10 minutes at 4 °C, washed once with cooled basal salts medium (see Section 2.2) and then solubilized by heating at 90 °C for 2 hours with 5 to 10 ml of 0.1 M sodium dodecyl sulfate (SDS) in 0.2 N NaOH. The exit gas was
passed through 10 ml of 4.0 N NaOH, so that the CO₂ evolved during the run was trapped as carbonates.

3.2.4 Liquid Scintillation Counting

0.5 ml samples of the SDS-solubilized biomass fraction were added to 15 ml of counting solution ACS, a Triton-xylene-based scintillant. 0.2 - 0.3 ml of the alkali-trapped carbonate fraction were added to 15 ml of 50% v/v ACS water solution, which, upon mixing, would form a gel-like homogeneous solution. Plastic vials were used for all samples. A Beckman model 133 liquid scintillation counter was used, and quenching corrections were made by the method of external standard channel ratio. All samples were prepared in triplicates, were counted to a maximum proportional counting error of 1-2% and were corrected for background counts.

3.2.5 Chemicals

All chemicals were of reagent grade, as described in Chapter 2. Sodium dodecyl sulfate was obtained from BDH Biochemicals, Poole, England. ¹⁴C-labeled formate (50-60 mCi/mmol) and carbonate (30-60 mCi/mmol) were obtained from ICN Pharmaceuticals, Irvine, CA; labeled methanol and glucose (all at 50-60 mCi/mmol) as well as ACS were from Amersham, Arlington Heights, IL.

3.3 THEORY
3.3.1 General Scheme

The reaction sequences in Figs. 1.1 and 1.2 can be condensed into a diagram as in Fig. 3.1 [50,58]. Two new features are added to complete the metabolism of carbon: decarboxylation and carboxylation reactions related to biosynthesis [59]. The $r_1$'s in Fig. 3.1 represent the reaction rates (or "flow rates" in analogy to flow distribution) at some sections of the carbon metabolism, and have units of C-moles per time per unit dry-cell weight. Note that $r_4$ represents the combined rate of CO$_2$ production from cyclic oxidation and from all other decarboxylation reactions. Although these two rates cannot be measured independently [50], they can be estimated based on the fact that decarboxylation reactions follow an approximately stoichiometric relation with total biosynthesis activities and can thus be treated as a fraction of biomass production rate, as will be detailed below.

From mass balances, the reaction rates are related as follows:

\begin{align*}
    r_1 &= r_2 + r_3 \quad (3-1) \\
    r_1 &= r_7 + r_8 \quad (3-2) \\
    r_3 &= r_4 + r_5 \quad (3-3) \\
    r_7 &= r_5 + r_6 \quad (3-4)
\end{align*}
Figure 3.1. Carbon flow diagram of *Methylophilus* L3. $r_1$: methanol or formaldehyde uptake rate, $r_2$: the linear oxidation rate, $r_3$: the rate of formaldehyde fixation into C$_6$ units, $r_4$: the combined rates of the cyclic oxidation and the general decarboxylation reactions, $r_5$: the direct biomass incorporation rate, $r_6$: the rate of the general carboxylation reactions, $r_7$: the overall biomass production rate, $r_8$: the overall CO$_2$ production rate and $r_9$: the cyclic oxidation reaction rate.
Since we have four equations for the eight reaction rates, three more equations are needed to determine all the reaction rates as a fraction of \( r_1 \). \( r_1 \) can be measured directly by performing a methanol balance over the reactor. Since there are three branch points in the metabolic pathways at formaldehyde, \( \text{CO}_2/\text{carbonate} \) and 6-phosphogluconate (6PG), three radioactively labeled compounds should be used as tracer to provide the additional information on the eight reaction rates.

There are three fundamental conditions that must be satisfied if any radioisotope tracer experiment is to be performed for the purpose of gathering quantitative information [56]. First, the metabolic pathways investigated must be unequivocally known, including the information about whether certain reactions are reversible or irreversible. Second, the addition of tracer must not disturb the existing metabolic state, and third, the radioactive tracer must follow the same metabolic pathway as the species that the tracer is intended to trace. These three requirements are satisfied by the system studied here. First, as implied in Fig. 3.1, the metabolc pathways are clearly defined: (i) formate is not directly incorporated into biomass nor reduced to formaldehyde. It can only be oxidized to \( \text{CO}_2 \) and then be incorporated into biomass via general carboxylation reactions. This is an amply tested hypothesis [1,11,33,50,60,61]. (ii) Glucose is taken up by bacteria, phosphorylated to glucose-6-phosphate (G6P) and
then assimilated through the KDPG/TA variant of the RuMP cycle [1,55], so that labeled glucose can be used to trace the incorporation of methanol or formaldehyde into biomass and their oxidation to CO₂ via the cyclic oxidation scheme and decarboxylation reactions. M. L3, like other obligate methanol utilizing bacteria, gave positive results for the key enzymes of the cyclic oxidation scheme and of the KDPG/TA variant, and negative results for the key enzymes of the FBP/SDP variant [57]. (iii) The arrows in Fig. 3.1 indicate a single direction for the reactions, i.e. irreversibility. Decarboxylation and carboxylation reactions are carried out by different groups of enzymes [59,62], and, for a given metabolic state, each individual reaction operates in one direction although the overall results may make these two groups of reactions appear two directional. Therefore in Fig. 3.1 these two groups of reactions are treated separately. CO₂ exchange reactions are found to contribute very little to the ¹⁴CO₂ flow compared to the irreversible carboxylation and decarboxylation reactions [1,50,59,63]. Degradation of biomass into smaller molecules may also cause problems because that would imply that the reactions which lead to biomass synthesis are not unidirectional. However, the biomass degraded is only about 2% of the total cell mass [56], and only the portion of that 2% which reenters the sugar-phosphate pool would violate the unidirectionness depicted in Fig. 3.1. Therefore this difficulty can be
neglected.

Since the amount of the tracer added is below the micromolar level and does not change the metabolic state, the second requirement is also met. Glucose was found to be taken up by the strain in small but consistent amounts, and it is known that glucose utilization by microorganisms always requires phosphorylation to glucose-6-P as the first step [17,64,65]. Thus the third requirement is also fulfilled. This set of requirements is apparently satisfied by other obligate RuMP-type methylo trophs, as well [1,50,55,60,66].

The tracer experiments, developed originally by Ben-Bassat et al. [50], are performed by adding a very small concentration of C-14 labeled compounds to the reactor during continuous, steady state growth of cells. The radioactivity distribution between biomass and CO$_2$/carbonates is determined by the procedures described in Section 3.2.

Let $Q$ be the ratio of the radioactivity in biomass over the radioactivities incorporated in biomass and CO$_2$/carbonates, and define

$$A = Q, \text{ when } ^{14}\text{C methanol is the tracer} \quad (3-5)$$

$$B = Q, \text{ when } ^{14}\text{C glucose is the tracer} \quad (3-6)$$

and

$$C = Q, \text{ when } ^{14}\text{C formate is the tracer} \quad (3-7)$$
It should be noted, in eq. 3-6, that $^{14}$C-glucose can be labeled in various manners, but there is only one correct labeling pattern that can give tractable results. Assume for the time being that the glucose tracer is labeled in the correct manner, which will be addressed later.

In view of the steady-state reactor operation, the ratios of radioactivity distribution between CO$_2$ and biomass are related to the various reaction rates by precise relationships depending upon the tracer. Thus, A, B and C are related to the key reaction rates by the following equations:

\[
A = \frac{r_7}{r_7 + r_8} \quad (3-8)
\]

\[
B = \frac{r_4 + r_5 r_6}{r_6 + r_8} / r_3 \quad (3-9)
\]

\[
C = \frac{r_6}{r_6 + r_8} \quad (3-10)
\]

Note that if $^{14}$C-carbonate were used as a tracer, then Q would be identical to the one obtained with the $^{14}$C-formate tracer. Using eqs. 3-1 to 3-4 and 3-8 to 3-10, all rates can be expressed in terms of the experimentally obtained quantities A, B, C and $r_1$ as follows,

\[
r_2/r_1 = (B-A)/(B-C) \quad (3-11)
\]

\[
r_3/r_1 = (A-C)/(B-C) \quad (3-12)
\]

\[
r_4/r_1 = [(A-C)(1-B)]/[(B-C)(1-C)] \quad (3-13)
\]

\[
r_5/r_1 = (A-C)/(1-C) \quad (3-14)
\]
\[ \frac{r_6}{r_1} = \frac{C(1-A)}{1-C} \]  
(3-15)

\[ \frac{r_7}{r_1} = A \]  
(3-16)

\[ \frac{r_8}{r_1} = 1-A \]  
(3-17)

### 3.3.2 Glucose Tracer

Although the general scheme is straightforward, the distribution of the radiolabel in glucose needs to be examined rigorously. This question cannot be resolved without closely examining the metabolic pathways shown in Figs. 1.1 and 1.2. It is essential to note that the glucose tracer serves not only as a tracer for 6PG in the metabolic reactions, but as a tracer for the formaldehyde carbons entering the RuMP cycle as well. In fact, it is the latter role of the glucose tracer that is considered in the formulation above. It is, therefore, necessary to know which positions in 6PG will be taken by those formaldehyde carbons when they first enter the cycle and this distribution must be matched by the labeled glucose carbons in order to fully represent the fate of those formaldehyde carbons. The carbons following the cyclic oxidation route occupy the first position in hexulose-6P, fructose-6P, glucose-6P and 6PG before being decarboxylated by 6PG dehydrogenase. On the other hand, those carbons that are converted into pyruvate and subsequently, into biomass or CO₂ (the latter by decarboxylation) occupy the upper three (1-, 2-, and 3-C) positions in the six-carbon skeleton.
This is so not just because pyruvate is derived from that part of the 6PG molecule but also as a result of RuMP cycle. Three formaldehyde molecules are required, by stoichiometry, to enter the RuMP cycle for each molecule of pyruvate produced, which, because of the steady-state conditions, implies that three consecutive condensations label the 1-, 2-, and 3-carbon locations. It can be argued that, because of the steady-state conditions, and in view of the rearrangement reactions, the probability that a methanol carbon will appear in the first, second or third position of the 6PG molecule is the same.

It is true that eventually the lower part the 6PG will be occupied by the incoming carbons as every carbon in the cell is made from them, but in steady state, continuous growth this can always be treated as downstream from pyruvate i.e., the lower portion of 6PG can be synthesized from pyruvate or its subsequent metabolites [1]. Indeed, species balances can readily show that unless glyceraldehyde-3P is produced from pyruvate via phosphoenolpyruvate [1,17,59,64,65], there can be no net production of glyceraldehyde-3P, ribose-5P, fructose-6P, etc., which are necessary for biosynthesis. Again, at steady-state, continuous growth, these intermediates which leave the cycle can be viewed as being synthesized from pyruvate or its subsequent metabolites, because there should be no net flow in or out of the pool of intermediates under steady state conditions. In view of the carbon flow pattern discussed above, uniformly-labeled
glucose is not a suitable tracer because of the label in the fourth, fifth and sixth position. The unsuitability of the uniformly-labeled glucose as tracer is discussed in more detail next.

Assuming that the fraction of carbons entering the RuMP cycle which follow the cyclic oxidation route is \( \alpha \), the fraction of the carbons which take the 1-C position at the 6PG branch point is:

\[
\alpha + \frac{(1-\alpha)}{3} \tag{3-18}
\]

and those for the 2-C and 3-C positions are identical and equal to:

\[
\frac{(1-\alpha)}{3} \tag{3-19}
\]

It is clear from the above derivations that the proper glucose tracer should be labeled at 1-, 2- and 3-C positions according to the fractions in eqs. 3-18 and 3-19 so as to accurately represent the fate of incoming carbons at that branch point. It is also clear why the uniformly labeled glucose is incorrect for this purpose. Specifically, it under-represents the 1-C position, since \( \alpha + \frac{(1-\alpha)}{3} \) is larger than one-sixth, and thus underestimates the extent of the cyclic oxidation scheme, and by so doing, overestimates the extent of the linear oxidation scheme.

The parameter \( \alpha \) cannot be estimated readily from experiments because the decarboxylation reactions complicate
the final \( \text{CO}_2 \) and biomass distribution. However, it is proposed that, with some approximations, \( 1^{-14}\text{C-glucose} \) be used as the tracer for 6PG and the cyclic oxidation route. Let us define

\[
\beta = Q, \text{ when } 1^{-14}\text{C-glucose is the tracer} \quad (3-20)
\]

and two quantities, \( B' \) and \( \beta' \), which describe the split at the 6PG branch point excluding the fraction reassimilated via carboxylation, i.e. \( r_5/r_3 \), for ideally-labeled and \( 1^{-14}\text{C-labeled glucose} \), respectively, by

\[
B = B' + (1-B') \cdot C \quad (3-21)
\]

and

\[
\beta = \beta' + (1-\beta') \cdot C \quad (3-22)
\]

In Fig. 3.2, the carbon flow beginning at \( 1-C \) is set to be 1, the flow of cyclic oxidation is denoted by \( \gamma \), and the decarboxylation flows for each of the three carbon positions are \( \delta_1 \), \( \delta_2 \) and \( \delta_3 \) respectively. It follows that

\[
\beta' = 1 - \gamma - \delta_1 \quad (3-23)
\]

The total carbon flow to the biomass is given by:

\[
3(1-\gamma) - (\delta_1 + \delta_2 + \delta_3) \quad (3-24)
\]

and the total flow to \( \text{CO}_2 \) is given by:

\[
\gamma + \delta_1 + \delta_2 + \delta_3 \quad (3-25)
\]

By definition,
\[ B' = \frac{[3(1-\gamma) - \epsilon]}{(3-2\gamma)} \quad (3-26) \]

where \( \epsilon \) is the sum of \( \delta_1, \delta_2 \) and \( \delta_3 \).

The decarboxylation reactions associated with biosynthesis follow an almost stoichiometric relationship with the biomass formation, provided that the average composition of cell mass is constant. For carbon limited growth, for example, it is possible to estimate the amount of carbon released by decarboxylation quite accurately. The calculation is based on a detailed accounting of the reactions necessary to produce all the building blocks for a given amount of biomass [17] (see also Appendix B). Our calculations show that 0.80 C-moles are decarboxylated per 4 total C-moles (including general carboxylation) incorporated into biomass and that 0.315 C-moles are carboxylated (by general carboxylation reactions) per 4 total C-moles incorporated into biomass. Thus, 0.86 C-moles are decarboxylated per 4 C-moles directly incorporated into biomass (corresponding to the rate in eq. 3-25) and a net of 0.485 C-moles are decarboxylated by general reactions per 4 total C-moles incorporated into biomass. The latter figure is very close to the 0.48 figure calculated by Harder et al [12].

As will be detailed in Section 3.5, a value of 0.8 is used instead of 0.86 per 4 C-moles directly incorporated, in order not to underestimate the extent of utilization of the cyclic oxidation scheme. A ± 0.2 variation in the value of
Figure 3.2. $C_1$ incorporation into $C_6$ and $C_3$ skeletons in M. L3.
0.8 will also be shown to have a negligible effect on the calculations of the various rates according to eqs. 3-11 to 3-17. Thus, from eqs. 3-24 and 3-25, it follows that:

\[ \varepsilon = 0.5(1-\gamma) = 0.5(\beta' + \delta_1) \]  \hspace{1cm} (3-27)

With this assumption, the cyclic oxidation reaction rate can be calculated as

\[ r_9 = r_4 - 0.2 \cdot r_5 \]  \hspace{1cm} (3-28)

where \( r_9 \) is the cyclic oxidation reaction rate. From eqs. 3-23 to 3-27, it can be concluded that:

\[ B' = 2.5(\beta' + \delta_1)/(1 + 2(\beta' + \delta_1)) \]  \hspace{1cm} (3-29)

The decarboxylation carbon that originates at the 1-C position, \( \delta_1 \) is larger than \( \delta_2 \) and \( \delta_3 \) because most of the important general decarboxylation reactions decarboxylate the carbons which originate from at the 1-C position of pyruvate (see e.g. [62]). In addition, from the results of 2-\(^{14}\)C-glucose tracer experiments, \( \delta_1 \) is found to about 80-90\% of \( \varepsilon \) (see Section 3.5). Therefore, it is valid to assume that:

\[ 0.6 \varepsilon < \delta_1 < \varepsilon \]  \hspace{1cm} (3-30)

In order not to underestimate the extent of cyclic oxidation (which increases very weakly with decreasing \( \delta_1/\varepsilon \)-ratio values as will be detailed in Section 3.5), a value of
0.8 is assumed for the ratio $\delta_1/e$. Combining expressions (3-27) and (3-30), we obtain:

$$0.429 \beta' < \delta_1 < \beta'$$  \hspace{1cm} (3-31)

Using the above expression for the upper and lower bounds and assuming an average value for $\delta_1$ (i.e. $\delta_1 = 0.8 \epsilon$), we obtain,

$$\delta_1 = 0.667 \beta'$$  \hspace{1cm} (3-32)

which together with eq. 3-28, results in the expression relating the experimental $\beta'$ values to the idealized-ratio $B'$ values, namely,

$$B' = 4.17 \beta' / (1+3.33 \beta')$$  \hspace{1cm} (3-33)

$B'$ as determined by eq. 3-33 is plotted against $\beta'$ in Fig. 3.3 together with the uncertainty region calculated from the double inequality 3-30. The average uncertainty of $B'$ is found to be about $\pm 3\%$ by this method. The overall (experimental plus computational) accuracy of the results obtained by this procedure is about $\pm 3\%$ of $r_1$.

The value of $B$ obtained using eqs. 3-21 and 3-33, together with the experimentally obtained $A$, $C$ and $r_1$ values, can be used in eqs. 3-11 to 3-17 to determine the complete carbon oxidation and incorporation pattern.

3.3.3 Application of the Tracer Technique to Batch Cultures
Figure 3.3. $B'$ versus $\beta'$ for a range of $y$ values.
Although eqs. 3-1 to 3-4 are derived for steady-state reactor conditions, it does not limit the application of this procedure to chemostatic cultures only [56]. As long as the transients of the cultures under investigation are well characterized, this procedure should be applicable. However, cultures under transient conditions are generally difficult to characterize or even to reproduce experimentally in terms of metabolic properties (e.g. metabolite pool sizes). Therefore, only mid-exponential phase batch cultures, besides chemostatic cultures, have been investigated by this or other similar radioactive-tracer techniques [56]. This is due to the fact that batch-culture growth at mid-exponential phase is regarded as balanced growth, whereby all reactions operate at a constant rate, and, therefore, the pseudo-steady-state hypothesis for the various intermediates of interest (formaldehyde, 6PG, formate) can be argued to be valid. The pseudo-steady-state hypothesis is equivalent in stating that the rate of accumulation of these intermediates is very small as compared to the rates of production and consumption of the intermediates. Simple calculations (see e.g. [67]) can readily demonstrate the validity of this hypothesis even when small amounts of intermediates (e.g. formaldehyde) accumulate in the reactor. The duration of each labeling experiment should also be kept short in order to obtain a meaningful average and to ensure that the cultures do not deviate from the mid-exponential phase during the
experiment. The results obtained must be interpreted in an average (over the experiment time) sense.

3.4 RESULTS

A number of batch experiments were performed to investigate the effect of initial methanol and formaldehyde concentrations on the carbon oxidation and incorporation patterns in M. L3. Radioactivity counts and Q ratios, i.e. A, C and B values are presented in Tables 3.1-3.5 together with the initial concentrations of methanol and formaldehyde, specific growth rates and biomass yields for each experiment. The specific growth rates and biomass yields shown in those Tables are overall values measured for the one-hour duration of the tracer experiments. Biomass yields over the entire batch runs were much lower. The rates of Fig. 3.1 are presented as fractions of $r_1$ in Figs. 3.4-3.8.

As the data in Figs. 3.4-3.8 show, the extent of cyclic substrate oxidation varies significantly with the initial conditions of each cultures from 0 to 68% of the total substrate oxidized. This extent is low when the initial methanol concentrations are low or very high (Figs. 3.4 and 3.7) and high when the initial methanol is at an intermediate level (Figs. 3.5 and 3.6) for growth on methanol alone. The primary effect of the initial formaldehyde concentration in the medium is to slow the
Table 3.1. Label distribution in batch culture experiment #1.

\[(\text{MeOH})_0 \colon 0.63 \text{ g/l, } \mu : 0.19 \text{ l/hr, } Y : 0.44 \text{ g/g}\]

<table>
<thead>
<tr>
<th>tracer</th>
<th>million dpm</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cells</td>
<td>CO₂</td>
<td>broth</td>
<td>Q ratio</td>
</tr>
<tr>
<td>methanol</td>
<td>0.0304</td>
<td>0.0296</td>
<td>0.429</td>
<td>0.506</td>
</tr>
<tr>
<td>[1-C]glucose</td>
<td>0.0626</td>
<td>0.0297</td>
<td>10.4</td>
<td>0.678</td>
</tr>
<tr>
<td>formate</td>
<td>0.109</td>
<td>4.177</td>
<td>14.63</td>
<td>0.026</td>
</tr>
</tbody>
</table>

Figure 3.4. Carbon-substrate oxidation and incorporation pattern as calculated from batch experiment #1. **: r9, *: r4 - r9
Table 3.2. Label distribution in batch culture experiment #2.

(MeOH)_0 : 2.38 g/l, μ : 0.29 l/hr, Y : 0.45 g/g

<table>
<thead>
<tr>
<th>tracer</th>
<th>million dpm</th>
<th></th>
<th></th>
<th>Q ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cells</td>
<td>CO₂</td>
<td>broth</td>
<td></td>
</tr>
<tr>
<td>methanol</td>
<td>3.454</td>
<td>2.553</td>
<td>34.25</td>
<td>0.575</td>
</tr>
<tr>
<td>[1-C]glucose</td>
<td>0.00628</td>
<td>0.0107</td>
<td>1.70</td>
<td>0.370</td>
</tr>
<tr>
<td>formate</td>
<td>1.015</td>
<td>9.653</td>
<td>12.1</td>
<td>0.095</td>
</tr>
</tbody>
</table>

---

Figure 3.5. Carbon-substrate oxidation and incorporation pattern as calculated from batch experiment #2. --: r9, *: r₄ - r₉
Table 3.3. Label distribution in batch culture experiment 
#3.

\[(\text{MeOH})_0 : 2.38 \text{ g/l, } \mu : 0.50 \text{ l/hr, } Y : 0.50 \text{ g/g}\]

<table>
<thead>
<tr>
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<th></th>
<th></th>
<th>Q ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cells</td>
<td>CO₂</td>
<td>broth</td>
<td></td>
</tr>
<tr>
<td>methanol</td>
<td>4.80</td>
<td>2.25</td>
<td>61.5</td>
<td>0.681</td>
</tr>
<tr>
<td>[l-C]glucose</td>
<td>0.0137</td>
<td>0.0130</td>
<td>11.3</td>
<td>0.505</td>
</tr>
<tr>
<td>formate</td>
<td>1.45</td>
<td>7.71</td>
<td>9.38</td>
<td>0.158</td>
</tr>
</tbody>
</table>

Figure 3.6. Carbon-substrate oxidation and incorporation pattern as calculated from batch experiment #3. •: r₉, *: r₄ - r₉
Table 3.4. Label distribution in batch culture experiment #4.

\[(\text{MeOH})_0 : 14.45 \text{ g/l, } \mu : 0.17 \text{ l/hr, } Y : 0.15 \text{ g/g}\]

<table>
<thead>
<tr>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cells</td>
<td>CO₂</td>
<td>broth</td>
<td>Q ratio</td>
</tr>
<tr>
<td>methanol</td>
<td>0.0069</td>
<td>0.0342</td>
<td>0.455</td>
<td>0.167</td>
</tr>
<tr>
<td>[1-C]glucose</td>
<td>0.0103</td>
<td>0.0140</td>
<td>1.585</td>
<td>0.423</td>
</tr>
<tr>
<td>formate</td>
<td>0.365</td>
<td>6.421</td>
<td>1.915</td>
<td>0.054</td>
</tr>
</tbody>
</table>

Figure 3.7. Carbon-substrate oxidation and incorporation pattern as calculated from batch experiment #4. ---: r₉, *: r₄ – r₉
Table 3.5. Label distribution in batch culture experiment #5.

\[(\text{MeOH})_o : 2.80 \text{ g/l}, \mu : 0.18 \text{ l/hr}, Y : 0.35 \text{ g/g} \]
\[(\text{HCHO})_o : 0.07 \text{ g/l} \]

<table>
<thead>
<tr>
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<th>million dpm</th>
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<th></th>
<th>Q ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cells</td>
<td>CO₂</td>
<td>broth</td>
<td></td>
</tr>
<tr>
<td>methanol</td>
<td>0.938</td>
<td>1.56</td>
<td>7.226</td>
<td>0.375</td>
</tr>
<tr>
<td>[1-C]glucose</td>
<td>0.0031</td>
<td>0.0122</td>
<td>8.588</td>
<td>0.204</td>
</tr>
<tr>
<td>formate</td>
<td>0.872</td>
<td>12.0</td>
<td>0.733</td>
<td>0.084</td>
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</tbody>
</table>

Figure 3.8. Carbon-substrate oxidation and incorporation pattern as calculated from batch experiment #5. -- : r₉, *: r₄ – r₉
growth, enhance the cyclic substrate oxidation and reduce the biomass yield (Fig. 3.8). Although the extent of cyclic oxidation (r9) is low under the very high initial methanol concentration (Fig. 3.7), it is still significant when compared to the carbon flux through the RuMP cycle (r3). The pattern of carbon oxidation and incorporation for the lowest initial methanol concentration (Fig. 3.4) is quite similar to the patterns obtained in carbon-limited continuous cultures [57]. Carbon-limited continuous cultures are characterized by very low (0-20 mg/l) residual methanol concentrations. The different results shown in Figs. 3.5 and 3.6 indicate that the oxidation pattern depends not only on the initial substrate concentration but also on the physiological state of the inoculum, which can affect the culture behavior significantly in terms of the biomass yield and the specific growth rate. A similar influence of the inoculum on the culture behavior has been observed in chemostatic continuous cultures [57].

3.5 DISCUSSION

A sensitivity analysis was carried out to examine the effect of the assumptions regarding general decarboxylation (eqs. 3-27 and 3-31) on the values of the reaction rates calculated by the present procedure. The results of this analysis for the data of a chemostatic culture experiment are shown in Table 3.6. Variations in the value of x (moles
Table 3.6. Sensitivity analysis of the effect of the two assumed values regarding general decarboxylation on the calculated rate figures. $x$ and $y$ are defined in the text. The $Q$-ratio values employed in this analysis were obtained from chemostatic-culture experiments [57].

<table>
<thead>
<tr>
<th></th>
<th>$x = 0.8$</th>
<th>$x = 0.6$</th>
<th>$x = 1.0$</th>
<th>$x = 0.8$</th>
<th>$x = 0.8$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$y = 0.8$</td>
<td>0.23</td>
<td>0.23</td>
<td>0.24</td>
<td>0.19</td>
<td>0.27</td>
</tr>
<tr>
<td>$r_2/r_1$</td>
<td>0.77</td>
<td>0.77</td>
<td>0.77</td>
<td>0.81</td>
<td>0.73</td>
</tr>
<tr>
<td>$r_3/r_1$</td>
<td>0.12</td>
<td>0.12</td>
<td>0.12</td>
<td>0.16</td>
<td>0.08</td>
</tr>
<tr>
<td>$r_4/r_1$</td>
<td>-0.01</td>
<td>0.03</td>
<td>-0.04</td>
<td>0.03</td>
<td>-0.05</td>
</tr>
</tbody>
</table>

decarboxylated by general decarboxylation per 4 C-moles directly incorporated into biomass) have hardly any effect on the calculations. High $x$ values tend to underestimate the rate of cyclic oxidation. The effect of $y$ (percentage of CO$_2$ produced by general decarboxylation which originates at 1-C position of pyruvate) is more pronounced especially on the relatively low values of $r_2$, $r_4$ and $r_9$. Note that high $y$ values tend to underestimate the cyclic oxidation. As mentioned in Section 3.3.2, the value of $x$ calculated for an average bacterial-cell composition is 0.86 (see also Appendix B). However, this calculation is based on a carbon accounting which includes some CO$_2$ exchange reactions, i.e. it includes the decarboxylation reactions of the carbons that originally come from the CO$_2$ pool [17,62]. The
contribution from the exchange reactions should be excluded because those carbons do not originate at the 6PG-branch point. Again, based on average cell composition [17], the corrected value of x should be close to 0.8. Since variations in the x value from 0.6 to 1.0 do not affect the computational outcome significantly, the value of 0.8 can employed in the calculation. The assumption made on the y value was examined further by performing a similar experiment employing 2-\(^{14}\)C-glucose as a tracer. Due to the low radioactivity in the CO\(_2\) fraction and to the impurities of the 2-\(^{14}\)C-glucose tracer (99% purity), it is not possible to obtain very accurate results (see Table 3.7). Q-ratio values were found to be vary between 0.92 and 1.0, indicating that y is, approximately, between 0.7 and 1.0, if the carbons originating from the 2- and 3-C positions of pyruvate have the same probability of being decarboxylated. Similar experiments in continuous chemostatic cultures indicate a y value higher than 0.9 [57]. A value of 0.8 is used for y to avoid any possible underestimation of the cyclic oxidation rate. In view of the experimental accuracy with which the various Q-ratios can be measured, and in view of the assumptions regarding x, y and the computational procedure (eqs. 3-11 to 3-33), the computed numbers of the various rates can be trusted within ±3 percentage points of r\(_1\). Thus, while the relative error is small for rates like r\(_3\), which have typically high values, it is relatively large for rates like r\(_9\), which often have low values. However,
Table 3.7. Label distribution of $2^{-14}$C-glucose tracer. $2^{-14}$C-glucose was added to a batch culture of M. L3 with (MeOH)$_O$ = 2.8 g/l. The radioactivity showed up in the CO$_2$ fraction was corrected for the radioactivity coming from the impurities in the labeled compound, as determined by a blank (without cells) experiment.

<table>
<thead>
<tr>
<th></th>
<th>1000 dpm</th>
<th>Q ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>cell</td>
<td>4.28</td>
<td></td>
</tr>
<tr>
<td>CO$_2$</td>
<td>1.51 - (1.13)$^a$</td>
<td>0.92 - 1.0</td>
</tr>
<tr>
<td></td>
<td>1.51 - (3.43)$^b$</td>
<td></td>
</tr>
<tr>
<td>broth</td>
<td>5670</td>
<td></td>
</tr>
</tbody>
</table>

$^a,b$: the low and high bounds of the radioactivity in CO$_2$ due to the impurities.

the general pattern of carbon metabolism can be clearly observed despite these uncertainties.

The methodology developed here makes it possible to measure the in vivo rates of the carbon metabolic reactions in methylotherms. It is a valuable tool for the investigation of the bioenergetics and physiology of these bacteria under various growth conditions. The data obtained so far, from batch and chemostatic cultures, are not yet enough to propose a complete picture of carbon metabolic regulation in methylotherms. However, the limited data suggest that, in Methylomonas L3, methanol and formaldehyde can affect the extent of utilization of the cyclic-oxidation
pathway and that the cyclic-oxidation scheme perhaps functions like a detoxification route and is not utilized extensively under low residual methanol concentrations.
Chapter 4

COMPUTER SIMULATION OF METHYLOTROPHIC GROWTH

4.1 INTRODUCTION

Process control, process design and optimization in fermentation processes depend greatly on good mathematical models which can predict, estimate or simulate realistic process variables from a limited number of measurements, on-line or off-line, with reasonable computation time [68]. This is especially true in bioreactor design because most fermentation processes are sensitive to small variations in environmental conditions [69,70] and the reaction mechanisms are often complicated with many unmeasureable but essential variables [71]. Bioreactor design relies on the microkinetic models and transport models combined with macroscopic mass and energy balances [72]. Microkinetic models deal with the kinetics of cellular metabolic activities, such as conversion of substrates into various products, biomass growth, and the regulation of key enzymes. Transport models describe the mixing characteristics and mass transfer of substrates (particularly gaseous compounds) in the bioreactor as related to the power input, reactor configuration and properties of culture fluid. Although transport models are difficult to construct due to the complex fluid mechanics, empirical or semi-empirical
approaches can often be successfully applied because the elementary principles involved are well understood [73,74]. Microkinetic models, on the other hand, are much more complex and difficult to develop. This is because the reaction mechanisms, stoichiometry and other physiological factors that affect the microkinetics are usually poorly understood either qualitatively or quantitatively [70].

It is easy to appreciate the difficulty involved in the development of microkinetic models. As simple a life form as a bacterium, it is several orders of magnitude more complex than the inanimate catalyst or other chemical systems encountered in industrial processes. Within a highly organized cell, there are hundreds of reactions involving many complex molecules taking place in an orderly, self-regulating fashion. The interaction between the environmental (extracellular) factors and the cell are often too complex to be described in an exact manner. Thus, the major challenge in modeling microbial growth is how to simplify the microbial system to manageable complexity while retaining the necessary predictive capability.

The model relating the specific growth rate to the limiting sustrate concentration proposed by Monod [75] is still the most widely used one. It can be modified in various ways to account for the different characteristics exhibited by various microorganisms. For example, variable yields, maintenance energy concepts and substrate inhibition
of growth have all been incorporated into the basic Monod model in various forms [40,69,72,76,77]. This kind of model commonly treats a cell population as a homogeneous, unsubdivided entity whose kinetics resemble the autocatalytic kinetics of chemical reaction systems. These models are often referred to as unstructured models, while more sophisticated models that account for a more detailed description of the intracellular composition are called structured models [71]. Unstructured models are generally simple to handle mathematically, and allow the system to be treated analytically. Thus, they form a basis for a first, qualitative study of microbial systems, as, e.g. in reactor design and in stability analysis. The "structured" components commonly employed in the development of growth models are the RNA, DNA and protein pools, or some unidentified compounds. These models are also called compartmental models [71,76,78]. Detailed metabolic reaction networks, including genetic regulation of enzyme synthesis and substrate transport mechanisms, may also be incorporated in a structured model if necessary [79,80]. Many simple structured models, with two or three compartments, have been proposed and applied to problems in biochemical reaction engineering [81,82]. Unstructured, and simple structured models, in general, represent the steady state or balanced growth data quite well, but their simplified representation of the complex cellular machinery makes them unable to predict transient behavior in which
changes in the composition of biomass can not be ignored. A systems engineering approach to solve this problem has been suggested [29,83,84]. By introducing a certain disturbance and following the response, some empirical dynamic parameters, as, e.g. time constants and dead times, of the cellular growth process can be found. These constants can be used in dynamic equations, namely, transfer functions, to predict the transient behavior of fermentation processes. The physical meaning of the empirical time constant derives from the substrate diffusion process which is a first order process (in process control language), and the dead time corresponds to the time lag for cellular growth rate to respond to changes in the substrate concentration. This empirical approach is valuable in some cases when the two aforementioned processes, diffusion and growth lag, dominate the transient behavior. The parameters thus obtained have limitations however, because they can be applied, in general, only to the same or similar conditions from which they have been determined.

A number of highly structured models have also been proposed [80,85,86]. For example, Schuler et al. have proposed a rather complicated model to describe the cellular growth cycle of Escherichia coli [85]. In that model, key metabolic reactions as well as the detailed cell wall synthesis mechanism have been accounted for. As a result, a very large number of equations and parameters were needed in the model, a common characteristic of complex structured
models. Although highly structured models seem to be the only logical choice for describing the different physiological states of the microbes, there remain serious problems for it to be of practical use. Firstly, the computational time requirement of such large systems of equations is enormous, although this problem may be eventually solved by the availability of faster computers. The second problem is much more difficult to deal with. Unlike the unstructured models whose parameters can be easily determined by either independent experiments or various simple data-fitting methods [69,87], there is not enough biochemical information or good data-fitting methods to determine all the values of the huge set of parameters needed in complex models. When the number of the parameters that need to be fitted is large, it is difficult to obtain meaningful results, particularly when the number of the observable variables is small as compared to the number of fitted parameters. It has been demonstrated by Reich and Selok [88] that totally different models can produce the same result if appropriate parameters were used. This is to say that complex models with many fitted parameters add little to the knowledge of microbial kinetics save for the results of the experiments that were performed to fit those parameters. Esener et al. [71] have proposed rigorous criteria for judging the usefulness of complex structured models, namely, that a model should be judged not only from its performance in predicting the commonly observable
variables, but also from its performance on the variables present in the model to justify the mechanisms included in the model. Nevertheless, with the advancement in various biosensors, as, e.g., in microfluorometers [89,90], more and more intracellular quantities can be measured and as the biochemical information about the cellular metabolism accumulates, structured models will become more and more manageable and attractive as the tool in elucidating the complex behavior of microbial cultures.

In most industrial fermentation processes, it is sufficient to treat the microorganisms as a homogeneous population, and the averaged behavior of this population can be related to the environmental parameters in an average sense [24]. Thus, most models employed in industrial fermentation processes are of this nature, and are referred to as nonsegregated models [91]. Since these models deal with the properties of a whole population they are also deterministic in their formulation. On the other hand, there are some cases where only a small number of cells is involved in the processes under investigation, such as sterilization or mutant selection. Then, it is necessary to account for the individual behavior of each cell. These models are called segregated models, and they are mostly stochastic in nature [79].

This chapter will focus on the modeling and simulation of the M. L3 growth in continuous cultures. An unstructured
model proposed by Chen et al., and DiBiasio et al. [30,92,93] will be modified to incorporate the maintenance energy concept and substrate inhibition kinetics. The simulation results of the modified model will be compared to the experimental transient data presented in Chapter 2.

4.2 MODEL

The kinetics of substrate inhibition of growth has been incorporated into a basic Monod-type model by many researchers [31,69,70]. A lengthy discussion about the possible mechanisms and the justification of such a formulation has been published [69]. Of particular interest is the finding that, in substrate-inhibited growth, there exist three steady states under certain process conditions, and that one of them is unstable [30,31]. The stability and the steady state multiplicity problems have been thoroughly dealt with [30,31,69,70]. DiBiasio et al. [30] used the data obtained from unstable steady state continuous cultures of L3 to fit expressions relating the specific growth rate and the biomass yield to the residual methanol concentration as follows,

\[ \mu = \frac{0.504S(1-0.0204S)}{0.00849+0.0406S^2} \]  \hspace{1cm} (4-1) \\
\[ Y = \frac{0.383S(1-0.0204S)}{1.0+0.296S+0.509S^2} \]  \hspace{1cm} (4-2)

where \( \mu \) is the specific growth rate (l/hr), \( Y \) is the biomass yield (g dry wt/g methanol) and \( S \) is the methanol concentration in g/l.
An open-loop chemostat is unstable at high residual methanol concentrations, due to growth inhibition by methanol, so that earlier data concerning high residual methanol levels were all obtained from batch experiments. DiBiasio et al. have designed a control system which made it possible to stabilize the previous unstable steady state [30], and thus their data represented a great improvement over earlier work. The physical meaning of the formulation used in the above two equations has been discussed by Chen et al. [92]. However, the biomass yield data of DiBiasio et al. on the stable-steady-state range were not comparable to the data reported by others for the L3 [33] and the data obtained during this research. Not only were they much lower, but also they did not show the dependence of yield on the dilution rate, which has been suggested to result from the maintenance requirements of the cells (9),

$$\frac{1}{Y} = \frac{1}{Y_{\text{max}}} + \frac{m}{\mu} \quad (4-3)$$

where $Y_{\text{max}}$ is the biomass yield with no maintenance requirement and $m$ is the maintenance coefficient.

It was, thus, necessary to modify eq. 4-2 to fit the steady state data first so that the model simulation results could be compared to the transient experimental data. It was found that the relation between the biomass yield and the residual methanol concentration can be best represented by the following equations,
\[ Y = 0.5(1 - 0.0204S)/(1 + 0.467S - 0.0089S^2), \]
\[ S > 55 \text{ mg/l} \quad (4-4) \]
\[ 1/Y = (1/0.549 + 0.1/\mu), \]
\[ S < 55 \text{ mg/l} \quad (4-5) \]

Figs. 4.1 and 4.2 present the comparison of the modified version of the biomass yield expression (eq. 4-4,4-5) to that of eq. 4-2 together with the original data used by DiBiasio et al. This set of modified equations means that when the residual methanol concentration is below 55 mg/l, i.e. in the range of stable steady states, the biomass yield is best correlated to the specific growth rate and the maintenance energy requirement (eq. 4-4). A value of 0.10 (g MeOH/g dry wt -hr) for \( \mu \) was used. This value is in reasonable agreement to published data [94] for other methylotrophs (0.09-0.12), and fits very well the yield data obtained in this study. Fig. 4.3 shows the correlation of the biomass yield to the dilution rate.

A model for continuous culture can be constructed, by combining eqs. 4-1, 4-4 and 4-5 with the mass balance equations for biomass and methanol, namely,

\[ \frac{dX}{dt} = \mu/X \quad (4-6) \]
\[ \frac{dS}{dt} = D(S_f - S) - \frac{\mu X}{Y} \quad (4-7) \]

\( X \) represents the biomass concentration (g dry wt/l), \( D \) denotes the dilution rate (l/hr), and \( S_f \) is the feed
Figure 4.1. Biomass yield versus residual methanol concentration.
Figure 4.2. Biomass yield versus residual methanol concentration. (At lower methanol concentrations)
Figure 4.3. Biomass yield versus dilution rate.
methanol concentration (g/l).

This model constitutes an initial value problem with nonlinear ordinary differential equations. The package EPISODE from the Rice University, Department of Chemical Engineering computer system was used for the integration of eqs. 3-5 and 3-6. Phase plane analysis was carried out by plotting methanol concentration vs. biomass concentration to represent a transient in the fermentation process. Phase plane analysis is one of the most widely used methods for analyzing the dynamic behavior of nonlinear systems, especially for systems with two dependent variables [95].

4.3 RESULTS

Two dilution rates (0.46 l/hr and 0.2 l/hr) were used in conjunction with various feed methanol concentrations (0.79, 7.9 and 70 g/l) for the phase plane analysis.

The phase plane diagrams are presented in Figs. 4.4 - 4.7. In Fig. 4.4, where the methanol feed concentration is 0.79 g/l, there is only one steady state. Whether this steady state is the washout state, denoted by \( W \), or a non-trivial state, denoted by \( A \), depends on whether the dilution rate exceeds the maximum specific growth rate, about 0.48 l/hr, as predicted by eq. 4-1. When the feed methanol concentration is 7.9 g/l or 70 g/l, there are three steady states, one of it is unstable (B), and the other two are
Figure 4.4. Phase-plane diagram of a continuous culture, $S_f = 0.79$ g/l, Dilution rate = 0.46 l/hr.
Figure 4.5. Phase-plane diagram of a continuous culture, \( S_f = 0.79 \text{ g/l} \), Dilution rate = 0.2 l/hr.
Figure 4.6. Phase-plane diagram of a continuous culture, Sf = 7.9 g/l, Dilution rate = 0.46 l/hr.
Figure 4.7. Phase-plane diagram of a continuous culture, $S_f = 70 \text{ g/l}$, Dilution rate = 0.46 l/hr.
stable (A and W). These conclusions can be proved using stability analysis [30,69].

The trajectories on the phase plane describe the dynamic characteristics of the chemostatic reactor, and represent the transient behavior exhibited from the initial point of the trajectory toward the stationery point, i.e. the steady state. Before the model and the phase plane diagrams generated by it can be applied to the design of a control system or the startup procedure, their validity in predicting the transient behavior must be examined first by comparing the simulated results with the transient data presented in Chapter 2. The specific growth rate profiles of shift-up and shift-down transients are replotted in Figs. 4.8 and 4.9 together with the profile predicted by the present model. It is clearly shown that the simulated specific growth rate profiles in both case are overdamped while the experimental results exhibit damped oscillations. The behavior of the dilution rate shift-up and the methanol-pulse transients at different dilution rates and different substrate compositions are also depicted in their phase-plane trajectories as shown in Figs. 4.10 to 4.18. For single-substrate cultures at dilution rates lower than 0.46 l/hr, the observed trajectories fall on the left side of the model prediction, while those of the mixed substrate cultures fall on the right side. At dilution rates of 0.46 and 0.49 l/hr, the experimental data are in better agreement with the predictions, but the observed trajectories return
Figure 4.8. Comparison of the model prediction of the specific growth rate profile of a dilution rate shift-up transient to the experimental data (transient exp. # 1).

Figure 4.9. Comparison of the model prediction of the specific growth rate profile of a dilution rate shift-down transient to the experimental data (transient exp. # 2).
Figure 4.10. Comparison of the model prediction to the experimental data by phase plane diagram (transient exp. #1). □: experimental data, ---: model prediction.
Figure 4.11. Comparison of the model prediction to the experimental data by phase plane diagram (transient exp. #3). □: experimental data, — and — : model predictions based on the calculated pulse magnitude and the first measurement of residual methanol concentration after pulse, respectively.
Figure 4.12. Comparison of the model prediction to the experimental data by phase plane diagram (transient exp. #4). □: experimental data, — and —: model predictions based on the calculated pulse magnitude and the first measurement of residual methanol concentration after pulse, respectively.
Figure 4.13. Comparison of the model prediction to the experimental data by phase plane diagram (transient exp. #5). □: experimental data, — and ——: model predictions based on the calculated pulse magnitude and the first measurement of residual methanol concentration after pulse, respectively.
Figure 4.14. Comparison of the model prediction to the experimental data by phase plane diagram (transient exp. #6). □: experimental data, —— and ——: model predictions based on the calculated pulse magnitude and the first measurement of residual methanol concentration after pulse, respectively.
Figure 4.15. Comparison of the model prediction to the experimental data by phase plane diagram (transient exp. #7). □: experimental data, — and ——: model predictions based on the calculated pulse magnitude and the first measurement of residual methanol concentration after pulse, respectively.
Figure 4.16. Comparison of the model prediction to the experimental data by phase plane diagram (transient exp. #8). □: experimental data, —— and ——— : model predictions based on the calculated pulse magnitude and the first measurement of residual methanol concentration after pulse, respectively.
Figure 4.17. Comparison of the model prediction to the experimental data by phase plane diagram (transient exp. #9). □: experimental data, --- and ——: model predictions based on the calculated pulse magnitude and the first measurement of residual methanol concentration after pulse, respectively.
Figure 4.18. Comparison of the model prediction to the experimental data by phase plane diagram (transient exp. #10). □: experimental data, — and —: model predictions based on the calculated pulse magnitude and the first measurement of residual methanol concentration after pulse, respectively.
to the original steady state more readily, and in one case, at 0.49 l/hr, the model predicts washout while in reality the culture returned to the non-trivial steady state. In summary, the low dilution rate, single-substrate cultures show more severe growth inhibition effect (after the methanol pulse) than what is predicted by the model, while the opposite applies to the mixed-substrate cultures. At high dilution rates, model predictions are very sensitive to the parameters used. As will be discussed later, this may explain the discrepancies observed between the observed and predicted results. In Figs. 4.19 and 4.20, methanol concentration is plotted vs. the growth rate for the transient experiments #3 and #7, together with the model predictions. A phenomenon called growth rate hysteresis [96,97] is observed for the transient data obtained from these methanol-pulse transient experiments.

4.4 DISCUSSION

The present model is better than the original model proposed by DiBiasio et al. [30] in that it offers a better correlation to the stable-steady-state biomass yields. Its performance is therefore much better in simulating the transient behavior in the stable-steady-state region, as for example, in the dilution-rate shift transients. It is however still difficult to predict other steady state properties. For example, at a dilution rate of 0.46 l/hr,
Figure 4.19. Comparison of the model prediction to the experimental data in the correlation of the specific growth rate versus residual methanol concentration. (transient exp. #3). \( \Delta \): experimental data, \(--\): model prediction.
Figure 4.20. Comparison of the model prediction to the experimental data in the correlation of the specific growth rate versus residual methanol concentration. (transient exp. #7) ∆: experimental data, ——: model prediction.
the model predicts a residual methanol concentration of 0.094 g/l, while the experimental data show less than 0.01 g/l of residual methanol. This problem arises from the scarcity of good experimental data at low methanol concentrations. In general, the model is capable of predicting the steady state biomass and methanol concentrations, and other parameters such as the biomass yield and the specific growth rate over a wide range of residual methanol concentrations with fairly good accuracy.

As was discussed in the Introduction, unstructured models are not expected to predict the transient growth behavior well because they do not account for the complexity of the intracellular events. For the present model of methylotrophic growth, this general observation is still applicable. The model performs better in dilution-rate-shift transients than in methanol-pulse transients. This is because of the more drastic perturbation employed in the latter type of transient. The overshoot and oscillating behavior observed in dilution-rate-shift transients reveals the complex dynamic mechanism involved in the regulation of the growth rate, which is not accounted for in the unstructured model. The discrepancy between the model prediction and observed results can be minimized by finding suitable time constants that can be used in an empirical dynamic equation [29,83]. The deviation of the model predictions from the actual methanol-pulse transient behavior is harder to eliminate through model modification.
There are two major events that the unstructured model fails to predict. The first is the unusual residual methanol profile, which indicates the existence of an active transport mechanism in methanol uptake as was discussed in Chapter 2. The second one is that the specific growth rate and biomass concentration are lower than what the model predicts in the single-substrate cases. The latter discrepancy is perhaps due to the toxic effects of methanol or its subsequent metabolites. As was discussed in Chapter 2, the toxic effect does not just depend on the concentrations of methanol or formaldehyde but also on the physiological state of cell, in other words, the intracellular composition of biomass. Since unstructured models assume a constant composition of biomass, it is not suitable to describe such situations. The results from the mixed-substrate experiments show a higher than predicted specific growth rate for a given methanol concentration. This again shows the inadequacy of the present two-variable model. DiBiasio et al. [30] have reported that when the feed-back control was removed from the bioreactor system operated at the middle (unstable) steady state (state B in Fig. 4.7), washout was observed in every case, even when a finite perturbation favoring the stable steady state (state A in Fig. 4.7) was introduced. They have attributed this phenomenon to the formaldehyde toxicity. They concluded that, since the unstructured model did not include formaldehyde concentration as a variable, it could not
predict that phenomenon. In view of the data presented here, however, the above observation may be oversimplified. Although formaldehyde is not an explicit variable in the unstructured model it is implicitly determined by the two state variables, biomass and methanol concentrations. Thus its effect is not totally disregarded by the unstructured model. Furthermore, the formaldehyde level at the unstable steady state is in the range of less than 5 mg/l, a level not uncommon during exponential phase of batch cultures (see Chapter 3), where the specific growth rate and the biomass yield are higher than those at unstable steady states. Therefore, the above phenomenon cannot be attributed to the formaldehyde toxicity alone; methanol and the physiological state of the cells are also apparently involved. An investigation of the substrate inhibition mechanism in a methanol-utilizing yeast using a model discrimination technique has also suggested that the inhibition effect cannot be attributed to formaldehyde alone, and that methanol is also growth inhibiting [98].

In view of the limitation of unstructured models, it would be interesting to contemplate on the possibility of constructing a structured model for methylotrophic growth. A conceptual, mechanistic model has been proposed earlier, based on the enzymatic profile of M. L3 under different growth conditions [60,99]. In this model, methanol induces of methanol dehydrogenase (MDH) and hexulose-6-phosphate synthase while formaldehyde represses both enzymes but
induces formaldehyde dehydrogenase. Formaldehyde can also inhibit MDH. This conceptual model no doubt will provide a basis from which a structured model may be constructed. However, because of the lack of quantitative data on those proposed mechanisms, this model cannot be evaluated or tested in a more quantitative way. Qualitatively speaking, this model cannot account for the specific growth rate drop in methanol-pulse transients either, just like the unstructured model. This is because methanol does not exert any adverse effects on the growth rate except via formaldehyde in this conceptual model, and the adverse effect caused by formaldehyde are somewhat cancelled out by methanol, so that high methanol concentrations do not cause much of an adverse effect on the growth rate or the biomass yield. A more detailed account of the adverse effect of methanol on the growth rate and biomass yield is necessary to improve this model. Another area where improvement is possible is in the energy metabolism. Both oxidation pathways (see Chapter 3) and their energetic characteristics and regulation mechanisms should be incorporated into the conceptual model.

Process economics require the use of high substrate feed concentrations in order to increase productivity and reduce harvesting costs. The Single-Cell Protein production process is found to be optimally operated under dual limitation of carbon substrate and oxygen [100], since the supply of oxygen is often limited by the configuration of
the bioreactor. Dual substrate limitation kinetics [101] should also be included in the structured model for this reason. In view of, (i) the fast development of various direct methods of measuring intracellular variables, (ii) the indirect estimation methodology for on-line measurement of important parameters, and (iii) the use of adaptive control strategies facilitated by computers, further efforts for the development of a simple, flexible structured model for methylo trophic growth would be worthwhile.
Chapter 5

SUMMARY

The dynamic behavior of strain L3 in continuous cultures was studied in order to understand not only the regulatory mechanisms of cell growth under various transient situations, but also to elucidate the energetic and physiological basis of the lower biomass yields obtained in large-scale bioreactors, where inhomogeneities in methanol concentration, both temporal and spacial, exist. Dilution-rate shifts and methanol-pulse additions were used to induce transients in a chemostat, employing single- and mixed-substrate (methanol plus formaldehyde) cultures. The values of the concentrations of biomass, residual methanol, residual formaldehyde as well as dissolved oxygen were measured during the transients, and the specific growth rate, the biomass yield and the methanol-uptake rate were calculated. At dilution rates lower than 0.46 l/hr, the methanol-uptake rate profiles of most experiments, single- or mixed-substrate, displayed an unusual phenomenon by showing negative MUR values for a time period following the methanol pulse. This phenomenon is probably a consequence of a methanol active transport system. In single-substrate cultures under a methanol-pulse transient, the specific
growth rate decreased immediately after the methanol addition (final concentration in reactor: 6.7 or 13.4 g/l) except in one case where a smaller amount of pulse was used (2.23 g/l). In mixed-substrate cultures, on the other hand, the specific growth rate increased after the methanol addition (6.7 g/l). The biomass yield, as expected, decreased after the methanol addition in all experiments. However, the drop in the biomass yield is more severe in the single-substrate experiments, especially at lower dilution rates, than in the mixed-substrate ones. The residual formaldehyde concentrations rose after the methanol addition. In general, this rise was larger at lower dilution rates or for larger methanol pulse. In mixed-substrate experiments, the rise was even larger, but did not seem to have any adverse effects on cell growth as in the single-substrate experiments. It appears that the mixed-substrate cultures were better adapted to larger fluctuations in methanol concentration. In dilution-rate shift experiments, overshoot and damped oscillatory behavior of the specific growth rate was observed for both shift-up and shift-down experiments. This reflects the adaptation mechanism of the cells to a new growth rate.

Radioactive labels were employed to elucidate the substrate oxidation and incorporation patterns of the strain L3. The understanding of such patterns is essential to a more precise prediction of the theoretical biomass yields as well as the understanding of the metabolic regulation
mechanisms. A thorough mathematical derivation of the proposed procedure was presented, which enables the calculation of the reaction rates of the oxidation pathways, the rates of the general decarboxylation and carboxylation reactions, as well as the rate of the direct biomass incorporation. $^{14}$C-labeled methanol, formate and [1-C]glucose were used as the tracers in a series of experiments with batch-grown, exponential-phase cells. The results showed that both oxidation pathways were operating in vivo, and that the extent of utilization of each pathway depended on the initial substrate(s) concentrations as well as on the state of the inoculum. The extent of the utilization of the cyclic oxidation scheme was larger in the cultures that had initial methanol concentration at 2.38 g/l than those having much higher (14.5 g/l) or lower (0.63 g/l) initial methanol concentrations. A mixed-substrate culture, with 2.8 g/l methanol and 70 mg/l formaldehyde initially, showed the highest extent of utilization of the cyclic oxidation scheme. The linear oxidation scheme operated to a significant extent in all experiments.

From the results of the tracer experiments mentioned above and those of the continuous cultures [57], where the cyclic oxidation scheme was minimally utilized, it is suggested that the cyclic oxidation scheme may serve a formaldehyde detoxification function, and it seems to be induced by formaldehyde or high methanol concentrations. This may in part explain the difference observed in the
dynamic behavior of the single- and mixed-substrate continuous cultures. The findings also suggest a possible way to increase the biomass yield by activating or inducing the cyclic oxidation pathway (which is more energetic than the linear one) under continuous-cultivation condition.

An unstructured growth model for strain L3 was constructed based on steady-state data. This model incorporates the basic Monod formulation with substrate-inhibition and the maintenance energy concept. The ability of this model to predict the transient cell behavior was tested by comparison to the transient data obtained. Based on the phase plane analysis, the model performed better at higher dilution-rate cases, and, in general, the observed growth rates were lower than the predictions in single-substrate experiments and higher than predictions in mixed-substrate experiments. The toxic effect of the methanol pulses to the cells was, therefore, underestimated for the cells growing at lower dilution rates, and overestimated for the mixed-substrate grown ones. This observation suggests that the toxic effect of the substrate(s) depends on the physiological state of the cells, and that a structured model is needed to account for the different physiological states.
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Appendix A

CHARACTERISTICS OF METHYLOMONAS L3

The nomenclature of the obligate methanol-utilizing bacteria has not been established officially [5]. Therefore, the name Methylomonas is just a preliminary one. It has been suggested that Methylomonas should only include the methane-utilizing bacteria [5]. Therefore, L3 should be given another generic name. However, the current name is retained until an official nomenclature is established. There has been only one validly published species of the obligate methanol-utilizing bacteria [102]. This is probably due to the lack of a widely acceptable battery of characterization tests. Some tests, including DNA/DNA hybridization [103,104], propose that there should be two distinct taxa in this group of bacteria, and were preliminarily named as Methylophilus and Methylobacillus [103,104]. Therefore, phenotypic properties and biochemical information of L3, either observed during this research or reported by others [33], is presented here in order to assist the determination of the taxonomic status of this strain.

L3 is a gram negative, oxidase and catalase positive, small rod, 0.5 μm by 1.0 to 1.5 μm in size and motile [33].
Chains of 2 to 4 cells linked end-to-end were usually observed during mid-exponential phase although single rods were more common. When grown on base salt plus 2% v/v methanol agar medium, L3 forms pale white colonies that are round, entire, convex and translucent [33]. A 48-hour old colony has a diameter of 1-2 mm and does not exceed 4 mm later. Characteristic scratch marks develop at the center of 48-hour old colony. Those marks were helpful in detection of contamination. Nitrate can serve as nitrogen source although ammonium is preferred [33]. Carbon substrates that support growth of L3 in liquid cultures include methanol, methylamine, formaldehyde and a mixture of methanol with formaldehyde or formic acid. Slight growth on glucose in liquid or agar medium was also observed. Aggregates of cells of the size of 1-2 mm appeared in liquid cultures when formaldehyde was used as substrate or co-substrate. Those aggregates also appeared in methanol-based cultures when the initial methanol concentration exceeded 1% v/v or when cultures were started fresh from an agar plate colony. No aggregates were observed in methylamine liquid cultures. Capsule formation was checked by the India-ink stain method. Cells grown in the presence of methanol, with or without formaldehyde as co-substrate, did not show any capsule formation whether in liquid or in agar medium. Cells grown on formaldehyde alone were found to have capsules. Optimal temperature for growth is 30 °C, and there is no growth observed at 37 °C [33].
L3 possesses the key enzymes of the KDPG/TA variant of the RuMP cycle, the linear oxidation and the cyclic oxidation pathways while no activities of the key enzymes for the serine pathway or the FBP/SDP variant of the RuMP cycle were observed [57].
Appendix B

DETAILED ACCOUNTING OF CARBON DIOXIDE'S INVOLVEMENT IN BIOSYNTHETIC REACTIONS

The amount of CO$_2$ produced via general decarboxylation reactions and the amount of its fixation via general carboxylation reactions can be estimated by studying those reactions in a model cell, *Escherichia coli* B/r, which can approximately represent average bacterial cells. Detailed information of the biosynthetic reactions of this model cell is readily available [105]. For example, the amount of every building block needed for a given amount of biomass has been determined. Therefore, with the information on every key biosynthetic reaction pathway available [62], it is possible to calculate the total amount of CO$_2$ decarboxylated and carboxylated for a given amount of biomass. For instance, 1078.9 µmol of α-oxoglutarate is needed for 1 g dry cell biomass [105], and to make 1 mole of α-oxoglutarate from pyruvate, there are two moles of CO$_2$ produced. Hence, a total 2157.8 µmol of carbon is decarboxylated in making this particular building block for 1 g of biomass. In Table B.1, a complete list of the results from such calculations is presented. It should be noted that, in RuMP-type methylotrophs, the TCA cycle is
incomplete and that they do not have the glycolytic pathway as *E. coli* does. Therefore, provisions have been made for these differences in the calculations presented. The total number of moles of carbon in 1 g dry biomass was calculated to be 41666 µmol, based on the elementary analysis of the cell, which shows that 50% wt. of dry biomass is made of carbon [105].

From the results shown in Table B.1, it can be calculated that 0.80 C-moles are decarboxylated and 0.315 C-moles carboxylated, or a net 0.485 C-moles decarboxylated per 4 C-moles incorporated into biomass. Therefore, 0.86 C-moles are decarboxylated per 4 C-moles directly incorporated into biomass, which is the value of x defined in Chapter 3. Because of the reasons detailed in Chapter 3, a value of 0.8 was used for x, instead.
### Table B.1 Carbon Dioxide Accounting in Biosynthesis

<table>
<thead>
<tr>
<th>Building block</th>
<th>Amount required (μmol/g-dry wt)</th>
<th>Decarbox. (mol/mol)</th>
<th>Carbox. (mol/mol)</th>
</tr>
</thead>
</table>

#### Amino acids
- Lys: 326
- Phe: 176
- Tyr: 131
- Leu: 428
- Isl: 276
- Val: 402

#### Nucleotides
- UTP: 136
- CTP: 126
- ATP: 165
- GTP: 203
- dATP: 24.7
- dGTP: 25.4
- dTTP: 24.7
- dCTP: 25.4

#### Polyamines
- polyamines: 59.3

#### Other compounds
- AcCoA: 3747.8
- α-Oxoglutarate: 1078.9
- Oxalacetate: 1786.7

Total (μmol/g dry wt): 8321.6 3283.7