Batch Production of Protein from Ethane and Ethane-Methane Mixtures

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A culture of *Graphium* grows upon natural gas and a mineral salt solution. Ethane is the preferred substrate but methane is co-utilized. A stirred-tank type fermentor was used to study batch growth. Maximum production rate of biomass was 80 mg/liter·hr, at pH 4, using simple synthetic supporting medium with ammonium sulfate as a nitrogen source. This rate was observed after 40 hr of fermentation. A doubling time of 3.7 hr was observed. The corresponding specific growth rate was 0.187 per hr. A magnetic drive fermentor was used to study the effect of continuous recycle of gases in a gas-tight system. The rate of oxygen utilization is approximately 2.1 times higher than for ethane. Oxygen must not be allowed to become limiting in recycle gases. The calculated efficiency of overall biomass synthesis averages 30%. Hyphal and unicellular tissue of *Graphium* contains 52% protein. It compares favorably with standard FAO protein in its content of amino acids.

Bacteria able to utilize methane as a sole source of carbon and energy have been known since 1906 (18). Methane utilizing bacteria were reclassified by Orla-Jensen (15) and new varieties of the most common strain *Pseudomonas methanica* were described by Leadbetter and Foster (12). Recently, research has indicated a potential use of methane oxidizing bacteria as a source of protein (9) either for food supplementation or as fodder. Mixed (20) as well as pure cultures (8, 23) have been proposed for this purpose. A critical review of the fermentation of methane was presented by Coty (2) who listed the advantages of the gaseous hydrocarbon substrates: (i) natural gas and methane are cheap, (ii) most sources are quite pure, (iii) it is readily available in most parts of the world, and (iv) gaseous hydrocarbons are easy to handle although the risk of explosion arises.

The current world deficiency in protein has created a need for additional production. Generally the microorganisms which have created interest as food sources have been yeasts, closely related fungi, and photosynthetic algae. Interest has settled on unicellular forms because of their rapid reproduction and growth rates as compared to higher plants and animals. Although bacteria are receiving consideration in terms of process development, bacterial protein is often more difficult to digest (17). Intact cells and non-digestible bacterial cell walls may reduce the nutritional value of bacterial protein, and much of the protein value of the cell is wasted by passing directly through the intestine. This same argument can be used for conventional plant and animal cells; however, the cell walls are more easily broken in higher plant and animal forms. Regardless of the source of protein, the most important criterion is a balanced amino acid composition which makes the protein nutritionally sound. In addition, no toxicity and full feeding efficiency are also absolute requirements.

The first fungal culture reported to grow well on gaseous hydrocarbons was identified as a species of *Graphium* (24). This culture utilizes natural gas or ethane as well as higher gaseous hydrocarbons as a sole source of carbon and energy. The fungal tissue containing approximately 50% of protein is a potential source of microbial protein (21).

The present report covers results obtained with *Graphium* sp. cultivated in submerged batch culture in stirred-tank type fermentor. Important physico-chemical parameters involved in the continuous-flow production of fungal tissue, as well as the efficiency of bioconversion of ethane into proteinaceous tissue by *Graphium* sp., were investigated.

**MATERIALS AND METHODS**

Culture. A pure culture of imperfect fungus identified as a species of *Graphium* was used in all the experiments. The volume of inoculum applied was 5% (v/v). Inocula were prepared by submerged shake-
flask culture. The *Graphium* sp. culture used was originally isolated from sewage by natural gas continuous-flow enrichment and using standard purification techniques (24).

**Medium.** A synthetic aqueous mineral salt medium described by Johnson and Temple (10) was used. This medium referred to further as C-medium contains: (NH₄)₂SO₄ or NaNO₃, 1 g; K₂HPO₄, 1 g; MgSO₄, 0.5 g; NaCl, 0.1 g; CaCl₂, 0.1 g; and FeSO₄, 0.001 g per liter of tap water. The pH was adjusted at desired level by adding either HCl or NaOH. A mixture of natural gas (60%) and air (40% by volume) was bubbled through the fermentation broth at the rate of 4 liters/min. No back pressure was maintained in fermentors. The gas mixture was passed once directly through the growing culture and exhausted except in gas-recycle experiments. The composition of the domestic natural gas used was: CH₄, 90.5%; C₂H₆, 6.0%; N₂, 3.0%; CO₂, 0.25%; and propane, 0.21%. In determining the rate of fungal tissue synthesis, the mycelial tissue withdrawn from the fermentor was separated from the supernatant by filtration, washed with distilled water, and dried overnight at 70°C. Fungal tissue to be further analyzed was freeze-dried.

**Equipment.** For studies on the recycle of gases, a Virtis magnetic drive fermentor was utilized. In all other experiments a model MF 14, stirred-tank type 14-liter fermentor (New Brunswick Scientific Co., New Brunswick, N.J.) was used. The fermentor was equipped with four baffles of standard geometry. A single orifice air sparger was located under the four-bladed disc-type impeller. The operating volume of the fermentor was 10 liters; it was agitated at 400 rev/min.

Temperature was maintained automatically at 31°C ±0.5. The pH was controlled with an accuracy of ±0.1 of a unit by means of automatic addition of either 3 N sodium hydroxide or hydrochloric acid.

The entire fermentation system (medium, vessel, air inlet, and exhaust filters, etc.) were sterilized prior to inoculation at 120°C, 15 psi, for 45 min.

The gas recycle fermentation system was slightly more complex. Figure 1 shows schematically the experimental arrangement of equipment used in gas recycle. A magnetically bottom driven Virtis fermentor with 10-liter glass jar operated at 6 liters was employed. This unit offers greater safety and is easy to maintain under gas-tight conditions. Baffles were omitted to avoid the surface growth of tissue. Two paddle-type blade impellers fixed on the central shaft were rotated at 300 rev/min. The gaseous phase was introduced with a diaphragm pump at the rate of 2 liters/min and dispersed into the broth by sintered glass sparger located under the lower impeller.

The recycle gas mixture consisted of pure ethane (Matheson Co., Inc., Rahway, N. J.; C. P. Grade, 99.0% and air slightly enriched with oxygen. As the fermentation proceeded, the CO₂ concentration increased. A steady pressure in the system was maintained by continuous controlled addition of either pure nitrogen (Liquid Carbonic Company of Canada, 99.96% purity) or by addition of a prepared mixture of O₂ + C₂H₆ as described.

Analysis of recycle gases for oxygen was accomplished by a model OA 150 paramagnetic oxygen analyzer (Servomex Controls Ltd.). An in-line gas-liquid chromatograph manufactured by F & M Scientific, Hewlett-Packard model 5750 was used for analysis of CH₂CH₄ and CO₂. This unit was operated using a TC detector and Porapak "Q" column (15 ft by 1/4 inch inner diameter; 4.57 m by 0.635 cm). Quantitative separation of these components was conducted at 115°C, using helium as carrier gas at 80 ml/min. Each sample was injected from a sealed loop possessing a volume of 1.7 ml.

The volume of the recycle gas phase was 25 liters. Excess CO₂ was removed as required by passing gases
through a column of Baralyne (National Cylinder Gas, Div. of Chemetron Corp.). The temperature of the fermentation medium was maintained at 31°C. The pH control was not utilized in the gas recycle experiments. C-medium with sodium nitrate as a nitrogen source was used in these experiments.

RESULTS

Culture pH requirements. A series of experiments were conducted in order to determine the pH giving the highest yield of mycelium as well as the highest rate of tissue formation. Experiments were conducted using C-medium containing either NaNO₃ or (NH₄)₂SO₄ as a source of nitrogen. Figure 2 shows the growth (production) rate and changes in pH which occur in typical batch fermentations without pH control. The initial pH was 4.5. The terminal pH of the culture was dependent upon the nitrogen source added. Addition of (NH₄)₂SO₄ caused the pH to decrease to 3.0 as the fermentation proceeded, whereas addition of NaNO₃ caused the pH to increase to 7.5. A medium containing (NH₄)₂SO₄ gave a lower cell concentration which was attributed to the steady decrease in pH during cultivation. After a short lag period the pH decreased to 3.0 during the exponential growth phase and remained at this value. As the fermentation proceeded into stationary phase of growth, the tissue adhered to all the stationary parts of the fermentor. The NaNO₃ medium gave a yield of 96 mg of tissue per 100 ml of medium when no pH control was applied. The pH during the fermentation decreases sharply from initial pH 4.5 to 3.2 followed by a steady increase to pH 7.5 during the exponential phase of growth. The pH does not change during stationary growth. The initial decrease in pH in a nitrate medium indicates organic acid synthesis and accumulation.

The pH is a very important parameter for the culture of Graphium growing on natural gas since the yield of tissue, as well as the type of growth is affected. Dispersion of mycelium growing in submerged culture is greatly influenced by pH value as well as the tendency of the biomass to form clumps sticking to all the stationary parts of the fermentor vessel. In order to collect enough data for further development of the process on continuous-flow culture basis, a number of batch culture experiments were conducted using controlled pH levels throughout the fermentation. As seen in Fig. 3 and 4, where pH values were controlled at 3.0, 4.0, 5.5, 6.5, and 7.0, there is always a period in the stationary growth phase in which the growing culture forms clumps and adheres to solid surfaces with a concomitant decrease in tissue from the broth. In many fermentations, the medium clears up completely with the tissue, forming a solid ring above the liquid surface adhering to baffles, electrodes, etc. The concentration of mycelium present in the liquid broth approaches zero and gives faulty data. Reasons for this clumping are not well known; however, clumping is commonly encountered in fungal fermentations.

The studies on the effect of pH control on growth of Graphium sp. were completed on C-medium containing NaNO₃. Natural gas was the only source of carbon and energy.

FIG. 2. Biomass concentration in the fermentation broth, the biomass production rate, and pH change in batch culture of Graphium sp. on natural gas. Synthetic aqueous supporting medium contained NaNO₃ as the source of nitrogen.

FIG. 3. Biomass concentration and Graphium sp. tissue production rate on sodium nitrate containing medium at pH levels of 3.0, 5.5, and 7.0, using natural gas as a source of carbon and energy.
Biomass concentrations at pH levels of 3.0, 4.0, 5.5, and 7.0 are shown in the lower plot of Fig. 3. Production rates were obtained by calculating the first derivatives of the cell concentrations. Tangents were drawn at selected points (time intervals) on the growth curves and derivatives obtained as their slopes. These values give the amount of tissue formed per unit of time and unit of volume. The upper plot (Fig. 3) obtained this way is showing biomass production rates as a function of time for the corresponding pH levels. The highest concentration of tissue was observed at pH 4.0. The minimal doubling time of 38.3 hr was observed at 100 hr. The corresponding specific growth rate was 0.0181 per hr. The doubling time decreased to 16.5 hr (specific growth rate, 0.042 per hr) at tests conducted at pH 5.5. A further decrease in doubling time to 5.5 hr (specific growth rate, 0.125 per hr) was observed at pH 7.0. However, cell concentrations decrease at the highest pH levels tested. The biomass production rate was 10 mg/liter-hr after 120 hr of cultivation. The low rate of growth has a considerable influence on production rate in long-term continuous fermentation. In comparison to results obtained on a medium with ammonium sulfate rather than NaNO₃, pH 5.5 did not give satisfactory results. Clumping was severe after 70 hr. On nitrate media, basically no growth was observed at pH 3.0. In terms of the highest production rate, the pH 7.0 was optimal, giving 20 mg/liter-hr in 40 hr. After 40 hr, tissue concentrations (400 mg/liter) decreased because of clumping. This decrease can be partially corrected in continuous-flow culture system by keeping the retention time below a critical value of 45 hr.

More encouraging results were obtained in experiments employing C-medium with (NH₄)₂SO₄ (Fig. 4) as a source of nitrogen. In experiments in which the pH was controlled over the entire fermentation period, the biomass concentration was higher and the production rate of biomass increased. Figure 4 shows the data obtained for the Graphium sp. grown at pH values of 3.0, 4.0, 5.5, and 6.5. Growth curves are plotted in the lower part of Fig. 4 and derivative functions representing production rates in the upper. The highest concentration of tissue was observed at pH 4.0, the maximum being 780 mg per liter. This pH also gave the highest rate of tissue formation of 75 mg/liter-hr in 40 hr. The doubling times and specific growth rates were calculated at 35, 40, and 43 hr. Doubling times were 8.3, 3.7, and 5.5 hr; specific growth rates were 0.0825, 0.187, and 0.125 per hr, respectively. They were lower at other time intervals tested. They also decreased at pH 5.5 and 6.5. In comparison with pH values of 5.5 and 6.5, pH 4.0 is the optimum. Basically no growth was observed at pH 3.0. Using a medium with ammonium sulfate as the nitrogen source also results in less adherence of the tissue to the stationary parts of the fermentor.

**Temperature effect.** It has been shown that a considerable amount of heat is evolved during the oxidative fermentation of hydrocarbon substrate (1). The quantity of heat theoretically evolved is approximately 2.5 times higher than that evolved during the fermentation of conventional carbohydrate substrate (1). For obtaining the proper operating thermal regime for the fermentation, the optimal temperature for the fermentation of natural gas by Graphium sp. was determined. The culture was grown at a controlled pH of 4.5 on C-medium with NaNO₃. Figure 5 summarizes results of temperature experiments. Growth curves for each temperature are plotted against time. Temperature definitely affects the biomass production rate. Rates during experimental growth were: 20 and 38 C; 7.5 mg/liter-hr; 25 C; 9 mg/liter-hr; and 32 C; 10.6 mg/liter-hr. Doubling times and specific growth rates at each temperature are summarized in Table 1. The shortest doubling time of 16.2 hr and best growth rates of 0.0428 per hr were observed at
Concentration of biomass was determined experimentally in a batch fermentation. The growth rate of Graphium sp. was calculated in an aqueous C-medium containing NaNO₃ as a source of nitrogen.

32 °C. Temperature affects growth rate more than it does terminal cell concentration in a batch fermentation.

In the exponential phase of growth simplified by approximate linearization, the production rates were calculated at each temperature and re-expressed as a function of temperature in the lower curve (Fig. 5). Additional studies on Graphium show an optimum temperature for growth on natural gas between 30 and 33 °C. This may vary slightly under certain cultural conditions, but it is the recommended temperature range for growth on gaseous hydrocarbons.

Batch fermentation with gaseous recycle. A batch system was installed in which complete control of the gases and recycle of gases could be accomplished. This arrangement enables one to measure the amount of ethane substrate utilized, amount of oxygen consumed during the fermentation period, and to find the rates of utilization of both. Fungal biomass concentration was determined by withdrawing samples of the fermentation broth. The amount of CO₂ evolved as a product of respiration was measured. In one experiment it was necessary to remove CO₂ in order to maintain its constant concentration. Data of this type were needed for calculating the efficiency of cellular tissue synthesis and comparing with calculations based on stoichiometric considerations.

Two types of experiments were completed at atmospheric pressure. In the first the gas mixture was recycled until oxygen became limiting, and in the second the oxygen and ethane concentrations were maintained at fairly constant levels by periodic replacement.

(i) Gas recycle with oxygen becoming limiting. The fermentation vessel was sterilized together with inlet and outlet gas-phase filters for 40 min in an autoclave at 120 °C (15 psi). A 5% by volume of pure culture of Graphium sp. was used to inoculate 6 liters of sterile broth. The composition of gaseous phase in the gastight system was adjusted initially to 17.3% by volume of ethane and 19.2% by volume of oxygen. Nitrogen was added to bring the gas volume to 100%. Approximately 0.2% CO₂ was present in the gaseous mixture. Nitrogen was used to replace gases utilized during the fermentation in order to maintain a steady pressure. The CO₂ produced by the growing culture was not removed. Parameters examined were cell concentration, concentrations of oxygen, ethane, and CO₂ in the recycle gaseous phase. Plotting these against the time of fermentation, a curve for each variable was obtained (Fig. 6). The upper part of the figure shows concentrations of the biomass and gases. After a 19-hr lag period, exponential growth was observed. Tissue formation was accompanied by a corresponding increase in CO₂ concentration. The amount of oxygen and ethane in the gaseous phase decreased. The rate of oxygen utilization was higher than the rate of ethane uptake.

This is shown more distinctly in the lower part of Fig. 6 where specific rates of oxygen and ethane utilization (milliliters per hour and liter of broth) are plotted against time. Also plotted are rates of CO₂ production and biomass production rate (milliliters per hour and liter of broth). The lower part of Fig. 6 is derived completely from the primary values measured and plotted in the upper diagram. All values are first derivatives (slopes) of the primary functions taken at selected points. The

| Temp (°C) | Doubling time** (hr) | Specific growth rate** per hour |
|----------|----------------------|--------------------------------|
| 20       | 22.5                 | 0.0308                         |
| 26       | 18.6                 | 0.0372                         |
| 32       | 16.2                 | 0.0428                         |
| 38       | 22.8                 | 0.0304                         |

* As estimated in exponential growth phase.
specific rate of oxygen consumption is approximately 2.1 times higher than that of ethane.

Since oxygen was not replenished in the system, it became limiting after 3 days of cultivation and on the fourth day the culture activity entirely ceased. At this time the entire gaseous phase was replaced. The concentrations of oxygen and ethane were adjusted to 19.8 and 22.2%, respectively. The level of CO₂ was lowered to 1.2%. Upon replenishing the gaseous phase and continued recycling, the exponential growth phase of the culture proceeded immediately. Oxygen limitation was not allowed to occur again. After 5.5 days of fermentation, the oxygen level was adjusted again and maintained within a range of 8 to 10% O₂. An adjustment of this type was made for ethane shortly after 8 days.

The fermentation was terminated on the ninth day when respiratory activity of the culture decreased. In the later stages of fermentation, notably after 7 days, the amount of tissue in the broth decreased due to the aggregation of biomass around the liquid surface and because of
adherence to the stationary parts of the fermentor vessel. By extrapolating the specific rates in the lower part of Fig. 6 over the period of oxygen limitation (dashed lines), it can be seen that in the latter stages of the fermentation, more oxygen is consumed and thus the ratio of oxygen to ethane consumption increases over the stoichiometric estimate for tissue synthesis. This is discussed later under efficiency. The rate of $\text{CO}_2$ production as compared to the ethane utilization rate remained close to a value of 1.2 throughout the whole fermentation period although a slight increase was observed toward the end.

(ii) Gas recycle with replenishment of oxygen and ethane. Another experiment was completed with Graphium sp. in 6-liter batch system with recycle of the gaseous phase. In this experiment the initial concentration of gases was adjusted to 23.2% oxygen, 19.5% ethane, and 0.2% $\text{CO}_2$ and the balance of the gas phase was adjusted to 100% with nitrogen.

The oxygen and ethane were premixed in a proportion 2:1 (by volume) and fed into the system as needed to replace the portions utilized in order to maintain the concentration of these gases within the desired range. The amount of the gaseous mixture metered in was measured on a time basis. The $\text{CO}_2$ produced during the fermentation run was periodically removed by absorption on Baralyme. The $\text{CO}_2$ concentration was maintained between 3 and 5%. Cell concentration and concentrations of gases in the system were measured. The plot of these parameters against time of fermentation is shown in Fig. 7. A third plot shows the rate of consumption in milliliters per hour of the gaseous mixture (2:1 oxygen and ethane). It corresponds to the first derivative of the consumption curve and is obtained by plotting its slope at selected time intervals against time. The peak consumption rate was 300 ml/hr as observed in the exponential growth phase. Calculated on a unit volume of broth basis, this value becomes 50 ml/liter·hr (of oxygen-ethane mixture, 2:1).

The dashed curve for biomass represents the actual yield of the tissue in the fermentation broth. Portions of the cellular mass adhered to the stationary parts of the fermentation vessel later in the exponential growth phase. Total yield of the biomass was 4.5 g of dry tissue/6 liters of broth.

The lower part of Fig. 7 shows the variation in concentration of oxygen and ethane during the fermentation. It was impossible to maintain them at constant levels; however, oxygen was maintained between 12 and 23% and ethane between 15 and 18%. Since there was a difference between the initial and final concentration of ethane, a correction was required. Knowing the consump-

![Graph](https://via.placeholder.com/150)

**Fig. 7.** Batch growth of Graphium upon ethane in a system in which gases were recycled and replenished to maintain concentration ranges. During the cultivation the following measurements were taken: biomass concentrations, amount of gaseous mixture consumed, and concentrations of ethane and oxygen. Rate of gaseous mixture consumption was calculated.

The culture of Graphium used was isolated in an attempt to find a potential source of a single cell protein from natural gas or gaseous hydrocarbon substrates. The fungal tissue was collected from the culture growing on pure ethane in which both $\text{O}_2$ and ethane levels were maintained during the fermentation. Fungal tissue was recovered by filtration and then freeze-dried. Analysis for organic nitrogen (micro-Kjeldahl) yielded 0.083 g of N in 1 g of dry biomass. This corresponds to approximately 52% of protein (N × 6.25) in the tissue.

A portion of the dry tissue was hydrolyzed (6 N HCl, 24 hr) and analyzed for amino acids using a Technicon Amino Acid Analyzer. Graphium possesses a well balanced amino acid composition (Table 2). The methionine concentration was 1.0% of the crude protein but it is still quite good. The lysine concentration in
Table 2. Amino acid composition of Graphium sp. protein compared to some other protein sources

| Amino acid | Graphium sp. Yeast BP* | Source of protein | Yeast BP* |
|------------|----------------|------------------|----------|
| Arginine   | 5.3            | Bacterial (Cell)  | 5.0      |
| Histidine  | 3.3            | 2.0              |
| Isoleucine | 4.0            | 5.3              | 4.5      |
| Leucine    | 6.0            | 8.4              | 7.8      | 7.0 |
| Lysine     | 7.7            | 6.4              | 7.8      | 7.0 |
| Methionine | 1.0            | 2.4              | 1.6      | 1.8 |
| Phenylalanine | 3.6   | 5.2              | 4.8      | 4.4 |
| Threonine  | 3.9            | 5.0              | 5.4      | 4.9 |
| Tryptophan | 3.0            | 1.3              | 1.4      | 1.4 |
| Valine     | 5.5            | 6.8              | 5.8      | 5.4 |

Essential amino acids

- Arginine
- Histidine
- Isoleucine
- Leucine
- Lysine
- Methionine
- Phenylalanine
- Threonine
- Tryptophan
- Valine

Nonessential amino acids

- Alanine
- Aspartic acid
- Cystine
- Glycine
- Glutamic acid
- Proline
- Serine
- Tyrosine

protein from Graphium was 7.7%, whereas the standard F.A.O. protein is 4.3%. All of the other essential amino acids in Graphium were higher than the F.A.O. standard except isoleucine.

DISCUSSION

Efficiency of fungal tissue synthesis. Cellular yield per unit weight of substrate utilized is a most important expression, particularly if biomass is the desired product.

Results on cell yields on hydrocarbon substrates reported by various authors vary considerably, ranging from 50 to 100% (weight of tissue per weight of hydrocarbon). Lower values of 50 to 90% weight of substrate utilization were observed for some bacteria (5). The highest cell yields of 110 to 130% were reported for the bacterial genera of Micrococcus and Nocardia (16, 22), whereas yeast grown on petroleum give a yield of 80 to 100% (4, 13, 19). Cell yields differ according to the microorganisms used and hydrocarbon substrate applied in the process. Of these values, 70% is more realistic for process calculations even though 100% cell yield by weight of hydrocarbon is more commonly used (3, 9, 14).

There has been certain confusion in literature as to the reports on cellular yield and the efficiency of biomass synthesis. The yield is usually expressed as weight of biomass per weight of substrate utilized or in terms of yield percentage calculated on the same basis. Data on the problem of efficiency calculation are insufficient and not treated uniformly. In this discussion, two ways are suggested to calculate the efficiency of biomass synthesis if the yield values are known.

In earlier work Darlington (3) developed a stoichiometric equation for bio-oxidation of paraffinic hydrocarbons. This was modified by Klass et al. (11) for use with methane.

A typical composition of microbial tissue grown on hydrocarbon substrate is given by Wagner et al. (22): C, 48%; H, 7%; N, 8%; ash, 8%; and oxygen, 29% (by difference). This agrees with the composition of Graphium sp. (not reported herein). Using this particular composition the general formula for cellular material is slightly modified being C_{4H_{1.81}}N_0.57 ash. Substitution to the original formula (3, 11) applied to ethane gives

\[3.33 \text{C}_6\text{H}_6 + 6.82 \text{O}_2 \rightarrow (\text{C}_{4H_{1.81}}\text{N}_0.57) + 2.66 \text{CO}_2 + 6.5 \text{H}_2\text{O}\]

However, this formula assumes the weight of tissue synthesized is equal to the weight of hydrocarbon substrate utilized (cell yield 100%) which agrees with only a few actual fermentation processes. This makes it of theoretical significance only.

From the experimental results obtained with Graphium sp. growing on ethane as a sole source of carbon and energy, the consumption of the gaseous substrate was 1.9 to 2.1 g of ethane utilized per 1 g of dry tissue synthesized. On weight basis, this corresponds to a yield between 48 and 53% of tissue formed per the amount of substrate utilized.

For the calculation of actual biomass synthesis efficiency a definition of 100% efficiency is needed. Hypothetical ideal conversion of all substrate carbon into cellular material would represent such 100% conversion efficiency. For ethane substrate and the given formula for cellular material, the hypothetical process equation would be:

\[2 \text{C}_6\text{H}_6 + 2.155 \text{O}_2 \rightarrow (\text{C}_{4H_{1.81}}\text{O}_{0.61}) + 2.5 \text{H}_2\text{O}\]
Expressing this on a weight basis, 60 g of C₂H₄ would be required for 109 g of tissue formed, which would include the weight of nitrogen and ash which contributes up to 16% of dry tissue weight (nitrogen and ash data are not included in the general formula).

Since neither of these formulas relates to practice, a simplified expression of the biosynthetic efficiency is presented based on known process cell yield data. This involves calculation of a factor \( f_b \) and \( \eta_T \) which describes the overall biomass synthesis efficiency.

\[
f_b = (60 \text{ g } \text{C}_2\text{H}_4/100 \text{ g of tissue}) \times 100\% = 60\%.
\]

The overall efficiency (\( \eta_T \)) of tissue biosynthesis is then expressed by:

\[
\eta_T = \left(1/\text{amount of substrate utilized in grams per gram of tissue}\right) \times f_b.
\]

\textit{Graphium} sp. grown on ethane utilizes 1.9 to 2.1 g of C₂H₄ per g of tissue synthesized. Using the above formula this corresponds to an efficiency of \( \eta_T = 28.6 \) to 31.6%.

The efficiency can also be calculated by relating the heat of combustion of the substrate utilized to that of the corresponding amount of \textit{Graphium} sp. tissue produced. The ratio of the heats of combustion should be again related by factor to the efficiency of 100% defined above in order to obtain the true figure for the overall biomass synthesis efficiency.

This calculation giving the average value of \( \eta_T = 29.9\% \) corresponds very well to that based on stoichiometric weight to weight conversion basis.

**Batch growth.** Growth rates, rate of ethane utilization, and certain other parameters have been studied to establish engineering requirements for proceeding from batch to continuous production. Although graphical solutions were used entirely to obtain derivatives used herein, some error is encountered. Mathematical expressions describing a sigmoid-type growth curve are known (6) and can be used in conjunction with computer to obtain derivatives on a purely mathematical basis. As sources of nitrogen, ammonium sulfate is far superior to sodium nitrate, particularly over a pH range of 3.5 to 6.0. For steady state cultivation, a pH between 4.0 and 5.0 was most effective.

To keep the growth medium simple, a synthetic medium was utilized in all studies reported herein. Previous studies have shown that yeast extract, beef extract, and other growth-supporting factor additions increase cell concentration over 10-fold (21).

The addition of growth factors can be expected to change some of the physical-chemical parameters reported herein. The amino acid composition of single cell protein from \textit{Graphium} is quite good in comparison with the F.A.O. standard protein (Table 2); however, until its nutritional value is assessed in forms of feeding tests, the true nutritional value will not be known.

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