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Methanol Inhibition in Continuous Culture of *Hansenula polymorpha*†

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Growth inhibition of *Hansenula polymorpha* DL-1 by methanol, formaldehyde, formate, and formic acid was examined to determine the causes of unstable behavior observed during continuous cultures on methanol. The much greater inhibition of growth by formic acid than by formate and the effect of formic acid excretion and assimilation on pH helped to explain culture dynamics observed after transitory oxygen limitations. Oxygen limitation caused by temporary reduction of agitation in a continuous fermentation caused methanol to accumulate to inhibitory concentrations. Immediately after resumption of agitation, formic acid was produced and caused culture inhibition. To ensure the stability of *H. polymorpha* in continuous culture, it was therefore necessary to prevent transient methanol accumulation.

Continuous-culture stability can be threatened by substrate toxicity (1, 3), particularly in high-productivity processes with high-inlet substrate concentrations. Such a case is the production of single-cell protein by yeast from methanol. The situation can be aggravated further by the excretion of oxidative intermediates of methanol catabolism which are toxic to growth. Methanol oxidation proceeds as methanol → formaldehyde → formic acid → CO_2 (13). The intermediates can be excreted, and with the yeast *Candida boidinii* Bh 11, Pilat and Prokop (9, 10) found that both formaldehyde and formic acid were inhibitory to growth.

During our study of the continuous culture of *Hansenula polymorpha* DL-1 with high-inlet methanol concentrations, several cases of culture instability were observed. To stabilize the culture, an on-line computer monitoring and control system is proposed. This paper describes experiments elucidating the basis for culture instability so that a more effective control system can be implemented.

Levine and Cooney (7) have indicated that, for *H. polymorpha* DL-1, methanol concentrations of >8 g/liter slow growth and concentrations above 80 g/liter stop growth. In this paper, growth inhibition by methanol, formaldehyde, and formic acid is more closely examined. These results help to explain culture responses to methanol accumulation caused by periods of oxygen starvation. The culture's response to periods of discontinuous feeding was also studied.

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MATERIALS AND METHODS

Organism and media. The organism used in this study was *H. polymorpha* DL-1 (ATCC 26012), isolated by Levine and Cooney (7). The culture was maintained on methanol-mineral salts agar slants transferred at 1- to 2-month intervals. The media used for shake flask and continuous cultures are presented in Table 1. Agar slants were prepared with 25 g of agar per liter added to the shake flask medium. The continuous culture medium was designed to satisfy the elemental requirements for yeasts as given by Harrison (4). Media were prepared by mixing the salts in distilled water, adjusting the pH to 4.5, and autoclaving for 15 min at 121°C for volumes up to 1 liter and for 45 min for volumes of 15 liters. After cooling, filter-sterilized vitamin solutions were added; 1 ml each of 8-mg/liter biotin and 800-mg/liter thiamine solutions were added to flasks; 1 ml each of 120-mg/liter biotin and 12-g/liter thiamine solutions were added to fermentors. Methanol was added without sterilization.

Continuous-culture equipment. The fermentor used was a 3-liter agitated vessel with a 2-liter working volume maintained constant by an electronic level controller which activated a peristaltic exit pump. Medium was fed by a Leeds and Northrup (North Wales, Pa.) peristaltic pump controlled either manually or by a PDP 11/10 digital computer (Digital Equipment Corp.).

The pH was monitored by an Ingold (Instrumentation Laboratories, Lexington, Mass.) autoclavable probe and a Radiometer (Copenhagen, Denmark) model 28 pH meter and controlled by the addition of 1 N KOH with a Radiometer model 11 titrator. Air flow rate was monitored and controlled with a model 5810 thermal mass flow meter and a model 5831 con-

TABLE 1. *Fermentation media for H. polymorpha DL-1*

Component	Concn (g/liter except where noted)	
	Shake flask medium	Fermentor medium
Methanol	4.0	59.0
$(NH_4)_2SO_4$	3.0	10.3
KH_2PO_4	2.0	2.0
$MgSO_4$	0.5	0.55
$CaCl_2 \cdot 2H_2O$	0.1	0.75
NaCl	0.1	0.11
$FeSO_4 \cdot 7H_2O$	1×10^{-6} M	15×10^{-6} M
$ZnSO_4 \cdot 7H_2O$	5×10^{-6} M	75×10^{-6} M
$CuSO_4 \cdot 5H_2O$	1×10^{-6} M	15×10^{-6} M
$Na_2MoO_4 \cdot 2H_2O$	1×10^{-6} M	15×10^{-6} M
$CaCl_2 \cdot 6H_2O$	1×10^{-6} M	15×10^{-6} M
$MgSO_4 \cdot 7H_2O$	5×10^{-6} M	75×10^{-6} M
Biotin	8×10^{-6}	1.2×10^{-4}
Thiamine-HCl	8×10^{-4}	1.2×10^{-2}

troller (Brooks, Hatfield, Pa.). The O_2 concentration in exit gas was measured with a model 7083 Leeds and Northrup paramagnetic oxygen analyzer, and the exit CO_2 was measured with a Mine Safety Appliances (Pittsburgh, Pa.) Lira model 303 infrared analyzer. Unimeasure 80 load cells (Pasadena, Calif.) were used to measure the weights of 8-liter medium and 1-liter base reservoirs.

Shake flask cultures. Experiments to examine growth inhibition by methanol, formaldehyde, and formic acid were done in 300-ml, side-arm, baffled flasks with nonsealing metal caps. Charged with 50 ml of medium at pH 4.5 (unless otherwise indicated), the flasks were inoculated with 2 ml of an exponentially growing culture and placed on a rotary shaker at 220 rpm. Growth at 37°C was followed by turbidity, using a Klett-Summerson colorimeter with a red filter. At a reading of 40 Klett units (e.g., after approximately two doublings), the inhibitory compound was added to all except two flasks, and the growth rate was monitored. During the additions, all flasks were removed from the shaker for 15 to 30 min. When replaced, the specific growth rate, μ, in the control flasks decreased by 20 to 40%. To compensate, all growth rates measured after inhibitor additions were corrected by multiplying by the following ratio: μ in the control flasks before addition/μ after addition. In the methanol and formic acid experiments, 2-ml samples were taken immediately after methanol and formate additions to check pH and methanol concentrations.

Continuous culture. Continuous-culture experiments were normally conducted at a dilution rate of 0.075 h^{-1} at 40°C, with the pH controled at 4.5. Normal agitation at 1,200 rpm maintained the dissolved-oxygen concentration above 30% of air saturation. Broth samples were rapidly removed to an ice bath and were centrifuged immediately at 12,000 × *g* for 10 min at 4°C. Total sample acquisition time was approximately 5 min.

Analytical methods. Dry cell weight was measured from 10- to 40-ml samples which were centrifuged for 10 min at 12,000 × *g*, resuspended, washed twice with distilled water, and dried in tared aluminum dishes at 80°C for 24 to 30 h. Methanol concentration was measured with a Hewlett-Packard model 5830A gas chromatograph (Avondale, Pa.) equipped with a model 18850A terminal and a model 7671A automatic sample injector. One milliliter of thawed sample was treated with 0.05 ml of 1 M H_2SO_4 (to lower the pH for formic acid analyses) and 0.2 ml of ethanol (to serve as internal standard). This mixture (0.7 μl) was automatically injected into a Chromosorb 101 column (0.125 in. by 2 ft [ca. 0.3 by 61 cm]). Peaks were detected by flame ionization, and conditions were as follows: column temperature, 110°C; injector temperature, 160°C; detector temperature, 225°C; and carrier gas (helium) flow rate, 30 ml/min. Peak areas were automatically integrated, and the methanol concentration was determined by using the ethanol peak as an internal reference. The lower limit of measurement was approximately 0.01 g/liter. Formaldehyde was assayed by the colorimetric method of Nash (8). Samples were diluted to formaldehyde concentrations within the linear 1- to 70-mg/liter region; the lower limit of formaldehyde detection was approximately 1 mg/liter. Formic acid concentration was measured by gas chromatography. Samples were prepared as described for methanol analysis; the addition of 0.05 ml of 1.0 M H_2SO_4 lowered the pH to 2.5 to ensure that at least 95% of the formate was in the acid form. A thermal conductivity detector (at 275°C) was used with two matching Chromosorb 101 columns (0.125 in. by 8 ft), one for the sample and one for a reference stream. The carrier gas flow rates were 70 ml/min in each column, with an injection temperature of 225°C and a column temperature of 170°C. The water peak was used as an internal standard, and the lower limit of measurement was approximately 0.1 g/liter.

RESULTS

Methanol inhibition. The influence of methanol on the growth of *H. polymorpha* DL-1 is shown in Fig. 1. Methanol concentration was measured in samples taken immediately after the addition of excess methanol and did not change significantly during the course of the growth rate measurement. The 30-min period of interrupted shaking at the time of methanol addition reduced the initial growth rate (measured over a period of several hours) in the control flasks from 0.20 to 0.14 h^{-1}. Therefore, all growth rates measured after methanol addition were normalized by multiplication by the factor 0.2/0.14 = 1.4. The results of Levine and Cooney (7) are shown in the same figure and are adjusted to reflect a maximum growth rate of 0.20 h^{-1}.

Methanol inhibition began at 6.5 g/liter, and the growth rate was linearly depressed between 6.5 and 58 g/liter according to the equation:

$$\mu = 0.225 - 3.87 \times 10^{-3} (S) \quad (1)$$

where (S) is the methanol concentration in

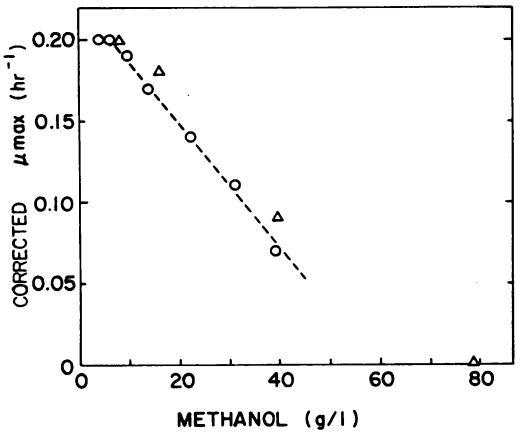

FIG. 1. *Growth rate of H. polymorpha DL-1 as influenced by methanol (growth rate was normalized for a perturbation introduced by culture transfer as described in the text). Symbols: △, results of Levine and Cooney (7); ○, this study.*

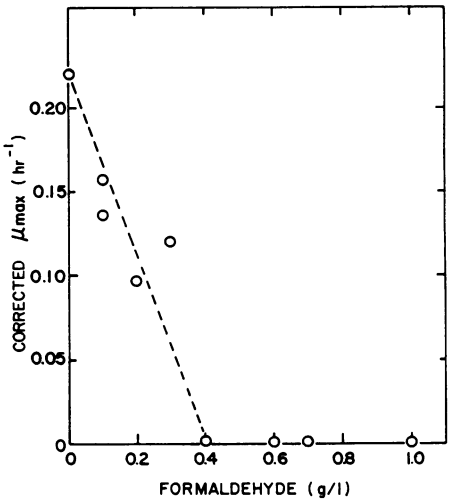

FIG. 2. *Inhibitory effect of formaldehyde on growth rate of H. polymorpha DL-1 in methanol; the growth rates were normalized as described in the text to reflect an uninhibited maximum growth rate of 0.22 h^{-1}.*

grams per liter. This equation implies that the maximum growth rate of *H. polymorpha* on methanol is 0.225 h^{-1} and that this value is approached as the methanol concentration approaches zero. However, at a low (e.g., <1 g/liter) methanol concentration, the normal Monod-type growth equation describes the dependence of growth rate on methanol concentration (D. Levine, S. M. thesis, Massachusetts Institute of Technology, Cambridge, 1972).

Formaldehyde inhibition. The inhibitory effect of formaldehyde on the growth rate of *H. polymorpha* in methanol medium is shown in Fig. 2. As little as 0.1 g of formaldehyde per liter caused a 30% inhibition, and 0.4 g/liter stopped growth completely. An interesting observation is that growth in the flasks containing 0.4 and 0.6 g of formaldehyde per liter was inhibited only temporarily; 6 h after the formaldehyde addition (0.4 g/liter), growth resumed at a rate equal to 83% of the control. In the case of 0.6 g of formaldehyde per liter, the lag was 10 h. At 0.7 and 1.0 g of formaldehyde per liter, no further growth occurred even after 30 h of incubation.

Formic acid inhibition. The relative concentrations of un-ionized formic acid and ionized formate depend on pH; the pK_a for formic acid is 3.75. Because of (i) the low buffering capacity of the medium at the initial pH of 4.5, (ii) the tendency of culture growth to reduce the pH by ammonium ion uptake and organic acid production, and (iii) the rise in pH accompanying formate uptake, the experiment to evaluate formic acid inhibition was conducted as follows: several flasks were inoculated with medium at initial pH's of 4.5, 4.75, 5.0, 5.5, and 7.0; in each flask, the initial growth rate was 0.20 h^{-1}. These results suggest that growth rate is insensitive to pH over the range 3.7 to 7 (see Table 2); however, this only reflects the initial growth rate after product addition and not a steady-state growth rate. When growth in each flask reached a density of 40 Klett units, various concentrations of sodium formate were added to the flasks originally at pH 4.5. To each of the others, 2 g of sodium formate per liter was added. The pH of each flask was measured immediately, and the initial growth rate was monitored by turbidity measurements. Formic acid concentration was calculated by the Henderson-Hasselbach equation. The results (Table 2) suggest that formate is not inhibitory at these concentrations, but that formic acid begins to inhibit growth at approximately 0.08 g/liter and completely stops growth at 0.2 g/liter.

The pH measured in each flask after growth had stopped is also shown in Table 2. In the control flasks and in the flask with the smallest formate addition, the pH dropped, whereas in all others, including those without further growth after 25 h of incubation, the pH rose. There are two opposing actions which influence pH; the first results from growing cells utilizing NH_4^+ ions and producing a hydrogen ion for each molecule of NH_4^+ assimilated as NH_3. The second results from formic acid assimilation. As formic acid is assimilated, hydrogen ions must be taken from the medium to restore the formate-formic acid equilibrium. The pH increase

TABLE 2. *Effect of formic acid and sodium formate on growth rate of H. polymorpha DL-1*

Initial pH	Total formic acid plus formate added (g/liter)	pH after addition	Calculated formate concn after addition (g/liter)	Calculated formic acid concn after addition (g/liter)	Corrected growth rate after addition (h^{-1})	Growth rate inhibition (% of control)	Final pH
4.5	0	3.70	—[a]	—	0.20	0	2.98
5.0	0	3.81	—	—	0.20	0	3.00
4.5	0.17	4.00	0.11	0.06	0.20	0	3.21
4.5	0.35	4.08	0.24	0.11	0.16	20	4.5
4.5	0.66	4.15	0.47	0.19	0	100	5.28
4.5	0.99	4.15	0.70	0.29	0	100	4.35
4.5	1.34	4.17	0.96	0.38	0	100	4.20
4.5	2.0	4.20	1.46	0.54	0	100	4.21
4.75	2.0	4.47	1.66	0.34	0	100	4.60
5.0	2.0	4.70	1.79	0.21	0.01	95	6.05
5.5	2.0	5.08	1.91	0.09	0.17	15	6.42
7.0	2.0	6.35	1.99	0.005	0.23	0	6.47

[a] —, Not done.

in cultures without growth suggests that assimilation of formic acid occurs even in the absence of growth.

Formaldehyde assimilation. In the experiments described above, inhibition of growth by formaldehyde concentrations between 0.4 and 0.6 g/liter could be overcome after a lag period. To examine this phenomenon more closely in continuous cultures, a sample of formaldehyde was added to a steady-state, methanol-limited continuous culture operating at a dilution rate of 0.075 h^{-1}. A formaldehyde addition of 0.5 g/liter was metabolized rapidly with first-order kinetics described by

$$dC_f/dt = 0.16\ C_f \qquad (2)$$

where C_f is formaldehyde concentration (in grams per liter) and t is time (in minutes) (Fig. 3).

The first sample, taken immediately after the formaldehyde addition, does not indicate 0.5 g/liter, but rather only 40% of that value. Equation 2 suggests that about a 5-min period is required to reduce the formaldehyde concentration by 60%; this is approximately the time elapsed during sample collection, cooling, and centrifugation. That 0.5 g of formaldehyde per liter did not inhibit growth in the continuous culture as in the batch culture is probably due to the higher ratio of cell mass to formaldehyde added. The higher this ratio is, the faster formaldehyde will be metabolized below inhibitory levels. Residual methanol increased (due to formaldehyde inhibition and growth) to 2.5 g/liter before the assimilation of formaldehyde reduced the formaldehyde concentration and allowed culture recovery. It is interesting that the methanol concentration continued to increase even though formaldehyde concentrations were less than those expected to cause culture washout. This suggests that the formaldehyde may have specifically affected some step early in methanol metabo-

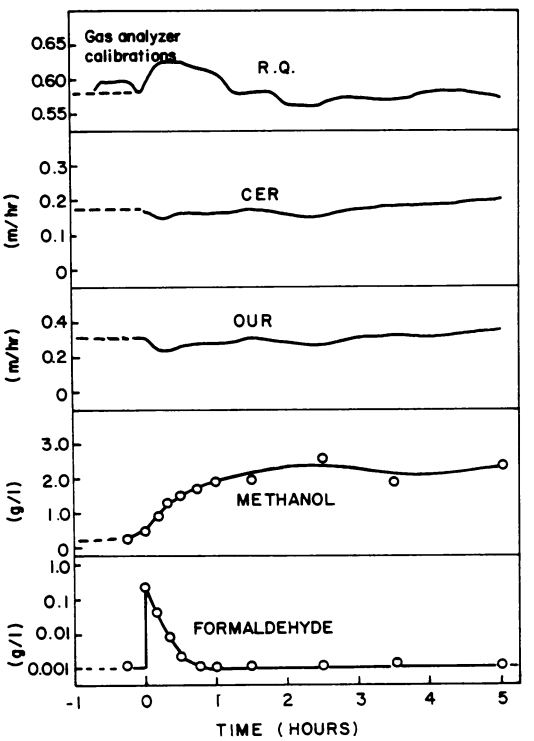

FIG. 3. *Metabolic response of H. polymorpha to the addition of 0.5 g of formaldehyde per liter in a methanol-limited continuous culture at D = 0.075 h^{-1}. The cell concentration was 13.5 g/liter. The formaldehyde concentration is plotted on a logarithmic scale. R.Q., Respiratory coefficient; CER, carbon dioxide evolution rate; OUR, oxygen uptake rate.*

lism; otherwise, methanol would be expected to continue to be used.

The increase in respiratory quotient may have been caused by either a reduced cellular yield on methanol (implying inefficient methanol metabolism) or oxidation of formaldehyde to CO_2. Both could have contributed; however, if the cell yield on methanol remained constant, the observed change in respiratory quotient could be accounted for by the oxidation of approximately 70% of the formaldehyde, with the remainder being used for cell mass synthesis.

Continuous-culture response to methanol accumulation. The response of *H. polymorpha* in continuous culture to transient increases in methanol concentration was examined. Such transient increases will occur if the culture becomes oxygen limited. This was examined by subjecting the methanol-limited continuous culture to a 3-h period of reduced agitation, e.g., from 1,200 to 100 rpm. Because of the obligatory role of oxygen in methanol metabolism, the resulting oxygen limitation led to methanol accumulation. The results of this experiment are shown in Fig. 4. The culture was originally at steady state at a dilution rate of 0.075 h^{-1} and a cell concentration of 14 g/liter. Although the rate of medium addition was under computer control, which was designed to prevent methanol accumulation, methanol, in this case, was allowed to accumulate to 7.6 g/liter before the feed was stopped. After 3 h, the agitation rate was increased to 1,200 rpm and the methanol concentration decreased. Both formaldehyde and formate then appeared in the fermentation broth (Fig. 4); between 3.0 and 3.5 h, these excreted products accounted for approximately half of the methanol utilized. Formic acid was by far the predominant product, with the sum of formate and formic acid reaching a concentration of 0.76 g/liter. At pH 4.5, 15% of the excreted formic acid will remain as the undissociated acid. Thus, the formic acid concentration (0.11 g/liter) was above the inhibitory

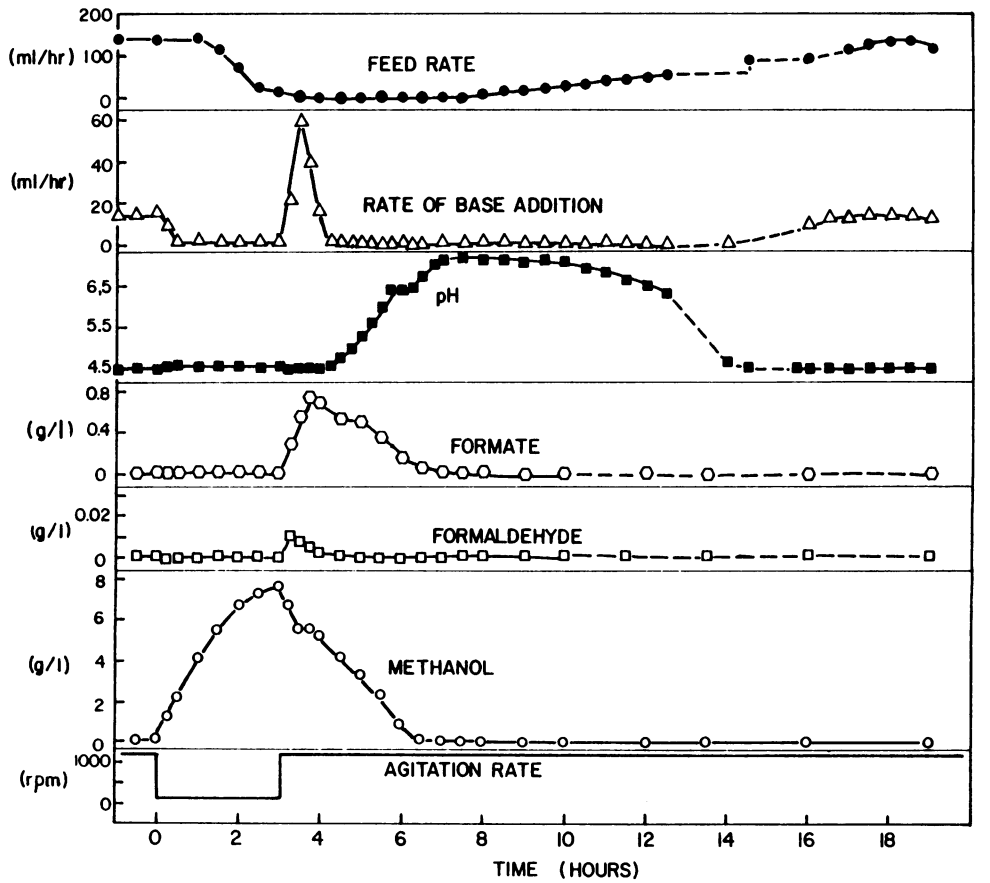

FIG. 4. *Response of a steady-state, methanol-limited continuous culture of H. polymorpha to a 3-h reduction in agitation rate.*

level and was probably the cause of the short plateau in the methanol curve observed at 3.6 h.

The rate of base addition rose sharply as formate accumulated. The rate of nitrogen uptake for growth during $t = 3$ to 4 h accounted for only about 4 ml (<10%) of the 1 N KOH added. This suggests that formic acid is the excreted intermediate and dissociates to require base addition for neutralization.

When formic acid was reutilized, the pH rose since hydrogen ions were needed to promote formate. Formic acid uptake thus provided a double benefit. Not only was the formic acid concentration decreased by assimilation, but also, as the pH rose, the formic acid-formate equilibrium shifted away from the inhibitory formic acid. As formic acid inhibition was decreased, methanol again began to disappear, and the culture recovered. After the accumulated methanol dropped to zero, medium addition was resumed under computer control.

Response to discontinuous feeding. Brooks and Meers (2) observed a decrease in the growth yield of *Pseudomonas methylotropha* in methanol-limited continuous culture subjected to discontinuous methanol feeding. They attributed the change to either inefficient use of excess methanol or accelerated protein turnover. Later, Pilat and Prokop (10) suggested that product formation may have been the cause. To study the effect of discontinuous feeding on *H. polymorpha* in continuous culture, the computer was programmed to provide a feeding pattern (Fig. 5). Over a feeding period, T (seconds), medium is fed at four times the desired average rate for $T/4$ s and is stopped for the remaining time of each cycle. A steady-state culture at $D = 0.075$ h^{-1} and a cell concentration of 14 g/liter was subjected to such feeding patterns with $T = 15$, 45, 120, 300, and 900 s. At a T of 300 s, the oscillatory feeding was continued for 12 h (approximately 1.1 residence times); for the other cases, the period was 4 h.

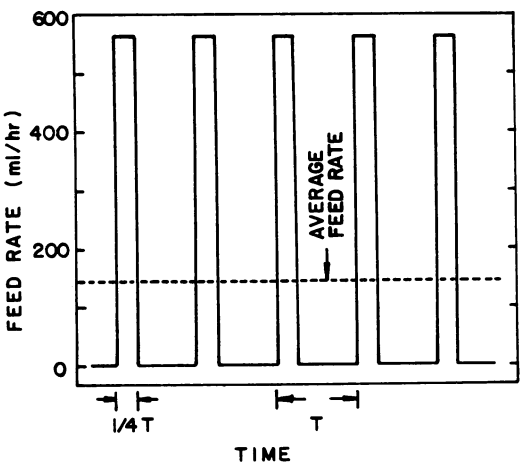

FIG. 5. *Pattern of oscillatory feeding for methanol addition to continuous culture. T is the length of one period of oscillation in which the feed rate was set at four times the desired dilution rate for one-fourth the period and stopped for the remainder of the period.*

The results are presented in Fig. 6. The methanol concentration was measured in samples taken at the end of a time period, T. The dotted line shows the slope of the cell concentration curve that would be expected if the cell yield on methanol had decreased by only 10%. In no case did a significant decrease in cell concentration or increase in methanol concentration result from the oscillatory feeds. Also, no detectable accumulation of formaldehyde or formic acid occurred. The events which occurred at days 3.1 and 4.7 both resulted from a computer software error which left the feed pump running continuously at a temporarily accelerated rate.

DISCUSSION

The pattern of growth inhibition by methanol observed here and reported by Levine and Cooney (7) follows the general pattern of substrate inhibition presented by Edwards (3), the pattern of methanol inhibition of *C. boidinii* Bh 11 (9), and the pattern of ethanol inhibition of wine yeast at pH 3.6 (5). The mechanism of methanol inhibition is not clear, but Pilat and Prokop (10) suggest that it is caused by an accumulation of formic acid or by methanol as it inhibits energy transfer and utilization by causing the disintegration of phospholipid sequences in the cell membrane (11).

Formaldehyde inhibition of *H. polymorpha* DL-1 is quite severe, as is formaldehyde inhibition of *C. boidinii* (9). Formaldehyde binds strongly to lysine amino groups of proteins (12) and also affects peptide linkages and several ring structures (11). Diel and Dellweg (*in* H. Dellweg, ed., Abstr. 5th Int. Ferment. Symp., Berlin, p. 390, 1976) found that formaldehyde binds strongly to homogenates of a methanol-utilizing *Kloeckera* sp. and suggest that it exerts its inhibition by changing the structure of membrane proteins.

The difference in inhibition between formic acid and formate is extreme and very important in understanding the dynamics of methanol cultures. Increasing the pH decreases the amount of formic acid. This may explain why the experiments of Pilat and Prokop (9–11), conducted at pH 4.8 to 5.0, indicated a much weaker formic acid inhibition than that observed here. Apparently, the formic acid concentration they report

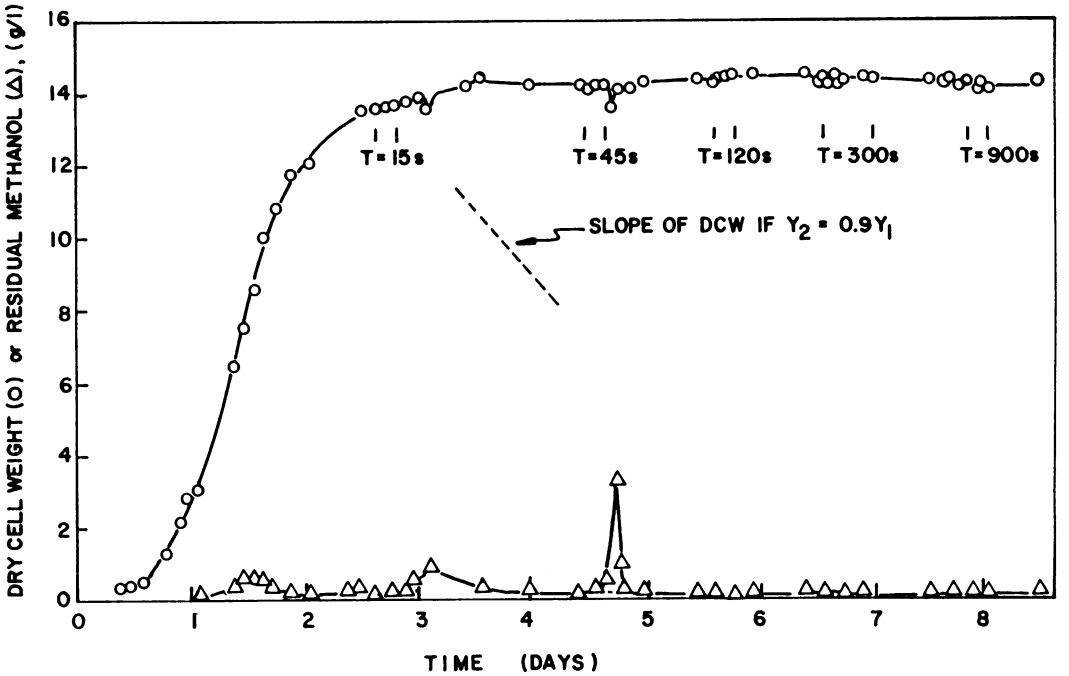

FIG. 6. *Cell mass concentration and residual methanol concentration for a continuous culture (dilution rate of 0.075 h^{-1}) which was subjected to oscillatory feed rates as described in the legend to Fig. 5 and in the text. Dashed line indicates the expected slope of the cell mass curve if the cell yield were decreased by 10%. DCW, Dry cell weight.*

is actually the sum of formic acid and formate. The site of action of formic acid is not clear, but low-molecular-weight fatty acids have been observed to inhibit both growth and respiration of *Candida* yeasts (6).

The response of the culture to the 3-h period of agitation reduction indicates that both formaldehyde and formic acid are excreted under conditions of excess methanol when oxygen is available in excess of the minimum requirement. This agrees well with the results of Pilat and Prokop (10) with *C. boidinii* Bh 11. In both cases, formate-formic acid accumulations are nearly two orders of magnitude greater than formaldehyde accumulations. It is tempting to attribute this to a rapid rate of formaldehyde reassimilation, but the observations of van Dijken (Ph.D. thesis, State University of Groningen, Groningen, The Netherlands, 1976) with *H. polymorpha* suggest another explanation. He showed that a hemimercaptal which spontaneously forms between formaldehyde and glutathione is most likely the true substrate of formaldehyde dehydrogenase and that *S*-formylglutathione is the product. Formaldehye forms the stable hemimercaptal as soon as it is released from the microbodies in the cell. The *S*-formylglutathione, however, can hydrolyze nonenzymatically to yield formate and glutathione. The free formic acid probably can traverse the cell membrane much more easily than can the formaldehyde hemimercaptal. Thus, more formic acid is released than formaldehyde.

In summary, it appears that the greatest threats to the stability of the *H. polymorpha* continuous culture are methanol and formic acid accumulation. Spicer (14) showed that a continuous culture is only stable as long as $d\mu/dS$ is greater than zero. Thus, whenever the residual methanol concentration exceeds 6.5 g/liter, culture stability is threatened. With high-productivity continuous culture, methanol can accumulate rapidly when assimilation is even transiently inhibited. Formic acid also can accumulate to inhibitory concentrations. Formic acid inhibition, however, is dependent on pH. The higher the pH of a culture is, the less susceptible it is to inhibition by excreted formic acid. Since formic acid excretion was always observed to follow periods of methanol accumulation, it appears that the key to stabilizing the culture is simply to prevent excessive methanol accumulation. The minimal effect of discontinuous feeding suggests that rapid changes in residual meth-

anol concentrations are not detrimental if the extent of accumulation is controlled. It is also important to consider the availability of oxygen, since methanol metabolism is dependent on it. The cells are probably more sensitive to methanol in the presence of oxygen than under oxygen-limiting conditions.

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