

# ***Bacterial Utilization of Pure and Mixed C<sub>1</sub> Compounds for Single Cell Protein Production***

## **INTRODUCTION**

The importance of a high biomass yield for an economically optimal production of single cell protein (SCP) on C<sub>1</sub> compounds has been well established.<sup>1-3</sup> Small quantities of formaldehyde and formate are usually found in methanol produced by a simple oxidation process from natural gas; this process is of vital importance for the production of methanol to be used for SCP production. It is therefore important to examine the possibility of using the crude methanol without removing formaldehyde and formate, for obvious reasons of lower carbon cost.

From the biomass yield viewpoint, yeasts are inferior to bacteria (see, e.g., Ref. 2) and among C<sub>1</sub>-compound-utilizing bacteria, those that possess the ribulose monophosphate (RMP) cycle<sup>4</sup> are energetically superior to those that utilize the serine<sup>5</sup> pathway (see, e.g., Ref. 2). Although all C<sub>1</sub> compounds are growth-inhibitory substrates, formate and especially formaldehyde are considerably more toxic to growth than methanol, both for yeasts<sup>6</sup> and bacteria.<sup>2,7,8</sup> Yet the effect of formate and formaldehyde on the biomass yield of RMP-cycle microorganisms and yeasts is not known, other than, perhaps, at the theoretical level.<sup>9-11</sup> As far as is known, however, no RMP-cycle microorganisms or yeasts can grow continuously on either pure formaldehyde or pure formate.<sup>8</sup> Thus, chemostatic biomass yields on mixed substrates are reported here for the bacterium *Methylomonas* L3.<sup>8</sup> Moreover, we show the significant effect that the culture pH has on the chemostatic biomass yield of the same microorganism grown on methanol alone. Some further observations, related to the utilization of C<sub>1</sub> compounds by species L3, are also discussed and comparisons are made with the behavior of yeasts.

## **MATERIALS AND METHODS**

Strain L3, an obligate methylotroph, RMP-cycle microorganism, was isolated in our laboratory and its characterization and enzymatic properties were reported in Ref. 8. The growth medium composition is also reported in Ref. 8. Methanol (99.85% pure) and formate (as the sodium salt) were filter sterilized and aseptically added to the autoclaved basal salts medium after it had cooled to room temperature. Pure, monomeric, sterilized formaldehyde was prepared as in Refs. 7 and 8. Chemostat cultures were established in a New Brunswick Bio-Flo fermentor with a working volume of 355 ml, equipped with automatic pH and temperature controls. Cultures were routinely kept at 30°C and pH 7.1 (unless otherwise stated) and the agitation and/or aeration rates were manually controlled to keep the dissolved oxygen at 50 to 60% of the saturation level. The gas chromatographic assay of methanol and the wet chemistry assays of formaldehyde and formate were reported in Refs. 7 and 8. Bacterial growth and dry weight determinations were performed as previously reported.<sup>7,8</sup>

TABLE I  
Biomass Yields, Feed, and Residual Substrate Concentrations for Growth of *Methylomonas* L3 on Pure and Mixed Substrates<sup>a</sup>

Dilution rate $D$ (hr <sup>-1</sup> )	Biomass conc. (g cells/liter)	Feed substrate concentrations (mg/liter)			Residual substrate concentrations (mg/liter)			Biomass yields	
		CH <sub>3</sub> OH	CH <sub>2</sub> O	HCOOH	CH <sub>3</sub> OH	CH <sub>2</sub> O	HCOOH	$Y$ (g cells/ g substrate)	$Y_m$ (g cells/ g mol substrate)
0.100	0.842	2000	100	1000	6	~0	130	0.40	12.83
0.100	0.041	100	0	1000	6	ND	370	0.41	13.12
0.100	—	—	—	—	—	—	—	0.38–0.40 <sup>b</sup>	12.16–12.8 <sup>b</sup>
0.190	0.441	1000	—	—	30	—	—	0.46	14.72
0.200	0.470	1000	—	—	~0	—	—	0.47	15.04
0.335	0.527	1000	—	—	23	—	—	0.54	17.28
0.375	0.346	800	100	1000	<4	2.5	101	0.38	12.30
0.375	—	—	—	—	—	—	—	0.55 <sup>b</sup>	17.60 <sup>b</sup>
0.430	0.356	800	100	1000	16	2.5	100	0.40	12.82
0.430	0.737	1600	100	1000	12	2.5	140	0.44	13.94
0.435	0.563	1000	0	0	16	0.4	ND	0.57	17.43
0.465	0.563	1000	0	0	7	—	—	0.57	17.43

<sup>a</sup> Temperature = 30°C, pH 7.1; ND = not determined.

<sup>b</sup> Extrapolated yield values from Figure 1 for growth on methanol alone.

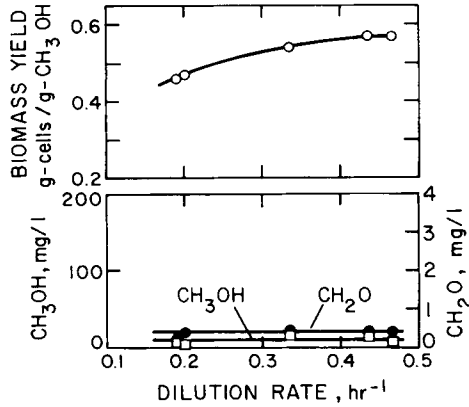


Fig. 1. Biomass yield, residual methanol and formaldehyde concentrations as functions of the dilution rate for growth of *Methylomonas* L3 on methanol. Temperature = 30°C, pH 7.1.

## RESULTS

Table I presents biomass-yield data of strain L3 on pure and mixed substrates, methanol, formaldehyde, and formate, for different feed compositions and dilution rates. The feed, residual, and cell-mass concentrations are also reported. Since formate is not incorporated into the cell mass (the RMP cycle) and it essentially provides only energy in the form of NADH<sub>2</sub> through oxidation to CO<sub>2</sub>,<sup>5</sup> we think it is appropriate not to consider HCOOH in the calculation of the biomass yield (also see the discussion in the first paragraph of the Appendix). Thus the yields ( $Y$  and  $Y_m$ ) shown in Table I were calculated on the basis of the combined methanol and formaldehyde consumptions. The reason for using lower formaldehyde than formate or methanol concentrations in the feed is that formaldehyde is considerably more toxic to growth than the other two C<sub>1</sub> substrates.

Figure 1 presents the biomass yield and residual methanol and formaldehyde concentrations as functions of the dilution rate for L3 grown on methanol alone at a culture pH of 7.1. Figure 2 presents the corresponding curves for pH 6.85. The biomass-yield curve of Figure 1 is also shown on Figure 2 (dashed line) for ease of comparison. The downturn part<sup>2,12,13</sup> of the biomass-yield function, for higher dilution rates toward washout, has not been obtained experimentally for the case of pH 7.1. In view of the smoothness of the biomass-yield curve of Figure 1, we take the liberty to interpolate and extrapolate from it biomass-yield values for dilution rates between 0.1 and 0.465 hr<sup>-1</sup>; the latter is the largest dilution rate value that was tried experimentally with a culture pH of 7.1.

## DISCUSSION

Figure 2 shows that pH largely affects the biomass-yield behavior as a function of the dilution rate. However, the largest (optimal?) biomass-yield value (0.57) is not

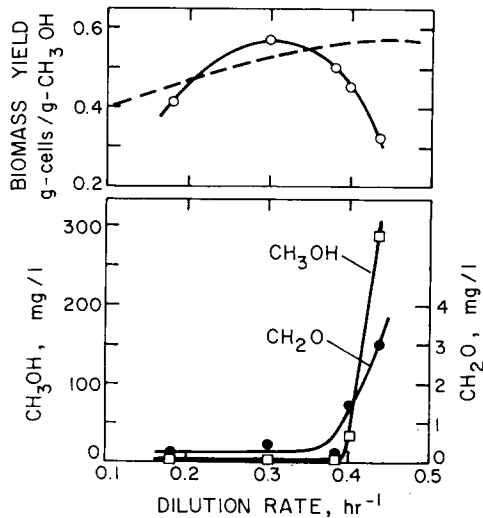


Fig. 2. Biomass yield, residual methanol and formaldehyde concentrations as functions of the dilution rate for growth of *Methylomonas* L3 on methanol. Temperature = 30°C, pH 6.85. (---) Biomass-yield curve of Fig. 1.

affected, although it moved to lower dilution rate values for pH 6.85. The biomass-yield curve for pH 6.85 certainly has a more pronounced cup shape and the washout dilution rate has moved to a lower value. Thus, the productivity, the best measure of which is the quantity  $D \cdot Y$ ,<sup>7</sup> is considerably higher with pH 7.1 than with pH 6.85, while, perhaps, none of them are optimal. Thus pH emerges as a very significant parameter in the determination of the optimal productivity.

The results of Table I show that all three substrates—methanol, formaldehyde, and formate—are readily utilized by strain L3, while the high toxicity of formaldehyde to growth is demonstrated by its low residual concentrations. Indeed, the residual concentration of an inhibitory substrate is a measure of the tolerance of a particular species to this substrate<sup>2,7</sup>; thus, the lower the residual substrate concentration, the lower the tolerance of the microorganism to this substrate or the higher the toxicity of the substrate to the microorganism. Curiously enough, the residual formate concentrations are an order-of-magnitude higher than the methanol concentrations, although it has been suggested that formate is more toxic (for yeasts at least) to growth than methanol.<sup>6</sup> Despite the fact that formate provides  $\text{NADH}_2$ , and thus energy, through its oxidation to carbon dioxide, it appears, from the data of Table I, to have no effect on improving the yield on its cosubstrates; compare, for example, the second entry of Table I with the  $Y = 0.38\text{--}0.40$  value (for  $D = 0.1 \text{ hr}^{-1}$ ) suggested by Figure 1. This is, of course, in contrast to theoretical predictions.<sup>9</sup>

For low dilution rates formaldehyde and formate do not appear to largely affect the biomass yield; compare the first two entries of Table I with the value of  $Y = 0.38\text{--}0.40$  for  $D = 0.1 \text{ hr}^{-1}$  from Figure 1. For high dilution rates, however, formaldehyde and formate have a strong negative effect on the biomass yield as the last

three entries of Table I suggest. Also compare the third entry of Table I with the value  $Y = 0.55$  for  $D = 0.375 \text{ hr}^{-1}$  suggested by Figure 1. Moreover, it appears that formaldehyde is the most probable cause of the lower yields and that it is the ratio of formaldehyde-to-methanol concentration rather than the formaldehyde concentration *per se* in the feed that determines the biomass yield. The lower biomass yields cannot possibly be explained by the lower reduced energy content of formaldehyde, as a simple theoretical calculation<sup>9</sup> may show. After all, there appears to be abundant, unused reduction energy ( $\text{NADH}_2$ ) from the oxidation of formate as we discussed above. Had it not been for the encountered contamination problems,<sup>14</sup> the findings in Ref. 13 would be most interesting for comparing them with the present findings. In any case, those findings<sup>13</sup> do not contradict the herein reported ones.

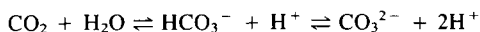
An interesting observation to comment upon is that both the methanol and formaldehyde residual concentrations increase as the biomass yield decreases for higher dilution rates toward washout (Fig. 2). Note how much lower both the above concentrations are in the increasing section (lower dilution rates) of the biomass-yield function of both Figures 1 and 2. The increase of the residual substrate concentration is typical of continuous cultures as the washout point is approached. It is the physiology of the cells to demand higher substrate concentrations in order to achieve higher growth rates. For the particular case of the methylotrophs, however, higher substrate concentrations have a negative effect on the biomass yield.<sup>7</sup> As the dilution rate increases toward washout, the biomass-yield decreases and the formaldehyde concentration increases to values similar to the batch-culture ones (Refs. 2, 7, and unpublished results). Indeed, we have found that with both strains L3 and EP-1,<sup>2,7</sup> batch cultures on methanol give consistently higher residual formaldehyde concentrations and significantly lower biomass yields. Perhaps the higher residual formaldehyde concentrations that accompany the lower biomass yields are another indication of the negative effect of the former on the latter. Furthermore, the simultaneous change with the dilution rate of the residual concentrations of both methanol and formaldehyde appears to violate the original notion of the one substrate (methanol) limitation of growth; this observation perhaps deserves further attention. A similar observation has been recently reported for growth of a yeast strain on methanol.<sup>15</sup> Curiously enough, for such different classes of microorganisms, the residual formaldehyde concentrations were similar to the ones reported herein. Although most probably  $C_1$ -compound-utilizing yeasts do not employ the RMP cycle,<sup>16</sup> formaldehyde appears to affect their growth and their biomass yield<sup>15</sup> in a way similar to that of RMP-cycle bacteria (see the present work and Refs. 2, 7, and 8).

It is appropriate also to discuss here the effect of formate and of the other substrates on the pH of the culture. When cells of either *Methylomonas* EP-1,<sup>7</sup> or L3 were grown continuously or batchwise on methanol and/or formaldehyde, pH control by alkali addition was required. However, when formate was used as a cosubstrate, the pH had to be controlled by acid addition to keep the pH from rising above 7.1. Without any pH control, steady states were achieved at pHs of 7.3–7.6 and the steady-state pH increased with the ratio of formate over the other substrates. It thus appears that there exists a process associated with formate utilization, that removes protons ( $\text{H}^+$ ) from the nutrient medium. For growth on methanol and/or formaldehyde the highest pH drop was observed with batch cultures, while with continuous cultures a comparatively little drop was observed; moreover, it was observed that the higher the yield, the lower the pH drop. A theory is proposed in the Appendix to explain the above effects of the various substrates on the culture pH.

## APPENDIX

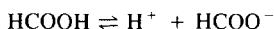
The relatively low activities of NAD-linked formate dehydrogenase in *Methylo-**monas* L3 which have been reported in Ref. 8 would naturally raise the question of whether formate is indeed oxidized to CO<sub>2</sub> through such a dehydrogenase. Yet the plain facts are that formate is readily oxidized to CO<sub>2</sub> and is *not* incorporated into the biomass, and as far as the authors know no other formate dehydrogenase exists. Further, whole-cell studies<sup>18</sup> have suggested that the cyclic pathway proposed by Ben-Bassat and Goldberg<sup>17</sup> does not operate, in an appreciable extent, in species L3, and thus formate oxidation would remain the only source of NADH<sub>2</sub> supply for biosynthetic purposes. It is in the light of the above facts that the authors believe that formate oxidation, shown by the data reported in Ref. 8 and presently (Table I), proceeds via an NAD-linked formate dehydrogenase, which, for species L3, appears to be unstable in free form. In view of the activities of the formaldehyde dehydrogenase reported in Ref. 8, the appearance of extracellular formate upon growth of L3 on either methanol and/or formaldehyde and the above discussion regarding the cyclic pathway of formaldehyde oxidation, it appears to us that formaldehyde oxidation proceeds predominantly through formate. Yet for this paper the exact mechanism of formaldehyde oxidation is totally immaterial. Finally, if the reader is not content with the arguments regarding the NAD-linked formate dehydrogenase, he or she may substitute NAD, throughout this paper, with any proton acceptor (coenzyme) he or she feels is suitable for the formate oxidation. No argument, conclusion, or discussion in this paper would change indeed, provided that *some* of the formate oxidation energy is eventually available to the cells through the reduced form of that coenzyme.

Lower biomass yields are caused by an ineffective coupling of energy production and utilization,<sup>11</sup> which is to say that more NADH<sub>2</sub>, and thus ATP, is produced through the complete oxidation of the carbon substrate than is required for carbon assimilation and growth. This was best demonstrated above by the ineffective supply of NADH<sub>2</sub> (through formate oxidation) and has been further demonstrated by the high oxygen demand and high carbon dioxide production rates that accompany lower biomass yields. In view of the fact (see main text) that lower biomass yields on methanol and/or formaldehyde are accompanied by larger pH drops, we speculate that this drop is caused by protons released from the ATP hydrolysis through the (reversible) ATPase system. Therefore, the oxidative phosphorylation theory<sup>18</sup> may predict a release of H<sup>+</sup> by the cells into the growth medium, so that upon growth the medium pH is expected to drop provided that there does not exist any process that requires a matching H<sup>+</sup> flow inwards. This may explain the pH drop observed during growth on CH<sub>3</sub>OH and/or CH<sub>2</sub>O. The peculiar phenomena associated with formate utilization (oxidation) are the transport process of formate and an excess production of NADH<sub>2</sub> and CO<sub>2</sub> through its oxidation. The NADH<sub>2</sub> overproduction will cause an increased H<sup>+</sup> flow outwards (i.e., a further pH drop), while the CO<sub>2</sub> transport outwards is thought to be a uniporter process<sup>18</sup> not associated with energy expenditure or H<sup>+</sup> translocation. If the medium was not saturated with CO<sub>2</sub>, then CO<sub>2</sub> overproduction would make it slightly more acidic by the H<sup>+</sup> released by the equilibration:



Thus, the inward transport of formate must account for the inward flow of H<sup>+</sup>, which increases with increasing formate utilization. Formate mainly exists in the anionic

form,  $\text{HCOO}^-$ , in the medium but it is transported in the proton-coupled form  $\text{HCOOH}$  by an appropriate symporter system through the membrane. If the required  $\text{H}^+$  is furnished by the membrane, 1 mol  $\text{H}^+$  will be required for the transport of 1 mol  $\text{HCOO}^-$ , and since these  $\text{H}^+$  will not be available for ATP synthesis, formate will be transported upon the expenditure of 1/2 mol ATP. Such a transport system is generally believed to exist coupled to  $\text{Na}^+$  import (Ref. 19,  $\text{H}^+/\text{Na}^+$  antiport). A similar system is believed to exist where the  $\text{H}^+$  is furnished by  $\text{NH}_4^+$  ( $\text{NH}_4^+/\text{H}^+$  antiport, Ref. 19), i.e., in both cases the  $\text{H}^+/\text{HCOO}^-$  symport is coupled to an appropriate antiport system. However, such a transport system would not lead to a pH increase but rather to a small decrease due to the protons freed by the equilibration



Another suggested transport mechanism is by means of a cyclic translocation of phosphate which causes an inward flow of  $\text{H}^+$ ,<sup>19</sup> and this mechanism or a similar one could only account for the observed pH increase due to formate transport. Clearly, such a mechanism does not suggest any energy expenditure for the transport of formate.

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ELEFTHERIOS PAPOUTSAKIS\*

Department of Chemical Engineering  
Rice University, Houston, Texas 77001

WILLIAM HIRT  
HENRY C. LIM

School of Chemical Engineering  
Purdue University, West Lafayette, Indiana 47907

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\* To whom all correspondence should be addressed.