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APPENDIX

Some of the kinematical quantities associated, directly or indirectly, with the model are defined here. Let

$\mathbf{r}$ = position at the present time t of an arbitrary point on the interface
$\mathbf{X}(s, \mathbf{r})$ = position at time $t - s$ of the material point whose position at time t is $\mathbf{r}$

A surface deformation tensor $\mathbf{C}$ can be defined by

$$\mathbf{C} = (\nabla_s \mathbf{X}) \cdot (\nabla_s \mathbf{X})^T$$

$\mathbf{C}$ is the surface analog of the Cauchy deformation tensor (Astarita and Marrucci, 1974). The physical significance of $\mathbf{C}$ is that

$$|d\mathbf{X}|^2 = d\mathbf{r} \cdot \mathbf{C} \cdot d\mathbf{r}$$

Another surface deformation tensor $\text{inv}_s\,\mathbf{C}$ or the surface inverse of $\mathbf{C}$ can be defined by

$$\text{inv}_s\,\mathbf{C} \cdot \mathbf{C} = \mathbf{C} \cdot \text{inv}_s\,\mathbf{C} = \mathbf{1}_s$$

$\text{inv}_s\,\mathbf{C}$ is not to be confused with the conventional inverse of $\mathbf{C}$, $\mathbf{C}^{-1}$, which does not exist. Furthermore, $\text{inv}_s\,\mathbf{C}$ is not the surface analogue of the Piola deformational tensor (Astarita and Marrucci, 1974) despite its definition. Such an analogue does not exist. Two surface strain tensors can now be defined by

$$\mathbf{G} = \mathbf{C} - \mathbf{1}_s$$

and

$$\mathbf{H} = \text{inv}_s\,\mathbf{C} - \mathbf{1}_s$$

$\mathbf{G}$ is the surface analog of the Cauchy strain tensor (Astarita and Marrucci, 1974). However, $\mathbf{H}$ is not the surface analogue of the Finger strain tensor (Astarita and Marrucci, 1974) since, again, such an analogue does not exist because two-dimensional spaces do not have a volume. The surface strain tensor $\mathbf{L}$ appearing in the model is defined by

$$\mathbf{L} = 1/2(\mathbf{G} - \mathbf{H})$$

Finally, the scalar ψ appearing in the model is given by

$$\psi = 1/2(tr\,\mathbf{G} + tr\,\mathbf{H})$$

For the behavior of the above kinematical quantities in different surface flows, see Gardner (1975).

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SCP Production on C_1 Compounds

Given the partial oxidation products of methane as the chemical feedstock for single-cell protein (SCP) production, their conversion efficiency is optimized by optimal selection of the pertinent microorganisms that can utilize the feedstock. The coenzymes of the three oxidation steps from methanol to formaldehyde to formate and carbon dioxide as well as the pathway involved in carbon fixation determine the cell-mass yield. Microorganisms which follows the ribulose monophosphate cycle and which have coenzymes which are linked to the maximum production of ATP (for example, NAD linked dehydrogenases), should be searched for economical production of SCP. Batch growth rate and yield data are limited in utilization and interpretation, and care should be exercised in their use. The subject of this case study, *Methylomonas* EP-1, gives an optimal cell mass yield of 65% and a stable chemostat operation at space velocities as high as 0.58 hr^{-1}.

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SCOPE

Great interest has been expressed in the last decade in the production of single-cell protein (SCP) from C_1 compounds as a sole carbon and energy source. Although a few processes are currently on stream, several university and industrial research groups are highly involved in the development of various alternative SCP processes. In all these new ventures, multidisciplinary teams are cooperating at various project stages from fundamental,

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exploratory research to product acceptability studies.

SCP production is a biological process in which organic and/or inorganic carbon together with inorganic nitrogen and certain essential minerals are converted into a product of which protein material is the major constituent.

The heart of any SCP process being the reactor system, it is instructive to approach its design by a systems analysis, starting with the study and understanding of the rates and the stoichiometry of the species involved in the biological reaction. This enables overall heat and mass balances to be derived in terms of the system variables, hence facilitating optimization of the unit operations. Topiwala (1975) shows, in diagrammatic form, such an analysis for the reactor system. From this analysis, important areas of research and development are identified, among which are:

1. Reaction stoichiometry; search for high protein yielding organisms.
2. Fermentation kinetics; understanding and control of biological reaction rates.
3. Reactor design for heat transfer; meaningful correlations for fermentation broths to enable scale-up and optimization.
4. Reactor design for mass transfer (of gaseous nutrients and products); meaningful correlations for real systems.

A continuous biochemical reactor can exhibit instability as a consequence of the sensitivity of microorganisms to a host of environmental factors. The most obvious variables, such as temperature, *p*H, and pressure, present conventional problems to the control/process engineer, but microorganisms can also be very sensitive to fluctuations in both major and minor constituents of raw material and product streams, such as trace elements and vitamins in the raw material stream and excreted metabolic byproducts in the product stream. Utilization of mixed cultures presents even more critical research problems concerning the reactor instabilities that these cultures may exhibit.

The ultimate problem, however, is the selection of the microorganisms. Even if the carbon and nitrogen sources have been chosen in advance, the selection of the microorganism is an ostensibly indeterminable problem. For there may exist an immense number of microorganisms which utilize the particular carbon and nitrogen sources. Thus, it is essential to understand the biological (or even molecular) basis of the cellular growth processes so that we can:

1. Assign the limits to the conversion efficiency of alternate chemical feedstocks and/or of microorganisms with given carbon substrate(s).
2. Set systematic and absolute criteria for the optimal selection of the microorganism(s).
3. Identify the important process parameters.
4. Develop methods to measure these parameters.
5. Formulate dynamic models.
6. Implement control strategies.

As a conclusion, it has been emphasized (Topiwala, 1975) that good mechanical design without regard to the biological features will not provide an efficient industrial SCP process.

Accomplishment of these research and development tasks may require appreciation of a great deal of sophisticated biochemical information and even acquisition of new biochemical information by utilizing techniques and strategies potentially unfamiliar to chemical engineers.

The present paper examines several aspects of the problems discussed above. Its aim is threefold: the first is to question certain traditional methodologies and data utilization and to compare techniques with respect to data reliability; the second is to relate cellular macrocharacteristics to microcharacteristics and thus to set safe criteria for the selection of microorganisms; the third is to put on record some important cellular characteristics of a uniquely potential microorganism for SCP production.

CONCLUSIONS AND SIGNIFICANCE

The selection of microorganisms for SCP production on C_1 compounds can be safely based on the biochemical microcharacteristics of the microorganisms. Three enzymes responsible for the complete oxidation of methanol can reveal the most important information about the cell-mass yield that can be obtained with the microorganism under optimal conditions. Microorganisms utilizing the ribulose monophosphate cycle are, in general, energetically superior to those utilizing the serine pathway. Biochemical information and techniques can go a long way to facilitate the engineering work.

Batch growth rate data are not good estimates of the steady state continuous data they are sometimes supposed to represent. Modeling for continuous reactors should not be based on batch data. However, batch data can yield useful information regarding the nature of substrate inhibition and its control mechanisms.

Cell-mass yield data from batch experiments are often misleading, as they may only represent a small fraction of the optimal yields. In addition, the techniques used for their acquisition involves serious errors due to the dependence of yield on substrate concentration and other factors. An understanding of these dependencies can lead to the elucidation of the physiology and kinetics of growth on inhibitory substrates.

Formaldehyde can be used as a cosubstrate of methanol, but given its inhibitory nature, its amount relative to that of methanol has to be carefully chosen as a function of the reactor cell density. The cell-mass yield on formaldehyde is expected to be lower than that on methanol, but still significant, as can be shown from a theoretical analysis similar to that of methanol. Since formate is not incorporated into cell mass when the ribulose monophosphate cycle operates, its yield is expected to be zero; however, it is a source of energy. Mixed cultures exhibit some attractive characteristics for SCP production. A slight reactor pressure and atmospheric carbon dioxide can enhance cell-mass productivity under certain circumstances.

The subject of this work, the C_1 compound utilizing

Methylomonas **EP-1, offers the most attractive characteristics ever reported for SCP production, namely, a yield as high as 65% and stable chemostat operation at a space velocity as high as 0.58 hr^{-1}.**

The advantages of methanol as substrate for single-cell protein (SCP) production among nonconventional substrates (that is, not sugars, pure or mixed with other carbonacious compounds) are well documented (Cooney, 1975). Small quantities of formaldehyde and formate are usually found in methanol produced by a simple oxidation process from natural gas. It is of particular importance to examine, therefore, the possibility of using the crude methanol without purification for obvious reasons of lower carbon cost. Formaldehyde and formate can serve as carbon sources by themselves for certain microorganisms. The yields and growth rates are usually low, however (Goldberg et al., 1976). The molecular basis behind this fact is examined rigorously in the light of accumulated biochemical knowledge, and experimental information and criteria for selecting microorganisms for SCP production are established.

From the major categories of microorganisms which can utilize C_1 compounds, yeast and bacteria have been examined extensively in the past. Fungi (other than yeasts) which can utilize C_1 compounds have been recently isolated (Sakaguchi et al., 1975), offering a stimulating alternative in addition to their unique properties. Yeasts have been chosen as a subject of extensive studies for possible SCP production on C_1 compounds, mainly with the rationale that they are potentially better acceptable for food by the public and the relative ease of yeast-cell recovery. On these bases a very large number of yeasts has been isolated and studied extensively. Usually, when the macrocharacteristics (such as specific growth rate, cell mass yield) of these microorganisms were reported, the biochemical characteristics (such as biochemical pathway of C_1 compound utilization and key enzymes involved) were not, and vice versa. Reuss et al. (1974) summarized to a great extent the generalized data on yeasts. Pilat and Prokop (1975), Ogata et al. (1975), Dellweg et al. (1975), and Fujii et al. (1975) added useful and more sophisticated micro and macro information on yeasts. The uniqueness behind their versatile characteristics became evident; they assimilate methanol through the ribulose monophosphate (RMP) cycle (Strøm et al., 1974), they all possess a methanol oxidase for the first oxidative step of methanol so that this step is not coupled with ATP production, they cannot assimilate formaldehyde and formate as sole carbon and energy sources, and they usually require vitamin supplements.

A major cost factor for SCP production being the carbon-energy source, the cell-mass yield is of a singular importance. On the basis of the same yield for both bacteria and yeasts, bacteria have an inherent advantage over yeasts for they contain 60 to 70% protein against 45 to 55% for yeasts. In addition, as compared to yeasts, bacteria generally have a higher content and better balance of the essential amino acids: lysine, methionine, and threonine. In light of the recent work by van Dijken and Harder (1975) and the fact that yeasts always possess a methanol oxidase, the maximum theoretical cell-mass yield for yeasts is 54%, while the yield for bacteria can be as high as 73%. Since bacteria grow faster than yeast, the productivity of bacteria is much higher than that of yeasts. The only drawback of bacteria as a SCP source is their high nucleic acid content (10 to 20%) as compared to that of yeasts (5 to 12%), since the faster a microorganism grows the higher the nucleic acid content.

The continuous culture of microorganisms in a chemostat involves the growth of microorganisms in a well-mixed vessel of constant volume, that is, in a continuous stirred-tank reactor (CSTR). The nutrient solution is fed to the reactor at a constant flow rate, and the microorganism suspension is removed from the reactor at an equal flow rate. Under appropriately chosen flow rates, the microorganisms in the culture reproduce as rapidly as they are depleted by the continuous dilution of the culture with a fresh nutrient medium. Thus, the density of microorganisms in the reactor reaches a constant value characteristic of the dilution rate; that is, the space velocity $D = F/V$ of the reactor under given culture conditions (that is, temperature, pressure, culture pH, nutrient composition, etc.). A nutrient or a substrate is limiting when a small change of its concentration in the feed increases the steady state cell density while the rest of the culture parameters are kept constant. Provided that sufficient amounts of mineral nutrient salts are supplied and that the pH, aeration, and temperature of the reactor are controlled, carbon and energy sources become the most interesting limiting nutrient to be studied for SCP production. A series of steady states are established, to each of which there corresponds a unique concentration of the limiting substrate (S) which, in fact, imposes the growth rate that the cells exhibit at a unique cell density.

Many of the substrates (except sugars) inhibit cell growth at high concentrations, thus giving rise to a bell shaped curve of specific growth rate (μ) vs. substrate concentration (S). Starting from zero for zero S concentration, the specific growth rate increases with increasing S owing to stimulation of the cell metabolism. Eventually a concentration S_m is reached at which further increases cause a decrease in μ due to growth inhibition. A maximum (or a maximal plateau) is thus achieved for μ. Severe inhibition at high substrate concentrations may cause the growth to cease. Often the initial selection of the candidate microorganisms is based on the maximum μ they exhibit under optimal conditions.

The problem of biochemical reactor stability is another important aspect in SCP production. Spicer (1955) has shown that a necessary condition for the stability of the aforementioned steady states in the chemostat is $d\mu/dS > 0$. This condition prevails only to the left of the maximum (or maximal plateau) in the μ vs. S curve. Because there are two different substrate concentrations that correspond to a given specific growth rate, two possible nontrivial steady states exist for a given flow rate. The steady state corresponding to the higher substrate concentration is unstable in the chemostat. Disturbances in the cell density or substrate concentration may lead the system away from the steady state, thus giving rise to instabilities which may lead the system to washout. Washout is the transient reactor operation in which the cells cannot grow as fast as the dilution rate imposes so that they are eventually washed out from the reactor vessel. Based on the information on the μ vs. S curve, several investigators have conducted studies concerning the stability and the dynamics of the CSTR (Andrews, 1968; Yano and Koga, 1969).

Since chemostat experiments can produce only the rising portion of the μ vs. S curve, batch culture experiments have been traditionally used to produce the entire curve. The initial specific growth rate, evaluated by varying substrate concentrations and following the cell-mass accumulation, is assumed to represent the steady state that corresponds to the particular substrate concentration. During a batch-culture experiment, the substrate concentration is not changing significantly from the initial phase of growth, while all other nutrients are supposed to be in sufficiently high concentrations so that they will not be limiting during the course of growth. Other culture conditions are kept practically constant.

In a SCP reactor, the carbon must eventually end up in the cell mass or in carbon dioxide. Ideally, it is expected that other undesired by-products, if there exist any, are minimized. The dry cell mass of bacteria is generally represented by a formula such as $C_4H_8O_2N$ (van Dijken and Harder, 1975; Harrison et al., 1972). Thus, the cell-mass production from methanol can be represented by

$$\left(3.75+\frac{a}{2}\right)CH_3OH+\left(\frac{3a}{4}-7.125\right)O_2+3NH_3 \rightarrow 3C_4H_8O_2N+\left(\frac{a}{2}-8.25\right)CO_2+a\,H_2O \quad (1)$$

where a is a number which depends on the pathway utilized and on the coenzymes involved in the oxidation steps. By definition, the cell-mass yield is

$$Y_{X/S}\equiv-\frac{r_X}{r_S}=\frac{(3)(102)}{(32)\left(3.75+\frac{a}{2}\right)} \quad (2)$$

while the specific oxygen demand is

$$Y_{O_2/X}\equiv-\frac{r_{O_2}}{r_X}=\frac{(32)\left(\frac{3a}{4}-7.125\right)}{(3)(102)} \quad (3)$$

Hence, by eliminating a from (2) and (3) we obtain

$$Y_{O_2/X}=\frac{1.5}{Y_{X/S}}-1.33 \quad (4)$$

Obviously, the heat of fermentation, which is the heat of reaction of (1), is directly proportional to the oxygen demand. Thus, the relationship between the specific heat of fermentation (that is, the heat load per unit cell mass to be removed by cooling) and the cell-mass yield is of the functional form of (4). Considering the fact that cell-mass production will be limited eventually by the rate of oxygen transfer to the cells (Harrison et al., 1972), the relatively high cost of aeration and heat removal, and the impact of the latter on the fermentor design, it becomes obvious that the cell yield is a very decisive factor for the economic operation of SCP production (Cooney, 1975).

There are two known biochemical pathways (mechanisms) that can account for the biosynthesis of cellular constituents from reduced C_1 compounds: the serine pathway (Quayle, 1972) and the ribulose monophosphate (RMP) cycle (Strøm et al., 1974). A key enzyme in the serine pathway is hydroxypyruvate reductase which exhibits high activities (Harder et al., 1973). Its presence can be regarded as a positive test for the involvement of the serine pathway. When the serine pathway is operating, the oxidation level for the incorporation of the C_1 compounds can be either formaldehyde or formate. In addition, for every 2 moles of CH_2O or HCOOH incorporated, 1 mole of carbon dioxide is fixed. For the serine pathway we can write the overall equation (Harder et al., 1973) for the production of 3-phosphoglycerate (3-PGA)

$$2CH_2O+CO_2+ATP+2NADH_2 \rightarrow 3\text{-PGA}+ADP+2NAD \quad (5)$$

The pathway of methanol oxidation is

$$CH_3OH \xrightarrow{E_1} HCHO \xrightarrow{E_2} HCOOH \xrightarrow{E_3} CO_2 \quad (6)$$
$$X^* \; X^*H_2 \quad Y^* \; Y^*H_2 \quad Z^* \; Z^*H_2$$

Let X^*, Y^*, Z^* also represent the equivalent moles of ATP formed during each oxidation step. We therefore write the equations

$$CH_3OH \rightarrow CH_2O+X^*H_2 \quad (7)$$

$$CH_3OH \rightarrow CO_2+X^*H_2+Y^*H_2+Z^*H_2 \quad (8)$$

It is well established that Z^*H_2 is always $NADH_2$ (Quayle, 1972) which can give 3 moles of ATP. Thus, we obtain from (5), (7), and (8)

$$3CH_3OH+ATP+NADH_2 \rightarrow 3\text{-PGA}+3X^*H_2+Y^*H_2 \quad (9)$$

In the RMP cycle, the incorporation of C_1 compounds occurs only at the oxidation level of CH_2O. The key enzyme of this cycle is 3-hexulose phosphate synthase (HUPS), which catalyzes the reaction

$$\text{D-ribulose-5-phosphate}+CH_2O \rightarrow \text{3-Hexulose-6-phosphate} \quad (10)$$

This enzyme accepts also as substrate D-Ribose-6-PO_4, with a 10% lower activity. In this situation the enzyme will be referred to as hexose phosphate synthase (HPS). The presence of this enzyme can be regarded as a positive test for the involvement of the RMP cycle. In addition, the specific activity of HPS will be considered as a direct measure of the rate at which the incorporation of formaldehyde takes place or the rate of growth of the microorganism on methanol.

A similar development for the RMP cycle gives

$$3CH_3OH \rightarrow \text{3-PGA}+3X^*H_2+NADH_2 \quad (11)$$

As it is explained elsewhere (van Dijken and Harder, 1975), the synthesis of cell material from 3-PGA can be represented by

$$4(\text{3-PGA})+3NH_3+5.5\,NADH_2+29ATP \rightarrow 3C_4H_8O_2N \quad (12)$$

Obviously, we can also write

$$CH_3OH \rightarrow CO_2+X^*H_2+Y^*H_2+3ATP \quad (13)$$

Eliminating 3-PGA from (12) through (11) and using the energetic equivalence $NADH_2 \equiv 3ATP$ we obtain

$$12\,CH_3OH+3NH_3+33.5\,ATP \rightarrow 3C_4H_8O_2N+12\,X^*H_2 \quad (14)$$

Given the X^*H_2 and Y^*H_2 equivalences to moles of ATP, the exact ATP (energy) requirements of (14) can be met through (13), so that we can write an overall equation for the production of dry cell mass ($C_4H_8O_2N$) from methanol. For example, if $X^*H_2 \cong 1$ ATP and $Y^*H_2 \cong 3$ ATP, Equations (14) and (13) with the addition of appropriate moles of oxygen to the left-hand side and water to the right-hand side give

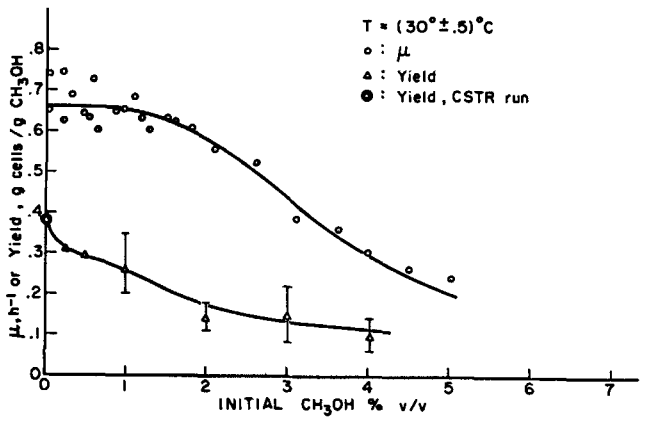

Fig. 1. Specific growth rate and cell-mass yield as a function of initial methanol concentration from batch experiments.

$$15.7\ CH_3OH + 3NH_3 + 10.605\ O_2 \rightarrow 3\ C_4H_8O_2N + 3.07\ CO_2 + 24.14\ H_2O \quad (15)$$

From (15), $Y_{X/S} = (3)(102)/(15.07)(32) = 0.63$.

Similar overall equations that permit the yield calculation can be written for the serine pathway, utilizing Equations (9), (12), and (13). Equations (9) and (11) show that the serine pathway requires energy for the production of 3-PGA, while RMP cycle produces energy with 3-PGA production. This suffices to conclude that microorganisms utilizing the RMP cycle exhibit always higher cellular yields than those that operate through the serine pathway. It is important to emphasize the (inherent) assumption made for the theoretical yield calculation, namely, that all the energy produced through complete methanol oxidation [Equation (13)] is used for cell synthesis according to Equation (12) or (14). Given that all the above reactions are enzymatic and that the amounts of corresponding enzyme are regulated according to the substrate concentrations (Papoutsakis et al., 1978), this important assumption will only hold for a unique substrate concentration. This prediction will be verified experimentally below.

The experimental values reported in recent years for the cell-mass yields of microorganisms grown on methanol have ranged from 0.25 to 0.54 (Whittenbury et al., 1970; Pilat and Prokop, 1975; Reuss et al., 1974; Cooney, 1975, with a collection of earlier data, Harrison et al., 1972; Battat et al., 1974; Goldberg et al., 1976; Dostalek et al., 1972).

For yeasts, the biochemical information necessary for the theoretical evaluation of the cell-mass yield is known (Reuss et al., 1974; Ogata et al., (1975); that is, $X^* = 0$, $Y^* = 3$, and the RMP cycle is involved. Thus, the yeast cell-mass yield is theoretically limited to 0.54. The highest experimental value ever reported for yeasts is 0.43 (Pilat and Prokop, 1975). For bacteria, owing to their versatility, it is impossible to make such a generalization with respect to the biochemical information needed to estimate the yield. This information was usually not available when the experimental yield figures were reported, with the exception of *Pseudomonas* C (Battat et al., 1974; Goldberg, 1976). An electron acceptor corresponding to $X^* = 3$ is very unlikely for a methanol dehydrogenase because the standard redox potential of the MeOH/CH_2O couple is not sufficiently negative for the reduction of NAD (Ribbons et al., 1970). However, such an enzyme has been reported recently to exist in a methanol utilizing fungi (Sakagouchi et al., 1975). The next most energetic bacteria correspond to $X^* = 2$ and $Y^* = 3$. The corresponding yield is 0.73, and the yield for the case of $X^* = 1$ and $Y^* = 3$ is 0.63. Such high yield figures have not yet been reported experimentally, posing the question of the existence of such bacteria. It is obvious that the discovery of a bacterium with a yield 10% higher than the one which is currently being used has a significant impact on the production of SCP. Not only will the substrate cost be reduced but also the processing (heat removal and oxygen supply) costs will be reduced very significantly.

EXPERIMENTAL MATERIALS, EQUIPMENT, AND METHODS

All the chemicals used were of analytical grade except for paraformaldehyde which was practical grade (Eastman Kodak Company, New York). All biochemicals have been purchased from Sigma Chemical Company (St. Louis, Missouri).

Methylomonas EP-1 used in this work was obtained as a contaminant of *Methylomonas albus* BG8 culture which was a gift of Professor J. G. Wilkinson (Edinburgh). The working medium contained $MgSO_4 \cdot 7H_2O$ 1 g/l, $CaCl_2$ g/l, NH_4Cl 0.3g/1, $FeSO_4 \cdot 7H_2O$ 0.01 g/l, $ZnSO_4 \cdot 7H_2O$ 0.025 g/l, 66 ml/l of *p*H 6.9 M phosphate buffer (final concentration: 0.0396 M) and EDTA-disodium salt 0.04 g/l. This medium was used for all the batch (shaking flask) experiments. In continuous culture experiments, the above medium was modified by using 0.9 g/l of NH_4Cl instead of 0.3 g/l and a *p*H 7.2 buffer instead of 6.9.

The reactor vessel of continuous culture experiments was 1 l with 300 to 350 ml working volume. The basic unit was the design of the New Brunswick Scientific Company (NBS) Bioflo model C-30 with the following modifications: a U tube was used to control the culture level using a peristaltic pump (30 to 600 rev/min) for the outflow. A low speed peristaltic pump (5 to 100 rev/min) was used to feed the medium. The *p*H was measured using a fitted Ingold *p*H electrode. Dissolved oxygen was recorded by a D. O. Analyzer model DO-50 (NBS Company) using an autoclavable galvanic D. O. probe. The reactor was fitted finally with a simple differential mercury manometer for pressure readings. The agitation rate was 550 to 650 rev/min. Small volume liquid inocula (0.1 to 0.8% v/v) were used to inoculate the reactor which was operated initially batchwise until the cell suspension became turbid to a reading of 0.6 to 0.7 O.D. at 550 nm, and then the operation was switched to a continuous mode.

Bacterial growth was determined by the measurement of extinction at 350, 450, or 550 nm in a Bausch and Lomb spectrophotometer (Spectronic 70). The preparation of formaldehyde monomer and the formaldehyde assay, the gas chromatographic assay of methanol, the bacterial cell dry weight determination, the preparation of cell free extracts, and the procedures for the enzymatic assays are described elsewhere (Papoutsakis, 1976).

RESULTS AND DISCUSSION

Growth on Methanol and Formaldehyde: Batch Data

Figure 1 shows the initial specific growth rates μ as a function of the initial methanol concentration S_o. It is appropriate to emphasize that the specific growth rates were determined during the exponential growth phase. Figures 3 and 4 show two growth curves. The initial *p*H was 6.9, and by the end of the exponential phase it was 6.80 to 6.83. The optimal *p*H with the working medium was found to be 6.7 to 6.9. Lag phases ranged from 3.7 up to 5.1 hr without any particular trend up to 1.5% (*v/v*) S_o. Thereafter, in general, the higher the S_o the longer the lag phase.

For S_o higher than 4%, the growth rates did not have very good reproducibility, since the effects of methanol evaporation became significant with the prolonged lag phases. The microorganism was able to grow on 6 and 7% (*v/v*)S_o, but very prolonged lag phases (of the order of 2 and 3 days) made the growth rate calculation

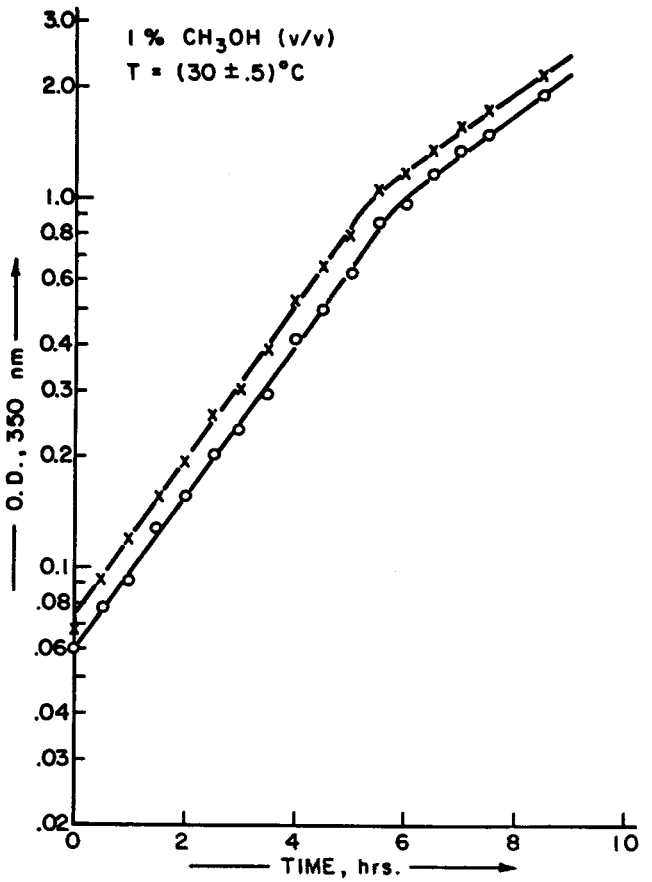

Fig. 2. Growth curves (duplicates) on 1% (v/v) $(CH_3OH)_o$.

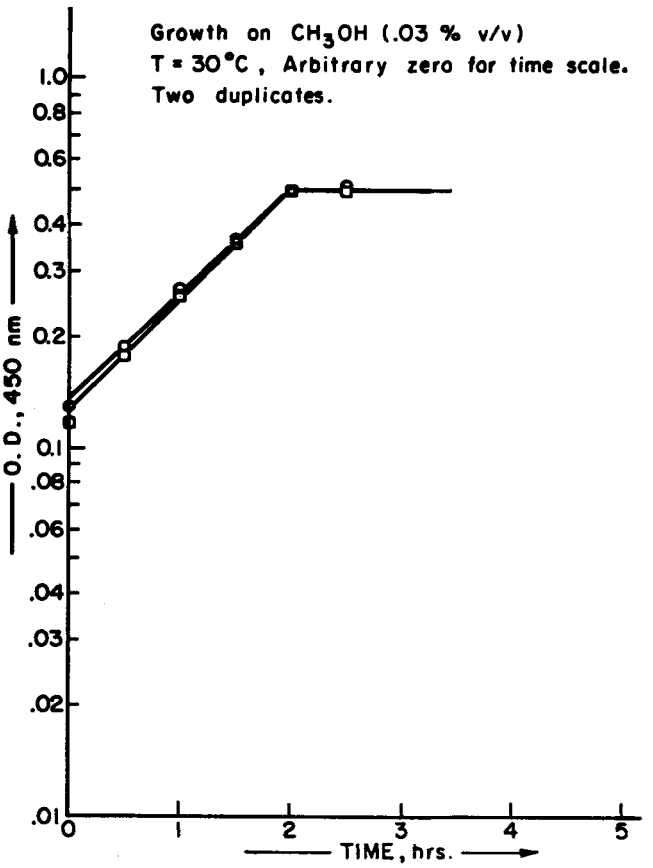

Fig. 3. Growth curves (duplicates) on 0.03% (v/v) $(CH_3OH)_o$.

difficult and inaccurate. In addition, by the time the growth was observed, the actual S_o was lower than the starting S_o. This indicates that substrate inhibition is in a sense reversible and not fatal as, say, antibiotic inhibition is.

The wide scattering of experimental points at the lower region of S_o reflects the effect of small differences in size and physiology of inocula and the degree of unpredictability of biological systems under nonsteady state cultivations. The batch data do not show the expected drop in μ for low S_o (that is, in the left section). A plateau in μ is seen for S_o in the range of 0.03% to 1% (v/v). Subsequent continuous culture data verified the descending portion of the μ curve at S_o lower than 0.03% (v/v).

The end of exponential phase occurs always at approximately the same O.D. value for S_o values in the plateau region and at somewhat lower values for higher S_o. With high initial substrate concentration, what follows the exponential region is another linear region on the semilogarithmic coordinates but with a smaller slope, that is, another quasiexponential region corresponding to a smaller specific growth rate, provided that the methanol has not been exhausted and that the rest of the nutrients are nonlimiting. By the end of the exponential phase the amount of methanol that has been consumed was 0.04 to 0.06% (v/v). The differences in the way in which cells went out of the exponential phase can be observed in Figures 2 and 3, where the initial methanol concentrations were 1 and 0.03% (v/v), respectively. From Figure 4 the excellent affinity of the microorganism for methanol becomes apparent. No residual methanol was detected at the end of the experiment.

The observation that the end of the exponential phase is not marked by any substrate limitation, a strong pH drop, or any other nutrient limitation poses critical questions: why are the cells going out of the exponential phase growth without any substrate limitation? What is the meaning of the second exponential growth? Similar behavior has been reported in the literature (Reuss et al., 1975; Dellweg et al., 1975). In the latter this fact was stated explicitly, but no explanation or comments were given. In general, no particular attention has been paid to this point, even though this may turn out to be general for many substrate inhibited microbial cultivations. However, some very important conclusions, from the theoretical and practical points of view, may be derived.

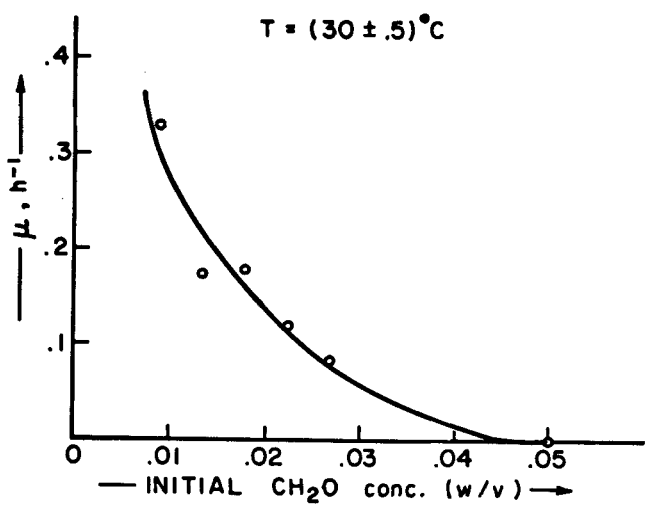

Fig. 4. Specific growth rate as a function of initial formaldehyde concentration from batch experiments.

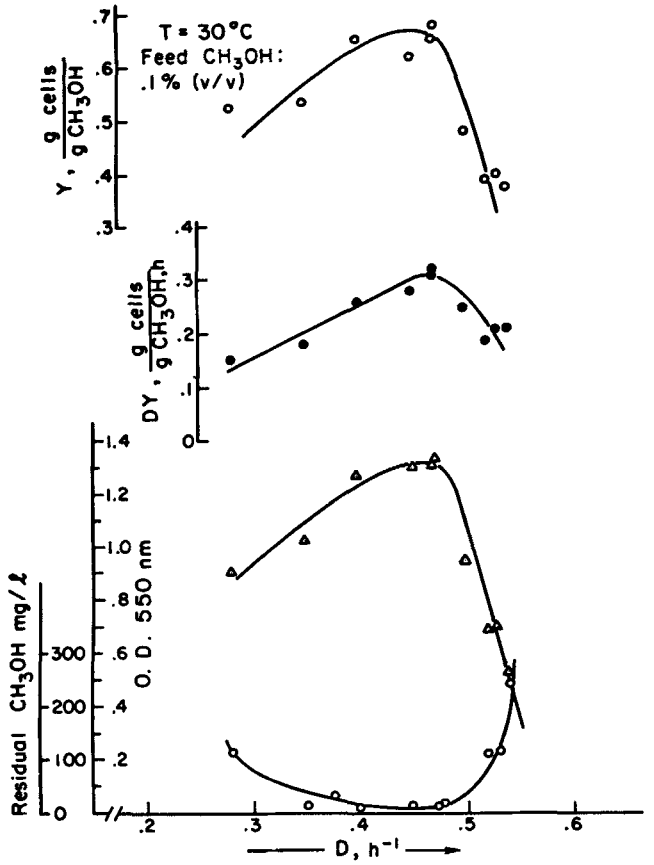

Fig. 5. Yield, productivity, reactor methanol concentration, and cell-mass density as a function of space velocity (D) from continuous steady state runs at 30°C. Pure culture.

In a turbidostat in which the methanol concentration and pH as well as the cell concentration are kept constant, to which portion of the batch run curve is the steady state μ more likely to be close? Certainly not to the first exponential phase, because eventually it will have to proceed to the next phase without any substrate limitation or pH effect. In fact, one should expect a higher growth rate to be exhibited since S is decreasing. If the next phase in the batch culture is an exponential one with a smaller slope, then this is expected to represent a state closer to the corresponding steady state. The same argument holds even if the next stage is not exponential but a prolonged deceleration phase not marked by substrate limitation. The question still remains, when does this quasiexponential phase terminate? Does it last until substrate exhaustion, or what happens after this?

For the modeling of growth in continuous cultures, a steady state continuity is assumed. However, the initial values of μ from batch experiments are used to represent the steady states in continuous cultures. The above discussion has shown that this approximation may not represent the real steady states at all. For realistic modeling we thus require more sophisticated data from continuous cultures. In light of the above, it is understandable why the stable steady states in a CSTR (data given below) correspond to lower μ's than those which had been predicted by the batch data. That is, the maximum point on the μ vs. S_o curve obtained from batch runs is not a realistic one, as it cannot be approached in chemostatic cultures (see, also, Dostalek et al., 1972); it is anticipated that the chemostat and turbidostat growth rates would be approached by the second exponential phase μ in the batch culture. An explanation is attempted in a companion paper (Papoutsakis et al., 1978).

In order to evaluate experimentally the yield during a batch culture, we write (2) as

$$Y_{X/S} \equiv \frac{r_X}{-r_S} = \frac{dX/dt}{-dS/dt} = \frac{dX}{-dS}$$

which can be integrated to give

$$-\int_{S_o}^{S} Y_{X/S}dS = \int_{X_o}^{X} dX = X - X_o \qquad (16)$$

Below is discussed that $Y_{X/S}$ is not constant during the course of growth of a batch culture. We thus define using (16)

$$\overline{Y}_{X/S} = \int_{S_o}^{S} Y_{X/S}dS \Big/ \int_{S_o}^{S} dS = \frac{X - X_o}{S_o - S} = \frac{X}{S_o - S} \qquad (17)$$

as $X_o = 0$, where the superbar is used to denote the average yield over substrate concentration.

Figure 1 shows the yield as defined above ($\overline{Y}_{X/S}$) as a function of initial substrate concentration. A point corresponding to 0.025% methanol concentration, which was obtained from a continuous culture run, is presented for comparison. The scatter in experimental points reflects the difficulties and errors associated with the experimental calculation of cell-mass yield. The methanol evaporation effect was critical, and blank runs did not always give reproducible corrections. Nevertheless, the trend is clear: the higher the initial methanol concentration, the lower the yield. For the initial substrate concentration of 0.2% the yield is 30%; continuous culture data show a 38% yield for 0.025% residual methanol concentration. A comparison of the shown batch

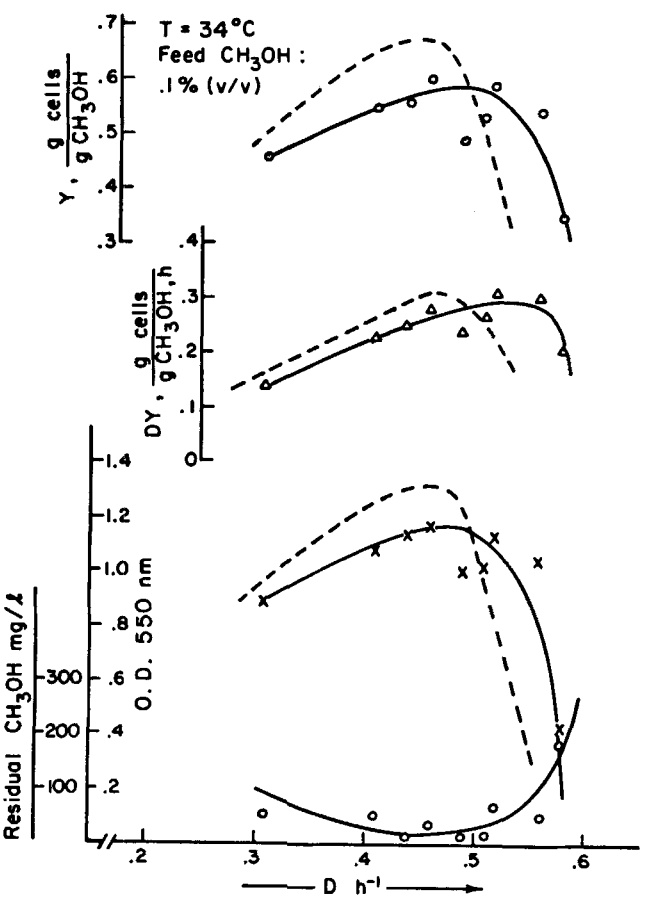

Fig. 6. Yield (Y), DY, reactor methanol concentration, and cell-mass density as a function of space velocity (D) from continuous steady state runs at 34°C. Pure culture.

yield values with the maximum yield of 65% obtained in CSTR shows clearly a very important advantage of continuous culture over the batch cultures. A similar yield function has been reported in the literature recently (Pilat and Prokop, 1975). Reuss et al. (1974) reported a linearly decreasing yield function with S_o; they also show the interesting function of average yield (that is, $\overline{Y}_{X/S}$) with time during the course of an extended batch experiment, that is, that yield has a maximum at the very early exponential phase dropping linearly from there on until the end of that phase.

Free extracellular formaldehyde was detected in the culture of cells that had been grown on methanol. The amount of formaldehyde varied, depending upon the initial methanol concentration and the stage of growth. The presence of extracellular formaldehyde indicates a considerable formaldehyde pool in cells, which can only be due to insufficient cellular regulation.

Formaldehyde supported growth, up to 0.05% (*w/v*) initially, as a sole carbon source. However, the growth was extremely poor. Lag phases (proportional to the initial formaldehyde concentration) lasted as long as 3 days. The actual growth phase lasted for very short period of time, while the final cell concentration never exceeded 0.2 O.D. units at 350 nm. Figure 4 shows the μ vs. (CH_2O) profile. It seems that the maximum μ corresponds to a very low formaldehyde concentration, of the order of free formaldehyde concentration detected during growth on methanol alone.

Continuous Cultivation

For steady state continuous culture, the mass balance on the cell mass gives

$$D = \mu \tag{18}$$

A mass balance on the limiting substrate gives

$$D(S_o - S) = \mu X/Y_{X/S} \tag{19}$$

where $Y_{X/S}$ has been defined in (2). Equations (18) and (19) imply that

$$Y_{X/S} = \frac{X}{S_o - S} \tag{20}$$

Figures 5 and 6 show the experimental data on pure culture of *Methylomonas* EP-1 at 30° and 34°C, respectively; pressure was slightly higher than atmospheric (1.08 atm), while dissolved oxygen tension was 60 to 80% of saturation. In Figure 6 the dotted lines represent the data at 30°C, while the solid lines represent those at 34°C.

A more meaningful measure of biomass productivity of a microorganism is the quantity $DY_{X/S}$. The conventional cell mass productivity $DX = DY_{X/S}(S_o - S)$ is a function of S_o and therefore depends on the actual operational conditions of the bioreactor. Since for a given value of $D(=\mu)$ there exist corresponding unique S and $Y_{X/S}$, which are independent of S_o, DX is proportional to $DY_{X/S}$ for each S_o. This fact, verified by us (data not shown) and by other researchers (Mateles and Battat, 1974), justifies studies at low-cell densities, for the sake of convenience, with results still valid at high-cell densities as those in industrial processes.

The effect of higher temperature was to lower the yield but increase the growth rate; that is, the washout was expected to occur at higher dilution rates. However, the productivity $DY_{X/S}$ has the same approximate maximum value at both temperatures. Higher temperature operation is advantageous because less heat removal is required. Lower yields, though, imply higher oxygen requirements and, thus, higher heat dissipation. Choosing the operation temperature, therefore, becomes a more complicated problem for this particular case. The experimentally calculated yields were remarkably high (65%). As D increased towards the washout, yield dropped to 35 to 38% approaching the batch yield data.

It is not very clear yet what was responsible for the decrease of yield and slight increase in residual methanol at lower dilution rates. It is possible that at very low residual methanol concentration a carbon dioxide fixation may play a role, increasing the yield slightly, while such effect is absent at appreciable residual methanol concentration. Should this be the case, we can check this out by feeding carbon dioxide with air instead of air alone. Otherwise, it could be an intracellular regulation effect. A similar situation has been reported in the literature (Battat et al., 1974). Another question that required further investigation was the extent of contribution to the yield value by carbon dioxide fixation. Conversion of carbon dioxide to protein is a very economically efficient process for protein production. Carbon dioxide is known to be fixed by many microorganisms; specifically, carbon dioxide fixation is an important intermediate step of C_1 compounds utilization through the serine pathway (Large and Quayle, 1963; Quayle, 1972).

When the ribulose monophosphate cycle is involved (Strøm et al., 1974), carbon dioxide fixation takes place at a much smaller scale through rather general enzymes (that is, enzymes not specifically associated with the particular pathway mentioned) (Quayle, 1972; Fujii et al., 1975). Babij et al. (1975) show that for *Pseudomonas* TB582, carbon dioxide fixation may account for as much as 45% of the cell-mass yield, depending upon the methanol feed concentration. No data have been shown for the dependence upon the dilution rate. Our experimental data on the effect of carbon dioxide on yield are presented in the next section.

During continuous cultivation, very small amounts of formaldehyde have been detected in the reaction system (0.2 to 0.6 mg/l), which is an order of magnitude smaller than those found in batch cultures. It therefore appears that even at balanced steady state growth, the presence of small amounts of formaldehyde is physiologically necessary.

To examine the utilization of formate in the presence of methanol by the *Methylomonas* EP-1, formate and more methanol were added to the feed tank to final concentrations of 0.28% (*w/v*) and 0.11% (*w/v*), respectively, after running the CSTR on pure methanol. Steady state was achieved within 30 hr with a dilution rate of 0.2 hr^{-1} at a *p*H of 7.45 (due to inefficient *p*H control). The *p*H rise is speculated to be due to the HCOOH transport process from the growth medium into the cells. At this nonoptimal *p*H, cell-mass yield on methanol was 48%; the yield on formate is zero. Attempts to switch the cultivation solely on formate failed; the *p*H went up to 7.7, and washout soon took place.

The fact that formaldehyde is present in different amounts at different stages of batch growth, decreasing towards the end of the exponential phase (Papoutsakis, 1976), indicates that this compound is readily produced and utilized by the microorganism in the presence of methanol. Formaldehyde therefore can serve as a cosubstrate in continuous cultivation, as long as its feed concentration is selected as a function of the reactor cell density, so as to keep its residual concentration in the reactor very low to avoid inhibitory effects.

Continuous Mixed Cultures

During a continuous run a mixed culture was spontaneously formed. Some preliminary batch runs showed that the contaminant bacterium grows well and fast on

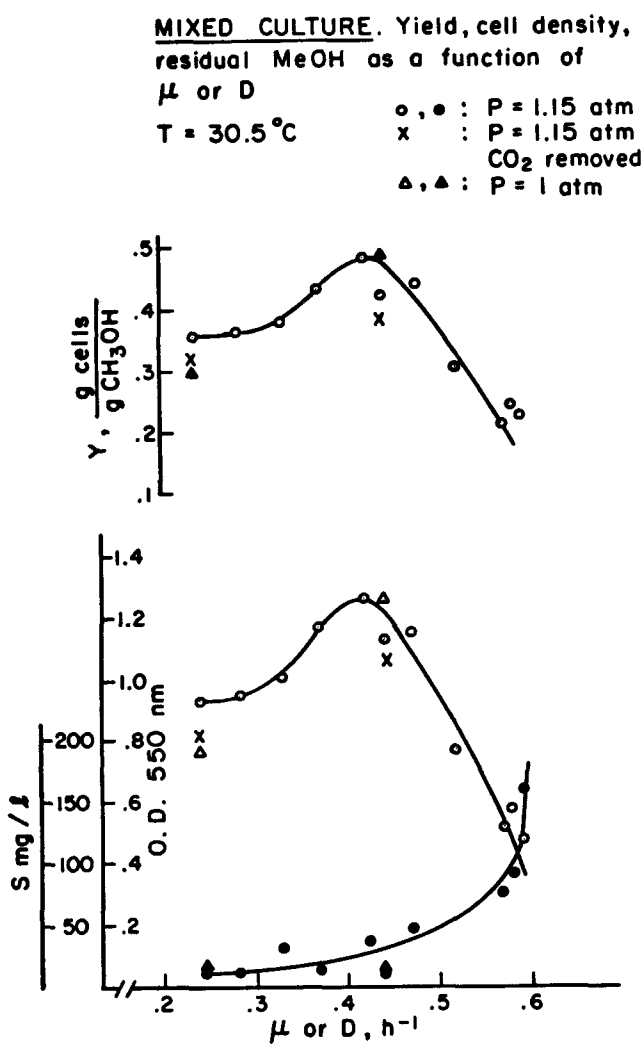

Fig. 7. Yield (Y), cell-mass density, and reactor methanol concentration as a function of space velocity (D) from continuous steady state runs at 30.5°C. Mixed culture.

methanol and is enriched in mixed culture growing on formaldehyde.

During the CSTR mixed-culture experiment, which ran for longer than a week, no particular trend was observed for enrichment of the one microorganism over the other; the ratio of two changed with dilution rate (space time), but, again, no particular trend was observed. Conservative yield calculations gave a maximum yield of 48%. As shown in Figure 7, the dependence of X (or O.D.) on D [feed methanol concentration 0.1% (v/v)] is similar to the one obtained for the pure *Methylomonas* EP-1. In the same figure, the effects of a slight pressure used (namely, 1.15 atm) and that of atmospheric carbon dioxide are shown. Carbon dioxide was removed from the feed air through a sodium hydroxide trap. The high pressure increased the yield at a low dilution rate, but it didn't have any effect at a higher dilution rate; atmospheric carbon dioxide is most likely fixed and to different extents at different dilution rates. It can be seen that the mixed culture, if it is indeed a stable system, exhibits faster growth characteristics than the pure one, but significantly lower yields. In light of the fact that formaldehyde is accumulated at higher methanol concentrations at which the specific growth rates drop, it is possible that the contaminant has better affinity for formaldehyde and therefore removes it partially, thus extending the range of dilution rates over which steady state chemostat operation can be achieved.

Enzyme Activities and Their Relation to the Macrocharacteristics of Growth

We adopt the convenient and elegant formalism suggested by Frederickson (1976) to define by C_j the concentration of an intracellular species j over the biomass volume. If $\hat{V}$ (a constant) is the biotic volume per unit biomass, $m(t)$ the biomass of the system, and r_{ij} the rate per unit biotic volume at which the j^{th} species appears because of the occurrence of the i^{th} process, then a mass balance on the j^{th} component of biomaterial gives

$$\frac{d}{dt}(m\hat{V}c_j) = m\hat{V}\sum_i r_{ij} \tag{21}$$

or, upon rearrangement

$$\frac{dC_j}{dt} = \sum_i r_{ij} - \mu C_j \tag{22}$$

where

$$\mu \equiv \frac{1}{m}\frac{dm}{dt} \tag{23}$$

From the foregoing equations we deduce that

$$\mu = \hat{V}\sum_i \sum_j r_{ij} \tag{24}$$

which states that μ is not an independent variable.

We shall denote by S_j the extracellular concentration of species j. We aim to derive expressions for μ, $Y_{X/S}$, dC_2/dt, and dS_2/dt for growth of microorganisms utilizing the RMP cycle, where the subscript 2 refers to formaldehyde. We shall derive these expressions under the condition (Papoutsakis et al., 1978) that formaldehyde diffuses freely through the cell wall membrane, that the kinetics is simple Michaelis-Menten type where the cosubstrates and/or coenzymes exist at constant intracellular concentrations, and that enzyme concentrations vary. According to (22), we write

$$\frac{dC_2}{dt} = r_{12} - r_{22} - r_{32} - r_{42} - \mu C_2 \tag{25}$$

in which the rate of formaldehyde production due to methanol oxidation is given by

$$r_{12} = \frac{k_{112}(E_1)C_1}{k_{212} + C_1} \tag{26}$$

where E_1 stands for the intracellular concentration of the enzyme which oxidizes methanol, C_1 the intracellular methanol concentration, and k's are rate constants. The rate of formaldehyde consumption due to oxidation to formate is given by

$$-r_{22} = -\frac{k_{122}(E_2)C_2}{k_{222} + C_2} \tag{27}$$

where E_2 stands for the concentration of formaldehyde dehydrogenase. The formaldehyde consumption rate due to incorporation into cell mass through the condensation reaction of (10) is given by

$$-r_{32} = \frac{k_{132}(\text{HUPS})C_2}{k_{232} + C_2} \tag{28}$$

where (HUPS) stands for the concentration of HUPS. Finally, the rate of diffusion of formaldehyde into the abiotic phase is assumed to be given by

$$r_{42} = k_{42\sigma}(C_2 - S_2) \tag{29}$$

A mass balance on CH_2O in the abiotic phase gives, for

a batch culture

$$\frac{dS_2}{dt} = \frac{k_{42}\xi m(C_2 - S_2)}{V - \hat{V}m} \tag{30}$$

which for low-cell densities reduces to

$$\frac{dS_2}{dt} = \frac{k_{42}}{C_3}\sigma X_M(C_2 - S_2) = \frac{r_{42}}{C_3}X_M \tag{30a}$$

Clearly, owing to the large number of chemical species in a cell, the relation (22) cannot be utilized directly to express μ. However, this is accomplished by writting, under the pseudo steady state assumption, for the CH_2O fixation into cell mass

$$CH_2O \xrightarrow{\text{HUPS}} 1/4\ C_4H_8O_2N \tag{31}$$

Then, by assigning the subscript 3 to $C_4H_8O_2N$, (22) can be written as

$$\frac{dC_3}{dt} = \frac{1}{4}r_{32} - \mu C_3 \tag{32}$$

But, the water content of cells is constant, or C_3 is constant. Thus, (32) reduces to

$$\mu = \frac{1}{4C_3}r_{32} \equiv k_{332}r_{32} \tag{33}$$

The yield may be expressed in terms of molar rates by

$Y_{X/S}$

= (moles of CH_2O converted to $C_4H_8O_2N$) $\left(\frac{102}{4}\right)\Big/$

(moles of CH_3OH consumed) (32)

$$= 0.80\frac{r_{32}}{r_{32} + r_{22}} = \frac{0.80}{1 + r_{22}/r_{32}} \tag{34}$$

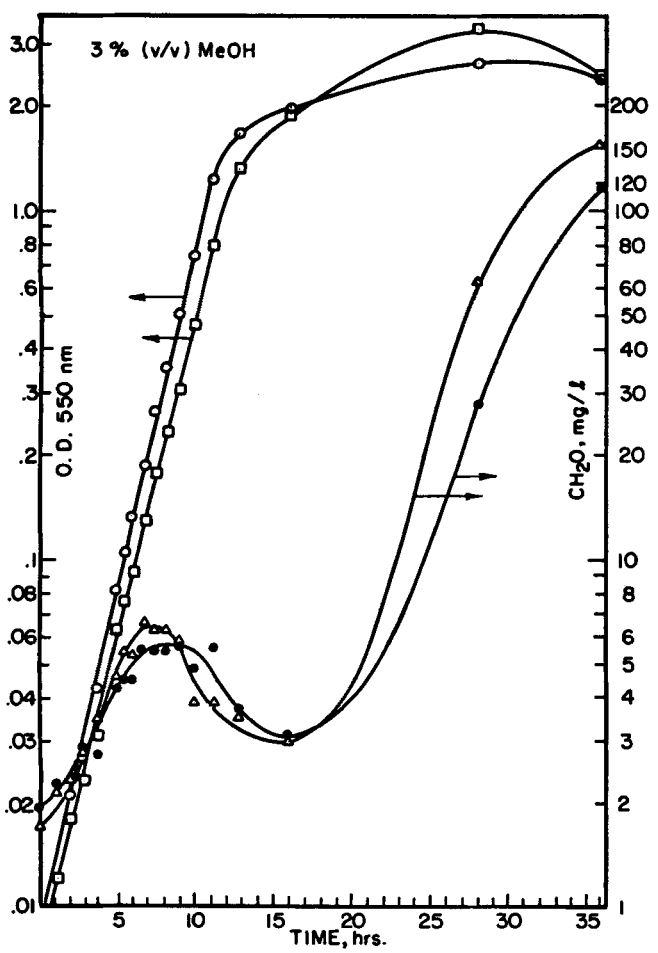

Fig. 8. Free formaldehyde concentration during the course of a batch culture on 3% (v/v) $(CH_3OH)_o$.

Enzymatic activities in cell free extracts expressed as (n moles substrate)/(minutes, milligrams protein) are directly proportional to the concentrations (E_1), (E_2), and (HUPS) defined over the biotic volume. It has been proposed (Papoutsakis et al., 1978) that these concentrations change according to methanol and formaldehyde concentrations.

The average specific activity of hexose phosphate synthase was found to be 3 027 (n moles CH_2O)/(min, mg protein) in the cell free extracts, although a fraction of the activity is also expected to be found in the particulate fraction of extracts (Lawrence et al., 1970; Goldberg and Mateles, 1975). Even so, the activities of HPS found are higher than any ever reported (Ogata et al., 1975; Goldberg and Mateles, 1975; Trotsenko, 1975; Lawrence et al., 1970; Strøm et al., 1974; Sahm and Wagner, 1974). Because of the location occupied by HUPS in the assimilation pathway of methanol, its specific activity is a direct measure of how fast a microorganism can grow on methanol via the RMP cycle as Equation (33) shows. *Methylomonas* EP-1 grows on methanol faster than any other microorganism ever reported. Therefore, selection of fast growing microorganisms can be accomplished by detecting high HUPS (or HPS) activities.

No hydroxypyruvate reductase activity was detected over a wide range of pH values. Thus, it may be concluded that *Methylomonas* EP-1 utilizes exclusively the RMP cycle.

Methanol dehydrogenase was found to be linked to the artificial electron-acceptor system, PMS-DCPIP. This same enzyme oxidized formaldehyde and was completely inhibited by higher concentrations of formaldehyde.

Only NAD linked formaldehyde dehydrogenase was detected in the cell free extracts, with an average specific activity of 9.1 (n moles CH_2O)/(min, mg protein) under standard assay conditions (Papoutsakis, 1976). The average activity of NAD linked formate dehydrogenase was found to be 19 (n moles HCOOH)/(min, mg protein), while the ratios of formate dehydrogenase to formaldehyde dehydrogenase for three different batches of extracts were found to be 1.98 and 3.35 for batch grown cells and 1.00 for continuously grown cells. If r_{14} is the rate per unit biotic volume at which formate is oxidized to carbon dioxide, then for continuously grown cells at steady state conditions, $r_{22} = r_{14}$. Thus, the ratio of 1.00 indicates that the enzymatic characteristics of the two enzymes are such that the in vivo rates of the catalyzed reactions are proportional to the enzyme concentrations, or that the enzymes operate in vivo at the saturation concentrations. Clearly, then, the ratios such as 1.98 and 3.35 express the dynamic unbalanced growth of batch cultures. Specifically, it has been proposed (Papoutsakis et al., 1978) that the enzymes E_1, E_2, E_3 are sequentially produced in that order in a batch culture, in response to the corresponding substrate concentrations. Namely, it was proposed that first methanol induces the production of E_1 (and HUPS), and that an intracellular formaldehyde pool is progressively formed inducing E_2 and thus resulting in a formate pool which induces the production of E_3. Moreover, formaldehyde and formate were proposed to repress the production of E_1 and HUPS. In other words, batch growth is a transient dynamic growth towards a steady balanced growth. Figure 8 depicts this fact. Extracellular formaldehyde concentration S_2 reaches a maximum at the midexponential phase, then a low minimum, and then rises again accompanied by a cessation of growth. As Equation (30a) shows, this is resulting from

a similar pattern of intracellular concentration C_2. In fact, further analysis based on Equation (30*a*) can show that C_2 is approximately constant up to an O.D. of 0.04 and that the pattern it follows is similar to that of S_2 with a small phase difference. In addition, under appropriate initial methanol concentrations, a second local maximum on the S_2 profile appears followed by a leveling off and an eventual decrease to zero (Krug, 1977).

In view of (33) and (30*a*) and upon rearrangement, (25) reads

$$\frac{dC_2}{dt} = r_{12} - r_{22} - (4C_3 + C_2)\mu - \frac{C_3}{X_M}\frac{dS_2}{dt} \quad (35)$$

It can be shown for the data of Figure 8 that $d/dt\ (1/X_M\ dS_2/dt) < 0$ until some time past the time that corresponds to the local S_2 maximum. Therefore, during the rising part of the C_2 profile, the dominant term is r_{12} owing to low (E_2) in the expression (27) of r_{22}, which becomes increasingly significant and becomes the dominant term after C_2 reaches its local maximum. It appears that macroscopically r_{32} is constant up to the tenth hour, while r_{22} is progressively increasing. However, although (HUPS) is expected to have its maximum concentration at the early exponential phase, owing to maximum C_1 and low C_2, r_{32} is expected to reach a maximum later in the early exponential phase owing to C_2 according to Equation (28). Thus, r_{22}/r_{32} has an early exponential phase minimum, increasing henceforth up to the end of exponential phase, and thus $Y_{X/S}(t)$, according to (34), has an early exponential phase maximum decreasing thereafter up to the end of exponential phase. Therefore, the experimentally determined yield, according to (17), is a function of time during the course of a batch culture, explaining the experimental error depicted on Figure 2, as the time of its determination was not exactly the same for all experiments. The yield function $Y_{X/S}(t)$ that Reuss et al. (1974) show is in accordance with the above prediction. It appears also that yield in batch cultures is always lower than that in continuous steady state cultures. Moreover, as Figures 5, 6, and 7 depict, it has a maximum at a certain dilution rate, that is, at a certain residual substrate concentration, which corresponds to the conditions that make r_{22}/r_{32} a minimum. Clearly, at any values other than the minimum value of r_{22}/r_{32} more energy is produced through formaldehyde oxidation than the energy required to fix carbon into cell mass. Hence, the minimum r_{22}/r_{32} corresponds to the maximum yield as shown by the earlier analysis in this paper.

In conclusion, we have shown that $Y^* = Z^* = 3$ and that $X^* = 1$ or 2 ($X^* = 0$, corresponding to a methanol oxidase, is safely eliminated). For $X^* = 1$, the corresponding theoretical yield is 65%, while for $X^* = 2$, the yield is 75% [a value of 2 to 3% has been added to the calculated yield, as suggested by van Dijken and Harder (1975), to account for ash]. Comparing the experimental maximum yield of 65 to 68% to the theoretical values, we conclude that either we achieved the optimal yield ($X^* = 1$), or we approached it closely from below ($X^* = 2$). It is, therefore, very likely that the PMS-DCPIP dehydrogenases can yield 1 mole of ATP, which is in accordance with the findings that these dehydrogenases are related to cytochrome c (Widdowson and Anthony, 1975; Tonge et al., 1975) and with the direct experimental findings of Tonge et al. (1977).

Thus, the pathway through which C_1 compounds are utilized and the electron acceptors (coenzymes) of the three enzymes responsible for the complete oxidation of methanol are all the information we need for the selection of microorganisms that give high cell-mass yield for SCP production.

If the theoretical yield of a particular microorganism were not achieved during the course of experimental work, then growth conditions and medium improvements would be suggested. In fact, the complete energy conservation and coupling, intrinsic in the theoretical yield evaluation, can only be achieved in continuously grown cells, under optimal conditions, that is, temperature, pH, aeration, medium, and cell density. This theoretical yield will be the target for improvement of these conditions with respect to cell-mass yield. With the exception of yeasts, even when the biochemistry was known for a particular microorganism, no systematic studies on continuous culture yields had been reported. *Methylomonas* L3 (an RMP cycle bacterium) also gave experimental results in line with the conclusions of this paper (Hirt et al., 1978). In the case of *Pseudomonas* C (Battat et al., 1974; Goldberg, 1976), the experimental results do not lead to conclusive comparisons which may be due to its biochemical characteristics which are not typical of the RMP cycle utilizers (Ben-Bassat and Goldberg, 1977).

The only other microorganism known to give yields higher than 54% is *Pseudomonas* TB582 which was reported to give a yield approaching 100% (regardless of the high carbon dioxide fixation contribution to it). However, the unattractive and obscure characteristics reported, namely, the dependence of yield on methanol-feed concentration (for 0.5% feed methanol concentration the yield is lower than 40%) and the low dilution rate (therefore the growth rate) (0.1 hr^{-1}), render this microorganism inferior for industrial applications (Babij et al., 1975).

ACKNOWLEDGMENT

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NOTATION

ATP = Adenosine 5′-triphosphate
C_j = concentration of chemical species j over the biomass volume, g-mole/L^3
D = space velocity (or dilution rate), $1/\theta$
DCPIP = 2,6-dichlorophenolindophenol
EDTA = ethylenediaminetetraacetic acid
E_1 = methanol dehydrogenase or oxidase
E_2 = formaldehyde dehydrogenase
E_3 = formate dehydrogenase
F = volumetric flow rate, L^3/θ
HPS = hexose phosphate synthase
HUPS = hexulose phosphate synthase
k_{1i2} = constant, $1/\theta$
k_{2i2} = constant, g-mole/L^3
k_{332} = L^3/g-mole
k_{42} = permeability constant, L/θ
m = biomass of a reaction system, M
NAD = nicotinamide adenine dinucleotide (oxidized form)
NADH = nicotinamide adenine dinucleotide (reduced form)
O.D. = optical density
3-PGA = 3-phosphoglycerate
PMS = phenazine methosulfate
r_{ij} = rate per unit volume of biomass at which the j^{th} species appears owing to the i^{th} process, g-mole/$L^3\theta$
r_{O_2} = rate of oxygen appearance due to cell growth, $M/L^3\theta$
r_S = rate of S appearance due to cell growth, $M/L^3\theta$

r_X = rate of X appearance due to cell growth, $M/L^3\theta$
RMP = ribulose monophosphate (cycle)
S = substrate concentration defined over the total reaction volume, M/L^3
S_j = concentration of species j defined over the abiotic phase volume, g-mole/L^3
S_m = maximum S
S_o = initial or feed S
V = reaction (total) volume, L^3
$\hat{V}$ = biotic volume per unit biomass, constant, L^3/M
X = biomass concentration, M/L^3
X_M = biomass concentration, g-mole/L^3
X_o = feed X
$Y_{O_2/X}$ = specific oxygen demand, dimensionless
$Y_{X/S}$ = cell-mass yield, dimensionless
$\overline{Y}_{X/S}$ = average $Y_{X/S}$ over a period of time
X^*, Y^*, Z^*, X^*H_2,
Y^*H_2, Z^*H_2 = coenzymes of an enzymatic oxidation reaction and their reduced forms, or their equivalent ATP moles
( ) = concentration of a chemical species over the total volume; for enzymes, over the biomass volume, M/L^3 or g-mole/L^3
μ = specific growth rate, $1/\theta$
σ = surface per unit biotic volume, a constant, $1/L$
ξ = surface per unit biomass, constant, L^2/M

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