Production of Bacterial Cells from Methane

BRIAN T. SHEEHAN AND MARVIN J. JOHNSON

Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706

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A mixed methane-oxidizing bacterial culture capable of stable and predictable growth in continuous culture was isolated. The culture consisted of two types of gram-negative nonsporulating rods resembling pseudomonads. The culture grew well at 45°C on an inorganic medium without asepsis. Specific metal requirements for Ca²⁺, Cu²⁺, MoO₄²⁻, Zn²⁺, Mn²⁺, Mg²⁺, and Fe⁺⁺ (or Fe⁺⁺) were shown. The cells grown in continuous culture contained 11.7 to 12.1% total nitrogen. From an animal nutrition standpoint, the distribution of amino acids was satisfactory. The continuous fermentation was operated over a range of steady-state dilution rates from 0.085 to 0.301 hr⁻¹. The maximum specific growth rate for the culture, μₘₐₓ, was 0.303 hr⁻¹ (doubling time 2.29 hr). The average yield for all fermentations analyzed was 0.616 g (dry weight of cells per g of methane used and 0.215 g (dry weight) of cells per g of oxygen used. The yields on both methane and oxygen were higher for the oxygen-limited than for the methane-limited fermentations. The maximum productivity attained in the fermentor was 2.39 g (dry weight) of cells per hr per liter at a dilution rate of 0.187 hr⁻¹ and a cell concentration of 12.8 g (dry weight) of cells per liter. The limit on maximum cell productivity was determined only by the mass transfer rate of oxygen in the fermentor. The simultaneous volumetric mass-transfer coefficients (kₐ in hr⁻¹) for oxygen and methane were determined. The results appear to indicate an oxygen to methane mass-transfer coefficient ratio of approximately 1.4.

Within the past decade, considerable work has been done on the growth of microorganisms on hydrocarbons. If hydrocarbons can be economically converted to cellular protein, they might become a significant indirect source of food for man or domestic animals.

The most obvious advantages of using natural gas (methane) as a possible substrate for cell growth are its relatively low cost, abundance, and high purity and the ease of removal of unused methane.

The literature on methane-oxidizing fermentation has been reviewed recently by Vary and Johnson (12), Wang (13), and Coty (2).

Most past research on methane fermentation has been devoted to the metabolic pathways of methane oxidation and the taxonomy and physiology of methane-utilizing organisms. Published information on the microbial cells themselves has been fragmentary. Data have been incomplete or lacking on such significant factors as cell growth rate, yield per unit of methane and of oxygen, concentration of cells per unit volume, metal requirements for growth, oxygen and methane requirements, protein and nucleic acid content, and distribution of amino acids and vitamins. Vary and Johnson (12) reported cell yields from methane between 60 and 70% and generation times of approximately 3 hr for a mixed methane-oxidizing bacterial culture. No data for continuous culture of cells on methane have been published.

Research on the growth of microbial cells on methane was initiated with the following objectives: (i) isolation of a methane-oxidizing culture capable of stable growth on an inorganic medium in continuous culture without asepsis; (ii) rapid production of cells with good yields on methane and oxygen; (iii) determination of cell yields, medium, and oxygen and methane requirements.

MATERIALS AND METHODS

Media. Media for isolation and for continuous fermentation of the bacterial culture are shown in Table 1. The isolation medium was used both for isolation of the culture and for maintenance of the culture in gassed shake flasks (3% CO₂, 15% CH₄, and 82% air) during shutdowns of the continuous fermentor. The medium for continuous fermentation with pH control will support a cell concentration of 12 g (dry weight) of cells per liter when supplied with adequate oxygen and methane. An appropriate dilution must be used if lower populations are antici-
Table 1. Isolation and fermentation media

| Component       | Isolation medium (g/liter) | Continuous* fermentation medium (g/liter) |
|-----------------|-----------------------------|------------------------------------------|
| KH₂PO₄          | 1.60                        | 0.67                                     |
| Na₂HPO₄         | 1.16                        | 0.22                                     |
| NaNO₃           | 1.18                        | 9.55                                     |
| MgSO₄·7H₂O      | 0.080                       | 0.32                                     |
| FeSO₄·7H₂O      | 0.014                       | 0.029                                    |
| Ca(NO₃)₂·4H₂O   | 0.025                       | 0.18                                     |
| CuSO₄·5H₂O      | 4.0 × 10⁻³                   | 9.1 × 10⁻²                               |
| ZnSO₄·7H₂O      | 3.4 × 10⁻⁴                   | 1.1 × 10⁻²                               |
| MnSO₄·H₂O       | 3.0 × 10⁻⁴                   | 1.5 × 10⁻³                               |
| Na₂MoO₄·2H₂O    | 2.4 × 10⁻⁴                   | 6.4 × 10⁻⁴                               |
| CoCl₂·6H₂O      | 4.5 × 10⁻⁵                   | 2.7₉                                    |
| Concd H₂SO₄(56 N) |                             |                                         |

* For 12 g of cells per liter. For lower cell concentrations, proportionally lower concentrations of all constituents were used.

Gas analysis. The flow rates of the air, pure oxygen, and C.P. grade methane feed gases were determined from time of passage through burette bubble-tubes (9). The nitrogen, oxygen, and methane content of the dried fermentor exit gas was determined with a gas chromatograph on a molecular sieve 5A column [10 ft by 0.25 inch, (3.04 m by 0.64 cm) 20 to 60 mesh] at 40 C. The column packing was prepared by grinding 3½ inch (0.16 cm) Linde 5A molecular sieve pellets and collecting the 20- to 60-mesh fraction after screening. The detector was a thermal conductivity bridge with a helium carrier gas. The concentration of CO₂ in the dry exit gas was determined with a membrane-type oxygen probe (1) from its O₂ content with and without the removal of CO₂ with Ascarite.

Cell analysis. Cells from a methane-limited continuous fermentation (μ = 0.20 hr⁻¹) were centrifuged at 8,000 × g for 50 min, washed, recentrifuged twice with distilled water, and dried for 48 hr at 105 C. Cell samples were analyzed by Micro-Tech Laboratories, Inc., Skokie, Ill., for carbon, hydrogen, nitrogen, and phosphorus.

The amino acid content of the cells was determined with a Beckman-Spinco Automatic Amino Acid Analyzer (model 120) after acid hydrolysis of dried cells with 6 N HCl at 120 C for 22 hr. Cystine was determined as cysteic acid after performic acid oxidation and acid hydrolysis by the method of Schram et al. (10). Tryptophan was determined with and without an internal standard after basic hydrolysis by the method of Oelschleger et al. (7). Additional details on the analyses are given elsewhere (B. T. Sheehan, Ph.D. Thesis, Univ. of Wisconsin, Madison, 1970).

Dissolved oxygen and methane determination. The dissolved oxygen concentration in the fermentor was continuously monitored with a membrane probe as described by Borkowski and Johnson (1). The dissolved methane concentration in the fermentor, during fermentations in which the feed gases were O₂ and CH₄, was determined with a tubing probe based on the concept of Phillips and Johnson (8). The probe was made of Dow-Corning silicone rubber tubing (24 inches in length (61 cm) with an inner diameter of 0.062 inch (0.16 cm) and an outer diameter of 0.095 inch (0.24 cm)]. A stream of hydrogen (7.6 ml/min) passed through the tubing, sweeping out the gases which diffused through the walls of the tubing from the fermentation broth. Oxygen, carbon dioxide, and water vapor in the probe effluent were eliminated by suitable traps. The level of methane in the effluent was then determined with a thermal conductivity bridge.

Yields. Cell yields on methane and oxygen were determined on steady-state continuous fermentations from material balances on methane and oxygen and dry weights of cells produced. Yields were calculated as grams of dry cells per gram of methane and per gram of oxygen used.

RESULTS AND DISCUSSION

Bacterial culture. The methane-oxidizing culture M45 isolated at 45 C was a mixed culture. The culture consisted of two types of gram-nega-
Table 2. Summary of data for continuous fermentations

| Dilution rate (hr⁻¹) | Cell concn (g, dry wt/liter) | Limiting nutrient | Y(CH₄)ᵃ | Y(O₂)ᵃ | Feed gas rate | Fraction CH₄ usedᵇ | Carbon recovery (%) |
|----------------------|-------------------------------|-------------------|----------|---------|---------------|-------------------|-------------------|
| 0.220                | 6.83                          | CH₄               | 0.618    | 0.221   | 81.0          | 0.788             | 99.1              |
| 0.195                | 7.50                          | O₂                | 0.652    | 0.235   | 80.6          | 0.755             | 96.0              |
| 0.193                | 3.74                          | O₂                | 0.656    | 0.224   | 50.6          | 0.553             | 104.2             |
| 0.191                | 8.75                          | O₂                | 0.629    | 0.226   | 84.6          | 0.830             | 100.2             |
| 0.189                | 6.40                          | O₂                | 0.599    | 0.210   | 82.6          | 0.634             | 112.6             |
| 0.183                | 7.31                          | CH₄               | 0.591    | 0.205   | 72.3          | 0.823             | 101.8             |
| 0.181                | 5.33                          | O₂(?)             | 0.619    | 0.215   | 60.8          | 0.679             | 100.2             |
| 0.178                | 5.03                          | CH₄               | 0.574    | 0.194   | 61.2          | 0.671             | 98.5              |
| 0.176                | 7.03                          | O₂                | 0.595    | 0.214   | 81.0          | 0.678             | 97.0              |
| 0.176                | 5.98                          | CH₄(?)            | 0.614    | 0.215   | 66.7          | 0.680             | 102.3             |
| 0.174                | 6.08                          | O₂                | 0.593    | 0.203   | 87.3          | 0.543             | 102.8             |
| 0.154                | 11.74                         | CH₄               | 0.592    | 0.197   | 97.7          | 0.837             | 95.6              |
| 0.100                | 1.61                          | O₂                | 0.641    | 0.214   | 26.7          | 0.328             | 99.2              |
| 0.0985               | 1.64                          | O₂                | 0.606    | 0.225   | 27.7          | 0.324             | 98.6              |
| 0.0854               | 1.86                          | O₂                | 0.593    | 0.227   | 26.9          | 0.335             | 99.1              |

ᵃ Grams (dry weight) of cells per gram of substrate used.
b Fraction of feed CH₄ converted to cells and CO₂.

tive nonsporeforming rods resembling pseudomonads. The culture has not yet been characterized. One of the organisms was a short almost cocoid rod measuring, 0.6 by 1.0 to 1.3 μm. The other was a longer but thinner rod, 0.3 by 1.5 to 3.0 μm. The approximate ratio of five times as many short rods as long rods appeared to be maintained over the growth conditions used. All fermentations were carried out with total lack of asepsis.

The culture was stable in the sense that the same two types of organisms persisted for 3 years when subjected to continual contamination and to stresses from equipment and operator failure, such as loss of power, pH control, temperature control, and loss of liquid or gas feed.

The maximum specific growth rate, μmax, was directly determined by increasing daily the liquid feed rate to the fermentor by small increments until washout of the culture M45 occurred. The maximum specific growth rate was found to be approximately 0.303 hr⁻¹ (doubling time 2.29 hr).

Growth conditions. The mixed methane-oxidizing culture M45 was continuously grown at 45 C at cell concentrations as high as 12 g (dry weight) of cells per liter with the simple inorganic salts mixture given in Table 1 and adequate methane and oxygen. No requirement for an organic growth factor, such as a vitamin or amino acid, was found. Specific requirements were found for the ions Ca²+, Cu²+, MoO₄²⁻, Zn²+, Mn²⁺, Mg²⁺, and Fe³⁺ (or Fe²⁺). No conclusive evidence for a Co²⁺ requirement was found. Any possible trace requirement for Co²⁺ may have been met by the exposure of the fermentation to the stainless steel in the fermentor or by an impurity in one of the reagents.

The specific requirement for the ions Ca²⁺, Cu²⁺, MoO₄²⁻, Zn²⁺, and Mn²⁺ was determined by the following experimental method. When the methane- or oxygen-limited continuous fermentation had been at steady state for at least two residence times at a cell concentration greater than 6.0 g (dry weight) per liter, the feed line to the fermentor was switched to a medium to which the metal ion being tested had not been added. The concentration of the other salts and all other conditions remained constant. As the fermentation became limited by the decreasing concentration of the metal in question, the dissolved oxygen concentration in the fermentor rapidly increased and the cell concentration slowly decreased. When the required metal ion was added directly to the fermentor, an increase in growth rate and decrease in dissolved oxygen level were observed. The high cell concentrations used prevented the culture from meeting its metal requirements from reagent or equipment contamination.

The use of ammonia as the nitrogen source was unsatisfactory because of contamination of the nonsterile fermentation at 45 C with nitrifying organisms. Analysis of cell-free beer showed detectable amounts of nitric and nitrate. The nitrite produced appeared to inhibit the growth of the methane-oxidizing bacteria. The change in nitrogen source from ammonia to nitrate at 45 C...
produced a stable mixed methane-oxidizing culture under nonsterile conditions.

Levels of carbon dioxide in the exit gas of as much as 55% (v/v) were not found to be inhibitory.

No systematic investigation of the effect of pH on culture growth was made. The culture appeared to grow well between pH 6.5 and 7.0. All continuous fermentations from which yield data were obtained were operated at pH 6.8.

**Cell yields.** The yield results for a series of 15 gas-limited continuous M45 fermentations are shown in Tables 2 and 3. The average yield for all fermentations analyzed was 0.616 g (dry weight) of cells per g of methane and 0.215 g (dry weight) of cells per g of oxygen used, with a standard deviation of the mean of 0.0064 and 0.0032, respectively. The average carbon recovery was 100.5% of the total carbon entering each fermentation. The yields on methane and oxygen were both higher for the oxygen-limited than for the methane-limited fermentations (Table 3). The table also shows that if the methane analysis were ignored and the amount of methane used was calculated from experimental values for oxygen used and for cell composition, the cell yield of methane thus obtained was very close to that obtained from the methane analyses. These calculations were made to assess the internal consistency of the experimental results.

Analysis of cell-free fermentation beer with a dichromate-oxidizing agent (5) revealed no detectable organic matter. From the carbon recoveries given in Table 2 and the lack of detectable soluble organic matter, it appears that essentially all of the methane utilized is converted to cells and CO₂.

The use of the fermentor draft tube produced methane feed gas conversions to cells and CO₂ of as much as 83%. At this maximum conversion, only 17 g of each 100 g of feed methane was vented to the atmosphere.

**Fermentor productivity.** The fermentor productivity (dilution rate times cell concentration) for all gas-limited fermentations was proportional to the feed rate of the limiting gas. Relative feed gas rates of 1.35 volumes of pure O₂ per volume of CH₄ were found to produce maximum cell productivity at any given total feed gas rate.

The maximum productivity obtained in the fermentor was 2.39 g (dry weight) of cells per hr per liter at a dilution rate of 0.187 hr⁻¹ and a cell concentration of 12.8 g (dry weight) of cells per liter. The gas feed rates required were 153 ml of pure O₂ per min per liter and 112 ml of methane per min per liter (temperature of feed gas, 25 C; pressure, 740 mm of Hg). By contrast, a flow rate of 283 ml of air per min per liter and 51 ml of methane per min per liter produced 0.726 g (dry weight) of cells per hr per liter at the same dilution rate (temperature of feed gas, 25 C; pressure, 740 mm of Hg).

**Cell analysis.** Elemental analysis of M45 cells grown in an oxygen-limited continuous fermentation with a dilution rate of 0.19 hr⁻¹ gave the results 50.1% carbon, 7.14% hydrogen, 11.7% nitrogen, and 1.62% phosphorus. Replicate Kjeldahl nitrogen determinations on two different samples agreed within 0.1%.
M45 samples gave an average assay of 12.1% N. The total cellular amino acid nitrogen content was 9.23% N corresponding to 57.7% protein ($N \times 6.25$). Assuming all nonprotein nitrogen was in the nucleic acids, the cellular nucleic acid content was 17.1%. Assuming all cellular phosphorus was in the nucleic acids and a nucleic acid composition of 9.5% and 15.5% N, the cellular nucleic acid content was 16.5%. It appears, therefore, that the cellular nucleic acid content was approximately 16.8% at a specific growth rate of 0.19 hr$^{-1}$. The amino acid composition of the M45 cells is given in Table 4. The lysine, cystine, and histidine content of the cells was significantly lower than for soybean meal or yeast grown on gas oil (11). However, the methionine and tryptophan content of M45 cells was approximately twice the level in yeast and soybean meal (11).

**Methane and oxygen volumetric mass transfer coefficients.** The use of a membrane-type dissolved oxygen probe, the tubing-dissolved methane probe, and gas chromatograph fermentation exit-gas analysis permitted the simultaneous determination of volumetric mass transfer coefficients ($k_{lA}$) for oxygen and methane.

A dissolved methane concentration of $1.7 \times 10^{-4}$ m was found for a methane-limited continuous fermentation with a dilution rate of 0.18 per hr. Approximately the same level of dissolved oxygen was found for oxygen-limited fermentations at the same dilution rate.

The simultaneous volumetric mass transfer coefficients ($k_{lA}$) in reciprocal hours for both methane and oxygen were calculated for three methane-limited fermentations for which dissolved gas and yield data were available. The mass transfer coefficient ($k_{lA}$) for oxygen was found to be approximately 1.37 times greater than for methane under the same conditions. This result is in agreement with the observations from many culture M45 fermentations that a volumetric oxygen-to-methane feed gas ratio of less than 1.32 to 1.40 was necessary to insure an oxygen-limited continuous fermentation.

An approximate comparison of the oxygen and methane volumetric mass transfer coefficients can be made from reported diffusion coefficients of the gases in water. The diffusion coefficient at 45 C for methane in water was computed to be $2.51 \times 10^{-6}$ cm$^2$ per sec from the diffusion coefficient at 42.6 C reported by Witherspoon et al. (14). The diffusion coefficient for oxygen at 45 C was computed to be $3.75 \times 10^{-5}$ cm$^2$ per sec from the data tabulated by Gertz et al. (3) for the diffusivity of oxygen in water. It was assumed that the diffusion coefficients of the gases varied directly as the absolute temperature and inversely as the viscosity of the water.

The volumetric mass transfer coefficient between gas and liquid phases is directly proportional to the diffusion coefficient according to the two-film theory of Lewis and Whitman (6) and proportional to the 0.5 power of the diffusion coefficient according to the penetration theory of Higbie (4).

Therefore, the predicted theoretical ratio of oxygen to methane volumetric mass transfer coefficient is equal to 1.50 for the two-film theory and 1.22 for the penetration theory. The average experimental fermentation mass transfer coefficient for oxygen was approximately 1.37 times greater than for methane under the same conditions.

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