

Thermophilic Mixed Culture of Bacteria Utilizing Methanol for Growth¹

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Received for publication 21 January 1974

A thermophilic mixed population of bacteria, capable of utilizing methanol as its sole carbon-energy source at temperatures up to 65 C, was selected by enrichment and studied. A maximal cellular yield of 0.42 g per g of methanol was observed at 50 to 56 C. The maximal specific growth rate of the mixed population in continuous culture at 56 C was greater than 0.32 per h. The amino acid profile of the mixed culture indicated that a high quality protein was produced and the protein content was 71%. The properties of this culture and its ability to grow at elevated temperatures are discussed in terms of single-cell protein production and the treatment of industrial waste.

Methanol is an attractive raw material for fermentation processes that include the production of organic and amino acids as well as single-cell protein. The advantages of methanol are its low cost, high purity (>99.8%), complete water miscibility, and restricted use by certain microorganisms (1). When compared with more conventional raw materials, such as glucose or other carbohydrates, its disadvantages are the relatively high heat of fermentation and high oxygen demand. As a consequence, the removal of heat is of considerable concern in methanol-based fermentations. Since typical fermentations are run at 25 to 30 C with the average cooling water temperature at 20 to 23 C, the heat removal in a high rate process is a problem due to the low temperature driving force. One approach to overcoming this problem is to grow the microorganisms at higher temperatures; however, this is feasible only when the organism is thermophilic or thermotolerant.

We set out to isolate a microorganism(s) with the ability to use methanol for growth at temperatures above 50 C. Levine and Cooney (6) report a thermotolerant yeast, *Hansenula polymorpha* ATCC 26012, able to grow on methanol up to 50 C; however, both the cell yield and the maximal specific growth rate begin to fall at temperatures above 42 C. Foster and Davis (4) have isolated a strain of coccus which will grow at temperatures up to 50 C, but no other work has been published on the isolation of microor-

ganisms able to utilize methanol aerobically above 50 C. The value of such organisms would lie in their use for single-cell protein where high-heat loads are obtained as a consequence of high cell productivities and in the treatment of industrial waste when the waste is at an elevated temperature. In addition, such cultures offer advantages for application in hot climates.

MATERIALS AND METHODS

Media. The medium used for continuous culture studies contained, in grams per liter: methanol, 2.4; KH_2PO_4 , 4.54; $(\text{NH}_4)_2\text{SO}_4$, 3.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.8; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.3; also, in molar concentration, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1×10^{-6} ; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 5×10^{-6} ; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1×10^{-6} ; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 1×10^{-6} . The pH was controlled automatically to pH 6.0 by the addition of 1.0 N aqueous NaOH in response to a Radiometer (Copenhagen, Denmark) Model 26 pH meter. The medium for the enrichment culture was similar to the above, except that it contained 8 g/liter of methanol, only 2.27 g/liter of KH_2PO_4 , and 2.39 g/liter of Na_2HPO_4 .

Enrichment procedure. Soil samples were used to inoculate the enrichment medium, which was incubated at 51 C in stoppered flasks. The oxygen demand during initial enrichment is low, and there may also be a need for dissolved carbon dioxide for carbon dioxide fixation; for these reasons, we employed standing cultures and the flasks were flushed with air daily. At weekly intervals, one-tenth of the broth was used to serially inoculate a new flask of medium. Growing cultures obtained in this manner were then added to a continuous enrichment culture maintained at a dilution rate of approximately 0.05 per h at pH 7.0 and 56 C.

Continuous culture. A 1-liter Fermentation De-

¹Contribution No. 2317 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139.

sign fermentor (Allentown, Pa.) was used for these studies. A galvanic dissolved oxygen probe (Fermentation Design) was used to monitor the dissolved oxygen which was maintained above 20% of air saturation. An Ingold pH electrode with a Radiometer pH meter and titrator were used to control pH. The culture was grown at dilution rates ranging from 0.03 to 0.32 per h. The fermentor was run non-aseptically, and methanol was the growth-limiting nutrient. Air was sparged continuously at 0.3 to 1.0 liter/min, and the liquid volume was maintained constant at 1 liter by means of an overflow tube connected to an exit pump operating at a higher flow rate than the feed pump. The opening of the overflow tube was partially shielded by a tube of larger diameter, such that the liquid removed came from the bulk liquid and not the surface. In this way, we prevented surface concentration of microorganisms. The growth medium, however, was sterilized in 20-liter containers to prevent growth in the medium reservoir during storage.

Assays. Dry cell weight of the culture was determined in duplicate by centrifuging and washing the cells from 80 ml of broth and weighing after drying overnight at 110 C. Cellular yields in Table 1 were calculated from the amount of cells formed per unit weight of methanol consumed. These data are uncorrected for residual methanol in the broth or evaporation loss of methanol in the air stream.

The protein content was determined by the biuret reaction (10), and the carbohydrate content of the cells was determined by using the procedure of Seifter et al. (9). The total nucleic acid content was determined by the method of Munro and Fleck (7). Amino acid analysis was carried out with a Beckman model 121 amino acid analyzer. For this analysis, cell samples were hydrolyzed with 6 N HCl by sealing a suspension of washed cells in ampoules under vacuum and incubating for 20 h at 110 C. After incubation, the vial was cooled to room temperature and opened, and the contents were flash evaporated at 50 C. The residue was dissolved in 0.2 N sodium citrate buffer, pH 2.2, and filtered through a membrane 0.45- μ m filter (Millipore Corp., Bedford, Mass.) prior to amino acid analysis.

Electron microscopy. Samples were prepared by allowing a thin film of diluted broth to dry on a collodion-coated grid. The film was then shaded with chromium.

RESULTS

Culture isolation. A mixed culture with stable cell density utilizing methanol as its sole source of carbon and energy was obtained by using a continuous enrichment culture inoculated with samples preincubated in the methanol-mineral salts medium at 56 C. At least three morphologically distinct microorganisms (i.e., large and small rod shaped, and ellipsoid shaped) were distinguishable in electron micrographs (Fig. 1) of the culture growing at low dilution rates (less than 0.06 per h).

The culture appeared to be predominantly

gram-negative. However, prolonged incubation in the absence of methanol under aerobic conditions led to sporulation of at least one of the species, thus suggesting the presence of at least one gram-positive species.

We attempted to separate the individual species in the mixed population by plating culture samples on methanol-mineral salts agar plates and on nutrient agar plates and incubating at 56 C. Growth of mixed organisms on the methanol agar plates was very poor, and colonies of single organisms could not be found. On the nutrient agar plates there was good growth and we could observe three distinct colony forms; however, none of the colonies or mixtures thereof were able to grow when put back into a methanol-mineral salts medium. We also tried serial dilution of growing cultures in a manner used to obtain the "most probable number" of organisms: the culture was serially diluted until there was no growth at the greatest dilution. The tubes in which growth occurred after the greatest dilution contained a mixed population of bacteria as indicated from microscope examination, thus further suggesting a synergistic relationship between the individual components of the mixed culture.

Cell yield. Cellular yields, expressed as grams of dry cell weight per gram of methanol, were measured for the mixed culture over a range of temperatures and dilution rates. The results are given in Fig. 2 and Table 1. The maximum yield occurs at 50 to 56 C and then falls as the temperature is increased. The maximum yield, 0.42 g of cell per g of methanol, is actually a conservative value since any correction for residual or evaporated methanol would increase it.

The results in Table 1 indicate that the cell yield increases with increasing growth rate at both 56 and 60 C. Unfortunately, the yield at 60 C could not be compared with the yield at 56 C when taken at a dilution rate of 0.32 per h because the culture at 60 C would not grow that fast.

Cell composition. The cell composition of the mixed culture growing in continuous culture at a low dilution rate of 0.03 per h was analyzed on two separate occasions. Early in these studies, the culture would form a pink pigment and then within a few days lose the coloration. This occurred at low dilution rates (0.03 per h), and, to examine the apparent stability of the culture with and without the pigment, we measured the cell composition and found it to be independent of pigment formation. After these initial studies, the pink pigment no longer appeared, and its occurrence was not pursued. The average



FIG. 1. Electron micrograph of the thermophilic mixed population of bacteria grown on a methanol-mineral salts medium at 56 C. Magnification, 2,436 \times .

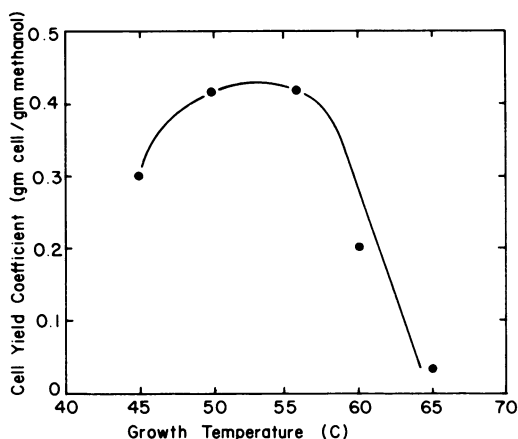


FIG. 2. Effect of growth temperature in continuous culture at a dilution rate of 0.2 per h on cellular yield. The point at 65 C was growing at a dilution rate of 0.033 per h and was computed from optical density measurements. Methanol was the limiting nutrient.

culture composition was 71% protein, 7.1% total nucleic acid, and 6.4% carbohydrate. The amino acid profile was determined for cells grown in continuous culture at a dilution rate of 0.05 per h. The results of this analysis are given in Table 2 and compared with the FAO reference level and a methanol-utilizing culture obtained by Haggstrom (5). Our mixed culture compares well with other sources of methanol-grown cultures; in comparison to the FAO reference our culture is limiting in methionine. A comparison

of tryptophan levels was not made because of acid destruction of tryptophan in our preparation of the culture for amino acid analysis.

Growth kinetics. In continuous culture studies, the most common method of measuring the maximal growth rate is to use the washout technique. This approach entails increasing the medium flow rate to slightly above the maximal growth rate; the maximal growth is then calculated from the culture washout rate. When we tried this approach, the calculated maximum appeared to be less than the previous steady-state growth rates. For example, a growth rate obtained by washout from a steady state in which the initial dilution rate was 0.32 per h was measured to be 0.27 per h from the washout data, and the rate declined even further as the washout proceeded. From this and many similar experiments, it became clear that the washout technique was inapplicable. The growth rate in batch culture at 51 C, for instance, was

TABLE 1. Effect of dilution rate on cellular yields on methanol in methanol-limited continuous culture

Temp (C)	Dilution rate (per h)	Cell yield (g dry weight/g of methanol)
56	0.11	0.30
56	0.21	0.39
56	0.32	0.42
60	0.06	0.12
60	0.23	0.20

TABLE 2. Amino acid analysis of a thermophilic mixed culture grown on methanol

Amino acid	Amino acid concentration (g/100 g of protein)		
	Thermophilic culture	TM20 (5)	FAO reference protein
Proline	4.32	3.81	
Lysine	6.50	5.30	4.2
Histidine	2.05	1.73	
Arginine	5.93	7.10	
Aspartic acid	10.54	8.47	
Threonine	5.50	4.52	2.8
Serine	3.58	3.62	
Glutamic acid	13.70	10.92	
Glycine	5.37	5.55	
Alanine	9.29	7.91	
Valine	7.98	5.85	4.2
Methionine	2.28	1.81	2.2
Isoleucine	5.20	3.90	4.2
Tyrosine	4.28	2.91	
Phenylalanine	6.08	4.18	2.8
Tryptophan	Not assayed		1.4
Cystine	Not assayed	0.32	2.0
Leucine	8.29	6.96	4.8

0.38 per h, corresponding to a mass doubling time of 1.8 h. The residual methanol concentration is normally low in continuous culture. However, as washout proceeds, the methanol concentration increases with time. We therefore hypothesized that the results might be due to an increasing growth-inhibitory effect of methanol.

Growth inhibition. To examine the growth-inhibiting effect of methanol on the mixed population, a series of shake-flask cultures were incubated at 51 C with varying initial concentrations of methanol, from 0.16 to 1.6 g/liter (Fig. 3). Each flask was inoculated from the same source, and the initial growth rate was monitored by optical density using a Klett-Summerson colorimeter with a red filter. The specific growth rate, up to an initial methanol concentration of 0.64 g/liter, was 0.35 to 0.38 per h. At 0.8 g/liter of methanol there was an initial lag of about 2 h, after which the culture grew with a specific rate of 0.35 per h. When the initial methanol concentration was increased to 1.2 g/liter, there was little or no growth. It appears that the minimum inhibitory concentration of methanol is about 0.8 g/liter. The observed lag in this experiment is likely due to the evaporation loss of methanol before growth began. An alternative possibility is that there was a selection of methanol-tolerant organisms. However, because little or no growth occurred at 1.2 g per liter of methanol, we feel this is a less likely explanation than methanol inhibition. Thus, in light of these experiments, it is possible that methanol inhibition is responsible for the above results in which the maximal growth rate

obtained by the washout technique is less than the prior steady-state growth rate.

DISCUSSION

Although a wide variety of microorganisms have been isolated for their ability to utilize methanol as their sole carbon and energy source, none has been able to grow at temperatures above 50 C. Our interest in the microbial utilization of methanol at thermophilic temperatures stems from the need to remove large amounts of heat during the production of single-cell protein from methanol. For instance, if one assumes a cell productivity of 5 g of cell per liter per h and an oxygen yield of 0.5 g of cell per g of oxygen, then the oxygen uptake rate is 310 mmol of oxygen per liter per hour. Using the correlation found by Cooney et al. (2) of 0.12 J per mmol of oxygen for predicting fermentation heat loads, the heat load in this example is 37 J per liter per h. To remove this heat load from a 100,000-gal (378,500-liter) fermentor, one would need approximately 100,000 ft² (9,200 m²) of heat exchange area, assuming a typical cooling water temperature of 20 to 23 C. This amount of surface area in a traditional stirred tank would fill the vessel with internal cooling coils. Thus, the heat removal problem becomes one of a low-temperature driving force and a low available surface area for heat transfer. The problem can be alleviated by using refrigeration to chill the cooling water or by raising the temperature of the fermentation. The latter approach is potentially less expensive. In addition to the benefit of heat removal, thermophilic operation also lessens the probability of contamination, enhances cell recovery as a consequence of decreased liquid viscosity, and provides a product low in nucleic acid. The microorganisms evolving from such a process would also be beneficial for industrial waste treatment for hot waste streams.

Our approach to this problem was to select a microorganism(s) from the environment which had the ability to utilize methanol as its sole carbon and energy source in a mineral salts medium at 56 C. The selection led to a mixed population of bacteria interacting in a symbiotic manner. Attempts to segregate individual methanol-utilizing species on methanol medium from the population were unsuccessful.

Growth inhibition by methanol concentrations over 1 to 2% have been reported for a number of methanol-utilizing cultures (1). Our mixed culture is more sensitive to methanol, displaying complete growth inhibition at con-

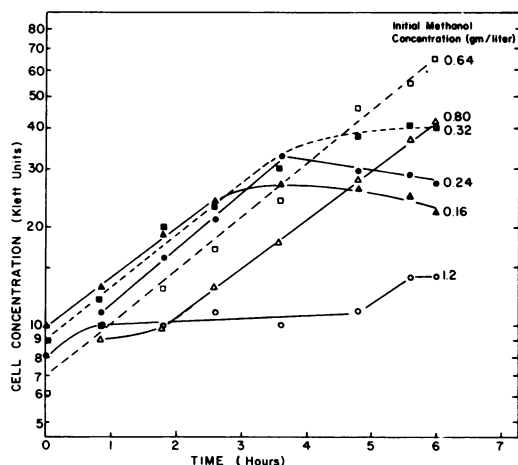


FIG. 3. Effect of initial methanol concentration on the growth rate of a mixed culture at 51 C in a pH 6.0 methanol-mineral salts medium.

centrations above 0.1%. In the context of single-cell protein production in continuous culture, however, this should not pose a problem since steady-state methanol levels were generally below 0.03 g/liter (0.003%), as shown by gas chromatographic analysis of the fermentation broth.

The maximal growth rate determined in batch culture at 51 C compares quite favorably with those values reported for mesophilic cultures at lower temperatures. The lowest doubling time for this thermophilic culture is 1.81 h, whereas the fastest doubling time previously reported for methanol-utilizing microorganisms is 2 h. The unique aspect of this mixed population is its ability to grow from below 45 C up to 65 C; thus, it is possible to operate a continuous culture process at thermophilic conditions without a sacrifice in growth rate.

In considering a microorganism as a source of single-cell protein, it is necessary to examine not only the growth rate but also the cellular yield of the culture. Sukatsch and Johnson (11) isolated a series of mixed cultures growing on hydrocarbons at temperatures ranging from 25 to 65 C and observed that the cellular yields of these mixed cultures decreased as the temperature of cultivation increased. In our work, the yield increased when the growth temperature was shifted from 45 to 50 C, and only above 56 C did the yield on methanol begin to fall rapidly with small temperature increases. Sukatsch and Johnson (11) found a decline in growth yield on hydrocarbons somewhere between 35 and 45 C for a mixed culture isolated at 55 C. It is likely that this is due to an increased requirement for maintenance energy to maintain the structural integrity necessary for growth at elevated temperature. The highest reported yield for bacteria growing on methanol is 0.5 g of cell per g of methanol at 30 C (M. Dostálek and N. Molin, *In* S. R. Tannenbaum and D. I. C. Wang, ed., *Single-Cell Protein*, in press). This compares with our maximal value of 0.42 obtained at 56 C, which is not corrected for residual methanol in the broth (shown to be negligible) and methanol lost by evaporation. Thus, the value is somewhat conservative.

It can be shown by calculation that the methanol losses by stripping are negligible. Using Henry's law and Dalton's law, the equilibrium concentration of methanol in the air stream can be computed. For a value of the Henry's law constant of 940 torr-mol of solution/mol of solute (derived from the work of Othmer [8]) and a typical steady-state methanol concentration of 30 mg/liter, the loss is 9 mg/liter of

broth per h for an aeration rate of 5 standard volumes of air per unit volume of culture per min. The MeOH feed rate is 900 mg/liter/h for a dilution rate of 0.3 per h and a relatively low feed concentration of 0.3% MeOH. Thus, even assuming a slow methanol feed, fast aeration rate, and equilibrium conditions, the loss of methanol due to stripping is only 1% of the feed. Under actual conditions it would probably be much less.

In addition to cell productivity, the protein content and quality of the culture must be considered when evaluating it for use as a source of single-cell protein. The total protein content of the culture is 71%, which is typical for bacteria. The total nucleic acid level is only 7.1% of the cell dry weight. This is relatively low and is most likely a consequence of the low growth rate of the cells analyzed and the high temperature of growth. Dicks and Tempest (3) found that the ribonucleic acid (RNA) content of *Aerobacter aerogenes* decreased with increasing temperature of growth; this comparison is made for constant growth rate. The reason for this decrease in RNA content is that the efficiency of protein synthesis by ribosomes increases with increasing temperature, and thus the cells need less RNA per cell to maintain a given growth rate. Thus, an added advantage of thermophilic operation is a lower nucleic acid level and increased protein to RNA ratio.

The essential amino acid levels in the protein compare well with other bacterial sources of single-cell protein and reasonably well with the FAO reference level. The limiting amino acid appears to be methionine which is typical for single-cell protein.

ACKNOWLEDGMENTS

This work was supported by the Undergraduate Research Opportunities Program at M.I.T., a grant from the Uniroyal Foundation, and National Science Foundation Undergraduate Research Participation Program Grants GY-9907 and GY-10680.

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