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# A Single-cell Protein From Natural Gas And Gaseous Hydrocarbons

Bohumil Volesky

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A SINGLE-CELL PROTEIN FROM NATURAL  
GAS AND GASEOUS HYDROCARBONS

by

Bohumil Volesky

Faculty of Engineering Science

Submitted in partial fulfillment  
of the requirements for the degree of  
Doctor of Philosophy

/

Faculty of Graduate Studies  
The University of Western Ontario  
London, Canada  
February 1971

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### ABSTRACT

In an attempt to find a new potential source of single-cell protein from natural gas, a fungal culture was isolated which utilized either natural gas or higher molecular weight gaseous hydrocarbons as a sole source of carbon and energy. The culture was identified as a species of Graphium. It grew well at 32°C on an inorganic medium with or without asepsis.

A mixed culture system in which Graphium sp. was the predominate culture was also studied. The fungus Trichoderma viride and two strains of acid-tolerant bacteria were the most persistent cultures in the mixed system.

In a series of shake flask experiments and in a 14 l stirred tank-type fermentor studies, some physico-chemical parameters were tested as to their effect upon the biomass concentration, the production rate and the optimal growth of Graphium sp. in submerged batch culture. The doubling time observed was ~4.7 hr., the biomass concentration ranging

from 2.5 to 4.5 g/l. Elevated CO<sub>2</sub> concentration, pH 4.0 - 4.8, yeast extract addition (0.1-0.4%) to the aqueous medium were shown to enhance the growth. Increased pressure of cultivation over 1 atp resulted in a marked growth inhibition. Methane co-utilization in the presence of ethane as a primary substrate was suggested. A preliminary pilot plant operation in a 400 l tank was carried out with the mixed culture system.

The growth of Graphium sp. in a continuous-flow culture was tested over a range of dilution rates from 0.025 to 0.031 hr<sup>-1</sup>. The biomass yield was 0.48 - 0.53 g per gram of ethane with the ratio of oxygen to ethane consumption rates approximately 2:1. A continuous-flow culture with complete recycle of the gaseous phase was devised and tested.

The biomass of Graphium sp. contains between 45 - 50% of crude protein (the mixed culture averages ~5% higher protein), 4 - 11% of lipids, ~10% of ash, and ~30% of carbohydrates and fibers. Neutral lipids and phospholipids (identified were phosphatidyl ethanolamine and phosphatidyl choline) constituted the lipid fraction. Oleic, stearic and palmitic acids are the major fatty acids averaging 47%, 20% and 19% of the total fatty acid content respectively.

A balanced amino acid composition of the proteinaceous fraction which compares favourably to the standard nutritional F.A.O. protein composition indicates the biomass is nutritionally sound. Preliminary feeding studies with rats were completed showing no toxic or deleterious effects on the animals over a 5 month feeding period.

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## CHAPTER I

### INTRODUCTION

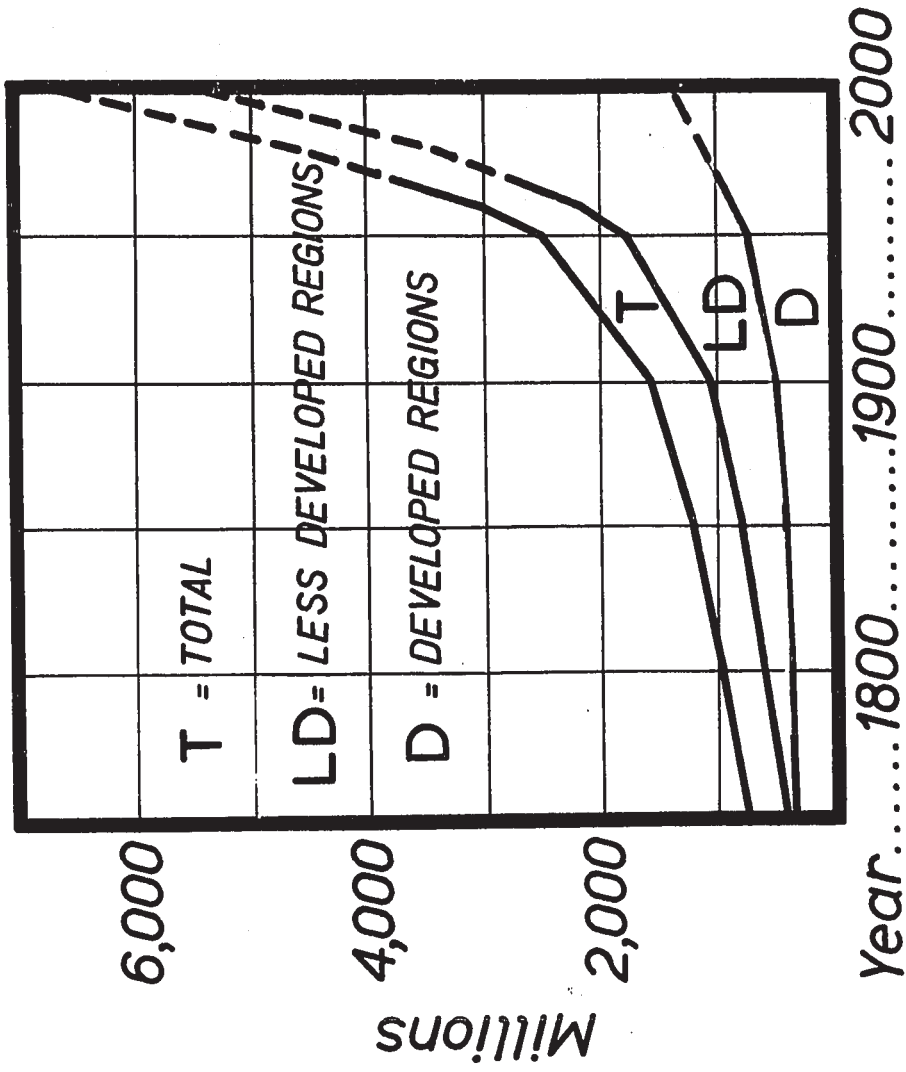
The present world shortage of protein, especially low cost animal proteins, for consumption by animals and humans is well known. The world population is now about 3,250 million and is increasing at the rate of 5,600 more births than deaths per hour. According to the latest estimates during the next thirty years the population will double. When the total population reaches 50 billion there will be an average of 1,000 people for every square mile of the Earth's land surface including both deserts and poles. At the latest present rate of population doubling this is likely to occur around the year 2100.

Currently, about 300 million people are already existing at starvation level and every day some 10,000 people die as a result. More than half the population of the developing countries receive less than 15 grammes of protein a day; and 80% of the world population receives less than 30 grammes. Most malnutrition takes the form of protein deficiency which in extreme cases results in death. More often protein deficiency leads to irreversible mental and physiological damage. There is an evidence (Stoch and Smythe, 1967) that from birth undernourished

children possess significantly smaller heads, lower intelligence quotients and poorer body coordination. Especially sensitive towards protein deficiency are infants between 6 to 18 months of age who suffer from irreversible brain damage if deprived of protein (Hunger USA, 1967). In examining the aspects of food availability, cost and acceptability are controlling. 71% of the world population is living in economically less-developed regions. This part of population produces 42% of the world's food but earns only 21% of the world's income. The requirement is not simply for more food but rather for low cost protein of the right nutritional value and a protein acceptable to the people. Even more critical is population control. The growth of world population is illustrated in Figure 1, while per capita food output in the developed and less developed parts of the world is shown on Figure 2.

As a logical extension of these figures and observations, the in toto problem of population nourishment becomes overwhelmingly important. To avoid mass starvation of an "insupportable population" new sources of food are being sought. With increasing population density, agriculture will reach a limiting production level. Statistics show the importance of development of new food resources. The methods most often suggested for the expansion of available food sources are (Pryor, 1969):

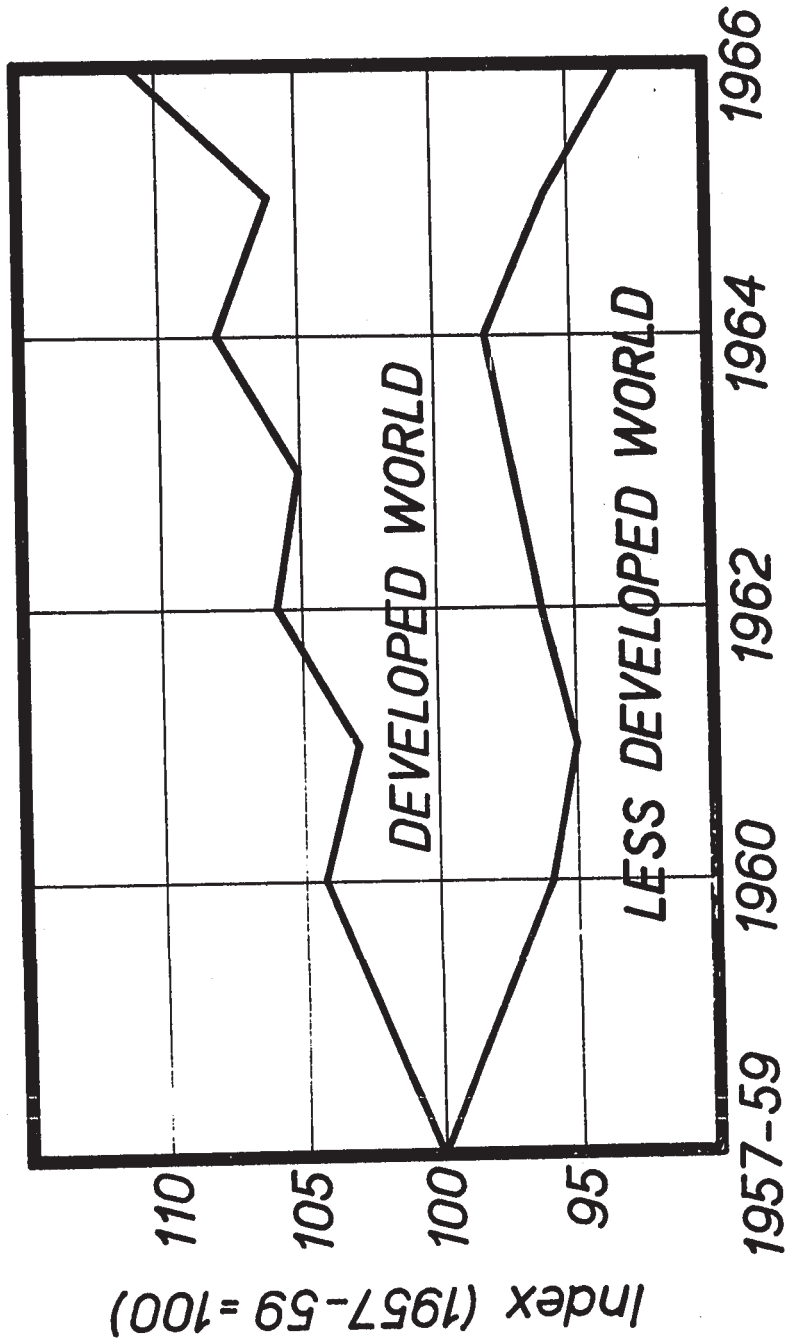
- 1) Increase the yield from existing farm land (limitation in supporting 10 billion people).
- 2) Utilize available but little used food resources.
- 3) Produce synthetic or manufactured food sources.
- 4) Develop new photochemical processes which exceed those utilized by plants in photosynthesis.



# World Human Population from 1750 - 2000

(Acc. to H. Mitsuda)





Per Capita Food Output in the developed and less developed world

(Acc. to L.R. Brown)

FIGURE 2

Item 3 is an area where intensive research activities and developmental work is being undertaken. Some essential food components are already being successfully produced in large quantities by purely chemical synthetic processes. Beside pure chemical synthesis (McPherson, 1961) a wide interest has been focused on the area of food biosynthesis by making use of various microorganisms (Humphrey, 1966; Keiffer, 1968). These are described under single cell protein (SCP) synthesis.

#### BIOSYNTHETIC FOOD PRODUCTION - MICROBIAL PROTEIN

The effort to improve human nutrition, particularly in its protein and vitamin parts, has led to intensified development of the field of biosynthesis over the last decade. Sources of microbial protein are ensured by growing various microorganisms upon diversified substrates, e.g. hydrogen, methane, methanol, mixed hydrocarbons, cellulose, sewage and industrial organic wastes (Mateles and Tannenbaum, 1968).

Efficiency of all bioprocesses is based upon the concept of doubling time. Microbes used for this purpose double in 2 - 8 hours while plants and animals require days and weeks, e.g.

- |    |          |             |              |
|----|----------|-------------|--------------|
| a) | plants:  | - soy beans | - 1-2 weeks  |
| b) | animals: | - chickens  | - 2-4 weeks  |
|    |          | - cattle    | - 2-4 months |

In addition to a high production rate, microbes contain a high protein content (40-70%). A yeast factory producing 10 tons of yeast per day (50% protein) might occupy an area of half an acre. To provide the same

amount of protein in the form of pork it would be necessary to kill 80 pigs a day. For production of equal amount of plant protein per year 8,000 acres of wheat would be required (Humphrey, 1969).

High growth rate of microorganisms stems from their relatively large surface area which is theoretically completely available for nutrient and product exchange coupled with growth of single or small clumps of cells or filaments which can be used to uniquely describe single cell protein.

Microbial protein - often called single cell protein (SCP) may be used as fodder protein supplement or consumed directly in foods. Extensive studies are being carried out to assess digestibility and nutritional efficiency of various microbial proteins considered as potential food supplements (Shacklady, 1969; de Groot et al., 1969). There is theoretically no reason why single cell protein should not become an established product consumed directly by man. There may be some social and psychological factors involved in introducing new chemo- and bio- synthetic foods. These should be properly assessed in order to obtain rapid acceptance of a new food by society (Brown, 1968; Bacigalupo, 1968).

## CHAPTER II

### LITERATURE SURVEY

#### SOURCES OF MICROBIAL PROTEIN

Among all the microorganisms the most widely used for food purposes are: 1) algae, 2) bacteria, and 3) fungi, particularly the yeasts. Algae have been known for centuries to some Asiatic countries as a food supplement (Volesky, et al., 1970). They grow freely in seas, oceans, and all natural bodies of water utilizing sunlight for energy and  $\text{CO}_2$  as a carbon source. For this reason these plants are a possible source of cheap microbial protein. Some unicellular algae (Chlorella, Spirulina) are very high in protein (50-60%) and their growth is very fast and efficient (Clement et al., 1968). The apparent low cost of the process has attracted a great deal of attention and stimulated considerable work on the production of algal protein. The possibility of growing algae on organic waste materials and sewage (McGarry, 1970; Oswald, 1969) has proven to be realistic and several pilot plants have been built already for this purpose. Another field where the advantage of algal growth can be readily seen is in space flight programmes. It has been calculated

(Norris, 1968) that production of 600 g of algae per day per man would not only be nutritionally adequate as regards to protein and vitamins, but would also supply sufficient oxygen while removing adequate amounts of CO<sub>2</sub> from environment. This multiple purpose for algal cultivation is still under intensive investigation. Algal culture in large scale is certainly one possibility particularly when the economy of the process becomes competitive to that of conventional sources of protein.

To date, most sources of SCP are by-products from other well-established processes, e.g. brewery, antibiotic, vitamin, organic acid fermentations, etc.

However, the possibility of valuable SCP biosynthesis by these same processes exists and offers excellent potential in alleviating a protein crisis. The most generally used are fermentations utilizing conventional carbohydrates as substrates. Most of these processes are not economic enough for solely the production of proteins since carbohydrate substrates are fairly expensive. Another disadvantage is the requirement for the addition of growth factors in many conventional fermentations. These are required to ensure high yields of the desired products.

This makes the economy of the SCP production from carbohydrates non-competitive, thus new cheaper fermentation processes are needed.

#### HYDROCARBONS AS SOURCES OF ENERGY

Even though there are abundant sources of surplus and waste carbohydrates, hydrocarbons are still even more abundant and cheaper. Large

reserves of liquid and gaseous hydrocarbons exist. Even though there are only a limited number of microorganisms able to utilize and grow on this type of substrate, the cultures which are already known are very promising (Humphrey, 1968). With single cell protein production being the major goal, additional effort must be made to find potentially marketable by-products from these hydrocarbon fermentations.

Comparing the prices of various carbohydrate and hydrocarbon substrates (Table 1) the advantage of hydrocarbons over the cheapest carbohydrate substrates can be seen.

The main advantage of using hydrocarbons as substrates is not only their low cost but their availability in large quantities for SCP production. Being non-agricultural products their production is independent of climatic conditions or seasonal fluctuations. The amount of hydrocarbons actually needed to fill the protein gap by production of SCP would be small and would have little impact on the overall hydrocarbon supply situation. It has been estimated that if 20% of the hydrocarbons produced were diverted into SCP synthesis, the world protein requirement could be met. Also, hydrocarbons are often available in, or close to, areas where protein deficiencies are quite pronounced. This is illustrated in Figure 3 where major oil and gas producing areas of the world are superimposed on the world map. Shaded parts show the protein deficiency areas (Norris, 1968). The most important factor for food distribution, i.e. transportation cost, would be reduced to a minimum. Another advantage of hydrocarbon substrates is their higher efficiency in utilization. There is approximately twice the amount of microbial

TABLE 1  
SUBSTRATE COSTS

HYDROCARBONS:	¢ / Lb.
Refined Hydrocarbons	2 - 4
Simply Separated Hydrocarbons (Paraffins)	1 - 2
Crude Oil, Gas Oil	1
Coal	0.5 - 1
Natural Gas	0.25
Flue Gas	0.04
CARBOHYDRATES:	
Refined Carbohydrates	4 - 8
Molasses	1.75
Cereal Grains	1.5 - 2.5
Cereal Grains Wastes	1

(after Humphrey, 1967)

**FIGURE 3**

Areas of crude hydrocarbons occurrence are superimposed on the world map with the shaded areas where protein deficiency is a permanent feature.



# THE RELATIONSHIP BETWEEN AREAS DEFICIENT IN FOOD AND AREAS OF OIL AND NATURAL GAS PRODUCTION

Adapted from P.R.Ehrlich & J.R.Norris, 1970.

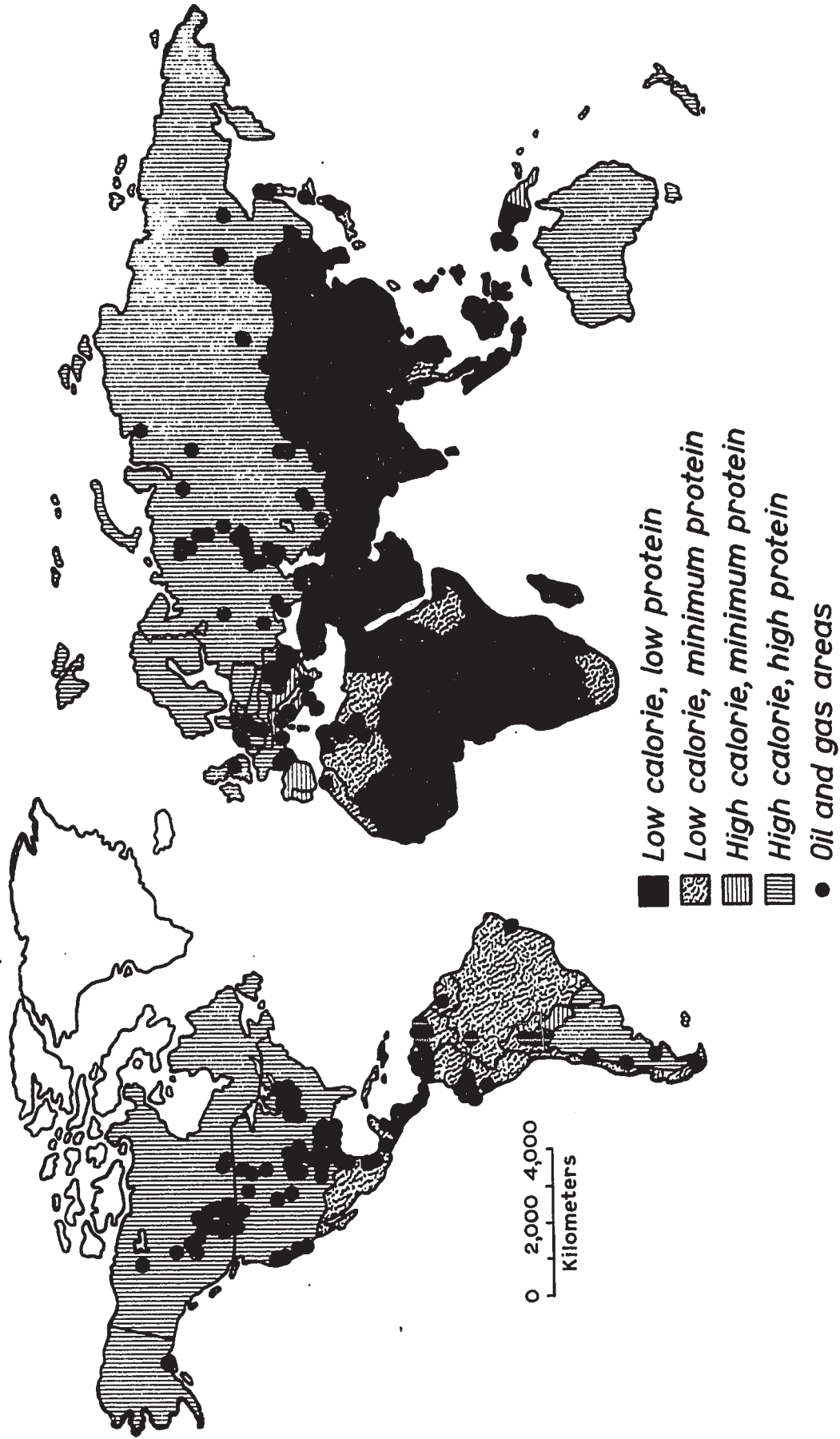
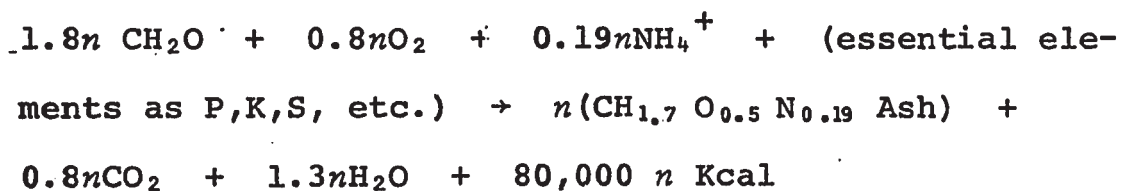


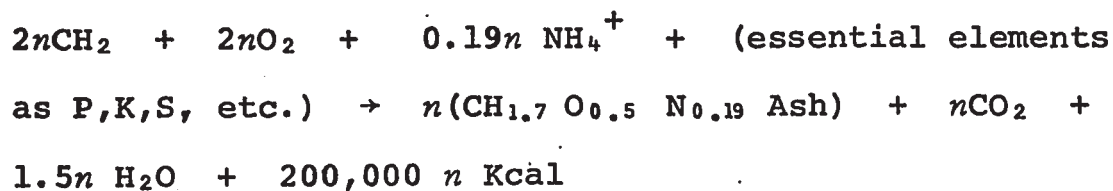
FIGURE 3

tissue synthesized from the hydrocarbons as compared to the same amount of carbohydrates utilized in a fermentation process. Hydrocarbon substrates do present certain disadvantages which should be emphasized in a critical analysis, e.g. their low water solubility, higher culture oxygen demand, a more complex  $O_2$  and substrate transfer system in the broth and greater exothermy during the oxidation of the hydrocarbon. Equations have been suggested (Bennett et al., 1969) which describe the fermentation process:

For carbohydrates:



For hydrocarbons:



It is seen that carbohydrates being a partially oxidized substrate need about 2.5 times less oxygen for the same amount of cellular tissue produced. The general composition of cellular tissue assumed is shown in brackets and is based on a typical average tissue composition.

The demand for supplied oxygen is much higher for hydrocarbon substrates and this contributes additional costs because of the increased aeration and increased energy for mixing in order to ensure sufficient oxygen transfer to the growing culture. The greater solubility of  $O_2$  in liquid hydrocarbons may partially reduce the expected

costs but no calculations have been made on benefits of this observation. The higher stirring rates for cultures grown on hydrocarbon require additional energy, a part of which is dissipated as heat. Heat itself evolved during the fermentation by degradation of the hydrocarbon is double or triple that evolved by a culture oxidizing an equivalent weight of a carbohydrate substrate. Total heat produced over the fermentation period may require mechanical refrigeration or at least a very efficient cooling system in order to maintain an optimal temperature for the growth which is in most cases  $\sim 30^{\circ}\text{C}$ . There have been reported some cultures isolated which grow well up to  $40 - 70^{\circ}\text{C}$  (Mateles et al., 1967; Sheehan and Johnson, 1970) and these new cultures or thermophilic mutants could mean considerable cost reduction for refrigeration or cooling.

Heat evaluation for single-cell production on various substrates is compared in Table 2 (Wang, 1968).

There is an additional problem which particularly complicates hydrocarbon fermentation. If a mixture of liquid hydrocarbons (e.g. gas oil) is used as a growth substrate, some of the components are not or are utilized only partially and the residue contaminates the biomass which is produced. This leaves an unpleasant odour and taste. The subsequent extraction step which is used in the processing line to free the biomass of hydrocarbon residue adds to the production costs. This disadvantage can be by-passed by using purified straight chain hydrocarbons which are entirely utilized by the culture. The biomass product thus requires only simple washing. Also, cellular lipids are removed in the extraction

TABLE 2

HEAT EVOLUTION FOR SINGLE-CELL PROTEIN  
PRODUCTION ON VARIOUS SUBSTRATES

SUBSTRATE	YIELD ON SUBSTRATE [g cell/g substrate]	HEAT OF FERMENTATION [Kcal/100g cell]	RATE OF HEAT EVOLUTION [Kcal/l·hr]	POWER COST (REFRIG.) [¢/lb cells]
<i>From existing data:</i>				
Carbohydrate	0.5	380	13.2	0.54
n-Paraffin (liquid)	1.0	780	27.0	1.03
Methane	0.6	1860	64.3	2.52
<i>With improvement:</i>				
Methane	0.8	1300	45.0	1.75
Methane	1.0	970	33.6	1.31
n-Paraffin (liquid)	1.2	590	20.4	0.79
n-Paraffin (liquid)	1.4	453	15.7	0.61

Basis: doubling time = 3 hr.  
 cell concentration = 15 g/l  
 refrigeration = 1.2 hp/12,000 BTU/hr.  
 1KWh = 1¢

(after Wang, 1968)

procedure which should improve the stability of the product. The cleanest operation of course is in the process where gaseous hydrocarbons are utilized. Gaseous substrates do not seriously contaminate the biomass and the solvent extraction step can be omitted.

In comparing the advantages and disadvantages of the production of single cell protein from hydrocarbon substrates, one can see that against the low cost and ready availability there are many disadvantages arising in the process of fermentation. It can be seen that the advantage of using a cheap substrate may be offset by the increased operational costs from their use.

It appears that a general consideration of costs cannot be made, and that an economic analysis of each process developed will be required. There are certainly some economical advantages in scaling up any process.

In examining the limits for economical operation of a production plant consideration must be given to

- (1) minimum size which is controlled by the cost of the product, and
- (2) maximum size which is controlled by the size of associated refinery and market potential.

A desirable economic size is considered to be 60,000 - 120,000 t/year, which is also in keeping with the size of the market (Decerle et al., 1969). In some cases advantage can be taken of dewaxing (Bennett et al., 1969) and therefore upgrading the crude gas oil substrate. Interest of

big oil companies which are very active in this area would suggest that there is high expectation in the price of the proteinaceous products becoming competitive. Comparison of anticipated prices of some proteins from various sources is given in Table 3.

If single cell proteins are to be used either as animal or human food supplements, the cost of production must be competitive with such products as oil-seed meals and fish-meals which are priced from 6 - 15 ¢/lb. Actual costs for the single cell proteins from hydrocarbons have not been disclosed by the companies working in the field, but it is possible to estimate that production cost may well be within the above range (Anonymous, 1967; Bennett et al., 1969; Llewelyn, 1967). Estimated production costs are shown in some detail in Table 4.

For human consumption, the price would probably range from 30 - 40 ¢/lb., since additional processing costs may include: (a) selective solvent extraction, (b) manipulation of cell properties for food formulation and (c) reduction of nucleic acid content.

#### LIQUID HYDROCARBON SUBSTRATE

From the world-wide research activities in the field of hydrocarbon fermentation it is apparent that the largest effort is being devoted to the process development using liquid hydrocarbons as a substrate (Fineberg, 1968).

This stems primarily from the numerous microbial cultures which are known to grow and give excellent yields on liquid hydrocarbons.

TABLE 3

COMPARISON OF ANTICIPATED PRICES OF SOME PROTEINS  
FROM VARIOUS SOURCES

	% PROTEIN	\$/lb	\$/lb PROTEIN	DEVELOP. STATE
<b>ANIMAL PROTEINS</b>				
Ocean perch	18	0.54	3.00	Commercial
Hamburger	20	0.54	2.70	Commercial
Dry skim milk	36	0.24	0.67	Commercial
Fish protein concn.	75-80	0.36-0.48	0.48-0.6	Semi-Comm.
<b>PLANT PROTEIN</b>				
Soy flour	44-47	0.07-0.11	0.16-0.23	Commercial
Soy concentrate	10	0.2	0.29	Commercial
Soy isolate	95-97	0.35	0.36-0.37	Commercial
Cotton seed flour	55-59	0.11	0.19-0.20	Commercial
Wheat gluten	80	0.30	0.38	Commercial
Peanut flour	50-60	0.12-0.15	0.24-0.25	Research
<b>MICROBIAL PROTEINS</b>				
Yeast- <u>Candida utilis</u>	50	0.15	0.30	Commercial
<u>Candida lipolytica</u>	50	0.15	0.30	Development
Algae				
<u>Chlorella, Scenedesmus</u>	45	0.20	0.45	Research
Bacteria				
Methane-utilizing	50	0.15	0.30	Research
Paraffin-utilizing	50-70	0.18-0.25	0.36	Development

(after Lipinsky et al., 1969)

TABLE 4

COST ESTIMATES FOR SINGLE CELL PROTEIN  
FROM PETROLEUM

(Basis: 100,000 t/year ~ 50% protein)

Reference:	(Wang, 1968)		(Bennett, 1969)	
	[\$/lb]	[% of cost]	(n-paraf.-BP-Gas Oil) [% of cost]	
RAW MATERIALS				
n-Paraffins	1.5-3.5		40	13
Others	0.5		18	30
SUBTOTAL	2-4	31-38	58	43
OPERATING & UTILITIES				
Aeration & Agitation	1.2-2.0			
Cooling	1.0			
Recovery	0.3-0.5			
Drying	0.4-0.6			
Others	0.2-0.4			
SUBTOTAL	3.1-4.5	42-50	18	25
CAPITAL INVESTMENT				
Fermentation	0.35-0.7			
Recovery	0.36-0.5			
Drying	0.05			
Cooling	0.22-0.44			
Others	0.2-0.4			
SUBTOTAL	1.18-2.09	18-20	24	32
TOTAL COST	6.3-10.6	100%	100%	100%



Many different microorganisms have been reported to oxidize and utilize liquid hydrocarbon substrates for growth, though most of the studies reported in the literature have been devoted to the propagation of yeasts such as Candida and Torulopsis. Some bacteria also give satisfactory yields when grown on hydrocarbons (Ko and Yu, 1968; Douros, 1968; Ertola et al., 1965, 1969; Perkins and Furlong, 1967; Wagner et al., 1969).

Table 5 lists the more efficient microorganisms which grow on liquid hydrocarbons.

Among the genera of yeasts, Candida seems the most important showing very high yields and having cellular protein content approximately 50%. Microorganisms are usually grown in pure culture, however, some authors claim that in some mixed culture systems yields were higher than in pure cultures. A patent has been issued for such a mixed culture system using Torulopsis and Brettanomyces (Takeda et al., 1965). Similar results were reported with Candida lipolytica and C. intermedia growing in mixed culture (Miller and Johnson, 1966).

For maximum cell yield the supporting medium composition is very important. Major chemical nutrients generally required by the hydrocarbon utilizers include:  $\text{NH}_4^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Fe}^{2+}$ , and anions of  $\text{Cl}^-$ ,  $\text{SO}_4^{2-}$ ,  $\text{PO}_4^{3-}$ , together with trace elements. These components are mixed in aqueous salt solution. Of particular importance are the concentrations of ferrous and magnesium ions (Takeda et al., 1965) which are critical for some yeast strains.

TABLE 5

## SOME MICROORGANISMS UTILIZING LIQUID HYDROCARBONS

ORGANISM	SUBSTRATE	YIELD (Cell concn.) g/l	CELL YIELD PER SUBSTRATE $\left[ \frac{\Delta S}{\Delta X} \times 100 \right]^{\circ}$	REFERENCE
<u>Candida lipolytica</u>	Gas Oil	13.5	90	Wagner et al.
<u>Candida lipolytica</u>	C <sub>16</sub>	3-5	102	Takeda et al., 1965; Dostalek et al., 1968
<u>Candida albicans</u>	n-paraf.		80-90	Tanaka et al., 1967
<u>Candida intermedia</u>			100	Miller & Johnson, 1966; Chepigo et al., 1967
<u>Pseudomonas No. 5401</u>				Ko & Yu, 1968; Yu et al., 1966
<u>Candida tropicalis</u>	C <sub>16</sub>			Miller & Johnson, 1966
<u>Candida sp.</u>	Gas Oil		72	Takeda et al., 1965
<u>Torulopsis sp.</u>	C <sub>14</sub> C <sub>18</sub>			
<u>Torulopsis magnolia</u>	C <sub>8</sub> -C <sub>19</sub>			Perkins & Furlong, 1967
<u>Pseudomonas fluorescens</u>	C <sub>8</sub> -C <sub>19</sub>			Perkins & Furlong, 1967; Takahashi et al., 1963
<u>Pseudomonas aeruginosa</u>	C <sub>8</sub> -C <sub>19</sub>			Perkins & Furlong, 1967; Ertola et al., 1965
<u>Pseudomonas desmolyticum</u>	C <sub>8</sub> -C <sub>19</sub>			Johnson, 1967
<u>Pseudomonas sp.</u>	C <sub>18</sub>		103	
<u>Micrococcus cerificans</u>	C <sub>16</sub>		130-113	Perkins & Furlong, 1967
<u>Nocardia NBS 28</u>	C <sub>14</sub> -C <sub>17</sub>	14.2	77	Wagner et al., 1969
<u>Nocardia NBZ 23</u>	C <sub>14</sub> -C <sub>17</sub>	(20-14)	111- 93	Wagner et al., 1969
<u>Nocardia opaca</u>	C <sub>14</sub> -C <sub>17</sub>	(24-9)	132- 60	Wagner et al., 1969
<u>Nocardia rubra</u>	C <sub>14</sub> -C <sub>17</sub>			Wagner et al., 1969; Perkins & Furlong, 1967
<u>Nocardia</u>				Perkins & Furlong, 1967
<u>Alcaligenes sp.</u>	C <sub>16</sub>	9/48 hours	22	Douros, 1968
<u>Pseudomonas ligustri</u>	C <sub>16</sub>	6/48 hours	51	Douros, 1968
<u>Pseudomonas pseudomallei</u>	C <sub>16</sub>	5.5/48 hrs	71	Douros, 1968
<u>Pseudomonas orvilla</u>	C <sub>16</sub>	6.3/48 hrs	62.5	Douros, 1968
<u>Cellulomonas galba</u>	C <sub>16</sub>	6.2/48 hrs	77	Douros, 1968
<u>Brevibacterium inspectiphilium</u>	C <sub>16</sub>	12.8/48 hrs	77.5	Douros, 1968
<u>Corynebacterium pourometabolum</u>	C <sub>16</sub>	9.5/48 hrs	75	Douros, 1968
<u>Corynebacterium sp.</u>	C <sub>16</sub>		95	Douros, 1968

Oxygen requirement, being higher than for conventional carbohydrate grown cultures, should be met by sufficient aeration accompanied by good mixing and dispersion of the gaseous phase within the fermentation broth. Design features of a fermentation process are dependent upon the substrate applied (Koichi, 1968; Ozaki, 1969).

The most widely used substrates giving the best biomass yields are the straight-chain hydrocarbons (n-alkanes). They are obtained from petroleum distillates and cuts contain fractions between  $C_{10}$  -  $C_{18}$  n-paraffins. A small fraction (less than 10%) of isoparaffins, cyclic paraffins and naphthenes are usually present but are not utilized by the current group of production cultures. Since they remain as a residue when the fermentation is completed, they must be extracted in subsequent purification steps. The cellular mass must be stripped of all the hydrocarbon residues. Solvent extraction of the biomass increases production costs but it does extract cellular lipids and adds to the stability of the final product.

This purification step is not required when pure n-alkanes, which are prepared by extraction procedures from petroleum by molecular sieves, are used as a substrate. These alkanes distill between temperatures used for diesel fuel and lubricating oil fractions. Containing high proportions of waxy paraffinic materials, gas oil may be up-graded by employing microbial cultures. These microbes feed upon the long-chain components and yield a proteinaceous biomass as a useful by-product. Under these conditions the conventional chemical process of dewaxing is

replaced by the biochemical process (Dostalek et al., 1968; Koch, 1969).

A process using either pure n-alkanes or gas oil as a substrate has been developed by BP (Bennett et al., 1969; Llewelyn, 1967) in production scale yielding a microbial high protein concentrate. Biomass produced from n-alkanes needs only to be washed with water, whereas residues when gas oil is used as substrate must be removed by solvent extraction which removes cellular lipids as well. Recovered solvent goes back into the process. Only about 10% of the feed gas oil is converted into cellular tissue and the remainder, with reduced pour and cloud points, is recovered for reuse in normal refinery product streams. Both versions of the process are operating on a continuous-flow basis.

#### FERMENTATION PROCESS USING LIQUID HYDROCARBONS

The fermentation takes place in an aqueous medium containing all the necessary inorganic nutrients with the hydrocarbon substrate being stirred into the medium as needed. The specific process used depends upon the substrate added which in turn determines the subsequent operations. Also the application of either aseptic or non-aseptic conditions during the fermentation is governed by the culture and substrate used. Figure 4 shows the general flow-sheet diagram of the hydrocarbon fermentation process (Bennett et al., 1969; Takata, 1969; Decerle et al., 1969) for both large pilot-plant and production operations.

The process utilizes gas oil as a feedstock which requires employment of an extraction stage. This fermentation process does not re-

FIGURE 4

General flow-diagram of hydrocarbon fermentation.

Fermentation process using liquid feed stock can be carried either

- 1) under aseptic conditions with purified n-paraffins as a substrate. Solvent extraction can be omitted, or
- 2) under non-aseptic conditions with gas oil as a substrate. No sterilization is required. Solvent extraction is employed to remove residual hydrocarbons from the biomass.

If gaseous hydrocarbon (natural gas) is used as a substrate no subsequent solvent extraction is needed and the process can be carried out under sterile as well as under non-sterile conditions as mixed culture system.

# Hydrocarbon Fermentation Flow Diagram

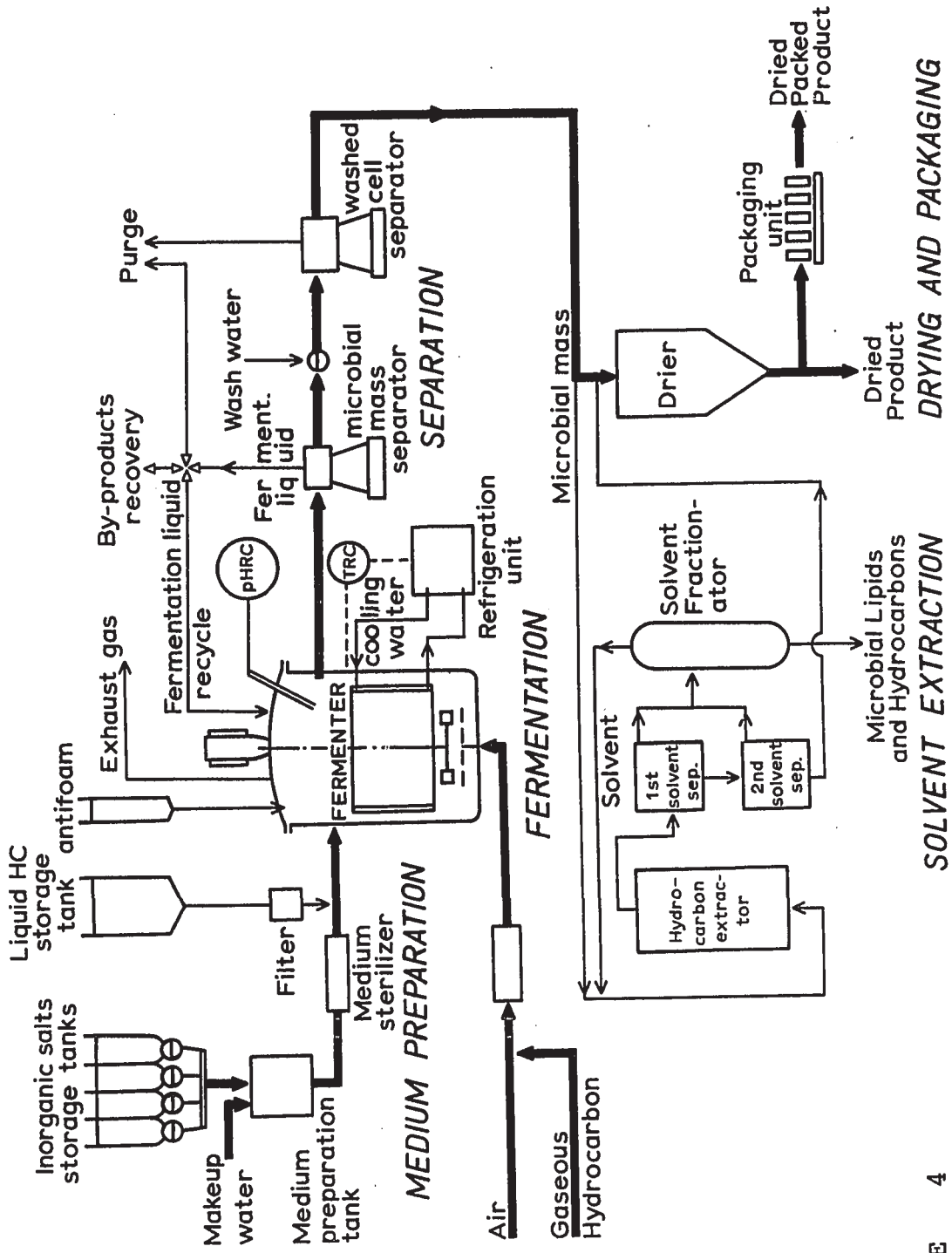


FIGURE 4

quire sterilization since the culture vessel does not operate under aseptic conditions. This approach has been successfully used by BP and the plant built in Lavera (France) operates with an output of 16,000 tons of BP protein concentrate per year.

Inorganic nutrients are mixed with water in predetermined proportions and the aqueous solution is fed continuously into the fermentor without being sterilized. Gas oil is metered into the fermentation vessel which is sufficiently aerated. pH and temperature controls are essential to maintain growth conditions in the fermentor at optimal levels. Subsequent separation and purification steps consist of: (1) separation of water phase, (2) separation of gas oil product, (3) predrying of the microbial mass, (4) extraction of residual hydrocarbons (and cellular lipids), (5) recovery of byproducts and solvent reuse, and (6) final product drying stage and storage. The final product contains less than 10% moisture and is reported to contain about 65% crude protein. Protein is expressed on a dry weight basis with the lipid portion removed.

The second alternative of the process which is depicted in Figure 4 uses n-paraffins as a pure feedstock. Otherwise it possesses the same features as the first alternative. However, the fermentation is carried out under entirely aseptic conditions. All the components entering the fermentor are sterilized. The sterilization is accomplished by holding the aqueous medium at approximately 120°C for 15-20 minutes or by flash heating for a few seconds to a temperature up to 180°C. The n-paraffin feedstock is sterilized by filtration after preliminary chemical treatment

which is required to provide a higher purity of straight chain hydrocarbons. The air is also sterilized by filtration. Aseptic conditions are maintained only in the fermentor. Normal precautions required by general food industry practice are observed in subsequent treatment of the harvested biomass. Cell recovery is an important operation which has been discussed in more details by several authors (Wang, 1968, 1969; Humphrey, 1967, 1968). The solvent extraction step may or may not be incorporated into the process. When possible, the tendency is to exclude it. In this modification the substrate is regarded as being entirely utilized leaving no significant residue. Optimal steady state operation is maintained in this type of continuous-flow production. The cell concentration in the harvest stream usually ranges from 10 to 35 g dry weight of tissue per liter of solution (Bennett et al., 1969). Provided the concentrations of all nutrients are kept above their respective critical limiting values in the broth, the exponential growth rate can be maintained. It has been shown theoretically that a continuous fermentor has far greater productivity than a batchwise operated system. In addition the continuous system is easier to control and the SCP produced has a more uniform composition. Ideally, commercial SCP production may be accomplished under non-sterile conditions where good practices of plant sanitation are applied (Laine and Hondermarck, 1968; Harada 1968).

#### GASEOUS HYDROCARBON SUBSTRATE

The use of natural gas as a substrate for SCP production possesses many advantages over that of liquid hydrocarbon substrates. The



principle one is elimination of the extraction stage since natural gas does not leave any hydrocarbon residue in the culture broth. Other advantages are wide occurrence, abundant supply, and availability in high purity which makes the price of natural gas the lowest of all chemical substrates. The main component of natural gas is methane (50-95%), ethane (5-40%), and lower amounts of propane and butane.

The natural gas process has not developed satisfactorily to date. The main reason for this being the lack of a microbial culture which would be efficient enough to promote industrial interest. Existence of methane-utilizing microbes was proved in 1906 by Sohngen (Bacillus methanicus) and many different bacterial cultures growing upon gaseous hydrocarbons as a sole source of carbon and energy have been isolated since. Extensive studies conducted by Zajic (1964) showed that about 450 different soil isolates are capable of taking part in this oxidation process. Among pure cultures a great deal of work has been devoted mostly to descriptive taxonomic studies and physiological characteristics, (Dworkin and Foster, 1956; Leadbetter and Foster, 1958, 1959; Johnson and Temple, 1962; Foster and Davis, 1966). In the past few years the interest in this field of fermentation has been increasing rapidly resulting in development of the process of SCP production from natural gas or pure gaseous hydrocarbons (Coty, 1967; Ribbons, 1968; Wolnak et al., 1967; Zajic and Volesky, 1970; Mueller and Walden, 1970; Mueller, 1969a, b).

The microorganisms used are isolated mostly from soils, pond muds, and sewage by enrichment techniques and applying both dilution and micromanipulative techniques. Cultures of importance reported to grow upon

pure gaseous hydrocarbons or natural gas are listed in Table 6.

It probably would be practical to give serious consideration to the possibility of working with mixed cultures of microorganisms oxidizing gaseous hydrocarbons or natural gas. From a pragmatic point of view it is easier to obtain and grow mixtures of microorganisms which would rapidly and efficiently oxidize the gaseous substrate. In terms of energy conversions and efficiency, a mixed culture system would assure that fewer biochemical intermediates would accumulate. In an actual process such a system would permit non-aseptic operation of the fermentation unit thus making the process considerably cheaper and simpler. The substrate used for this type of fermentation is selective to such an extent that outside contamination is limited (Sheehan, 1970).

It was observed, and this point should be of particular scientific interest, that the activity measured by growth rate was higher in some mixed culture systems than in pure culture ones (Vary and Johnson, 1967; Sheehan and Johnson, 1970; Volesky and Zajic, 1970).

Some strains of methane-oxidizers have been shown to possess the ability to co-oxidize other gaseous substrates (Leadbetter and Foster, 1960). Both co-oxidation and co-utilization may contribute considerably to new products and processes as new information is developed.

So far only bacteria have been reported as a potential source of single cell protein grown on gaseous hydrocarbons. The literature has been reviewed by Davis and Updegraff (1954) and Coty (1967). Some of the isolates possess favorable growth characteristics which may promote

TABLE 6  
SOME MICROORGANISMS UTILIZING GASEOUS HYDROCARBONS

MICROORGANISM	SUBSTRATE	YIELD	CELL YIELD PER SUBSTRATE Weight of cells X 100% Weight of HC	RATE OF GROWTH ( $t_d$ - doubling time)	REFERENCE
<u>Bacillus methanicus</u>	CH <sub>4</sub>	3-4	50	3-4 g/l/40 hrs	Söhngen, 1906 Stormer, 1908 Munz, 1915 Aiyer, 1920 Wolnak et al., 1967 Hutton, 1949; 1948 Dworkin and Foster, 1956 Brown, 1958; Ribbons, 1968
<u>Bacillus hexacarbovorum</u>					
<u>Bacillus methanicum</u>	NG	3	10-40 58	$t_d$ = 3 hours	Elizarova, 1963 Bogdanova, 1966 Bogdanova, 1966 Davis et al., 1964  Nechaeva, 1949 Nechaeva, 1949 Kersten, 1964 Kersten, 1964
<u>Bacillus (Pseudomonas) fluorescens</u>					
<u>Bacillus sp.</u>					
<u>Methanomonas carbonatophilla</u>					
<u>Pseudomonas methanica</u>					
<u>Methanomonas methanooxidans</u>					
<u>Chromobacterium</u>					
<u>Pseudobacterium</u>					
<u>Pseudomonas radiobacter</u>					
<u>Pseudomonas methanitricans</u>					
<u>Mycobacterium methanicum</u>	0.28	0.28	0.28/1/21 days	0.28/1/21 days	Hamer et al., 1967 Ribbons, 1968 Vary and Johnson, 1967 Enebo, 1967 Zajic and Volesky, 1969
<u>Mycobacterium flavum</u>					
<u>Mycobacterium rubrum</u>	CH <sub>4</sub> NG NG CH <sub>4</sub> NG	3 0.05	58	$t_d$ = 6.5 hrs $t_d$ = 3 hours 0.05 g/l/10 days $t_d$ = 5 hours	
<u>Mycobacterium lacticolium</u>					
<u>Methanomonas sp.</u>					
<u>Methanococcus capsulatus</u>					
<u>Pseudomonas (mixed cult.)</u>					
<u>Chlorella (Alga)</u>	C <sub>2</sub> -C <sub>4</sub> paraff.	0.05	58	0.05 g/l/10 days $t_d$ = 5 hours	
<u>Graphium sp. (fungus)</u>					

development of an industrial scale process. The occurrence of certain fungi has been reported in the areas of natural gas seepage (Davis, 1967; Zovarsina, 1968).

#### ANALYSES OF SCP FROM MICROBES GROWN ON HYDROCARBONS

Because of the supply, low cost and simple processing, many regard hydrocarbons as the ultimate source to make single cell protein. The composition of microbial mass grown on hydrocarbons does not differ significantly from that grown on conventional substrates. For SCP production purposes, microbial cells containing 40% and higher protein are of interest. The remaining portions of the cellular material are carbohydrates, lipids and mineral constituents usually expressed as ash.

Table 7 gives a general composition of various microbial cells grown on hydrocarbon substrates. Bacterial tissue contains usually 50-70% protein while yeast protein content is slightly lower averaging between 40 to 60%. The lipid portion of the microbial tissue varies considerably over the range from 2 to 25% according to the culture and culture conditions. Composition of the lipid fraction of cellular material and extracted intermediates studied in detail by some researchers (Klug and Markovetz, 1967, 1969; Wagner et al., 1969; Romero and Brenner, 1966; Raymond and Davis, 1960; Dunlap and Perry, 1968), helps to assess the mode of attack of the substrate by microbes and reveals additional information on the metabolic fate of hydrocarbon substrates. Many of these cells are high in poly- $\beta$ -hydroxy butyric acid which is an undesirable end-product unless a use can be found for it.

TABLE 7

## MICROBIAL MASS COMPOSITION

SOURCE	SUBSTRATE	PROTEIN %	LIPID %	CARBOHYDRATE %	ASH %	MOISTURE %	REFERENCE
Yeast BP	gas oil	68.5	1.5		7.9	5.0	Bennett et al., 1969
Yeast BP	n-paraffins	65.0	8.1		6.0	4.2	
Yeast (Japan)	n-paraffins	54.1	2.8	27.9	7.1	4.5	Takata, 1969
Yeast (ESSO)	n-alkanes	54	10	26	7		Anonymous, 1967
Yeast	n-paraffins	44	18	22	4		Miller et al., 1964
Yeast	gas oil	40-55	1-2	12-20	6-12		Valdakar, 1963 Iyengar, 1963
<u>Torula</u>	sulphite waste	47	4.8	32	9		Inskeep et al., 1951
Bacteria	n-paraffins	55-73	10-25	10	6-12		Anonymous, 1967
Bacteria	methane	50-60	6				Vary and Johnson, 1967
Fungus	natural gas	50	3-6		6-12		Volesky et al., 1970
Mixed culture	natural gas	50-60					Vary and Johnson, 1967
Bacteria	carbohydrates	40-80	1-30	10-30	1-4		Stoke, 1953
Yeasts	carbohydrates	40-50	1-2	32-40	6-10		Stoke, 1953

## AMINO ACIDS

Of the greatest interest is the protein portion of the cells. Distribution of amino acids of SCP serves to assess its potential nutritional value. A well balanced amino acid content of most of the SCP's produced from hydrocarbons makes them nutritionally adequate.

Table 8 summarizes the amino acid spectrum of some cultures used in SCP sources synthesized from hydrocarbons in comparison with those grown on carbohydrates. The composition of some conventional foodstuffs is shown in Table 9. The ideal protein composition for dietary purposes as recommended by FAO is also listed. Generally most SCP's are slightly lower in the sulphur-containing amino acids (methionine, cystine) than the FAO standard, but on the other hand most of the remaining amino acids, particularly the essential amino acids, exceed the required concentrations set by FAO. A high lysine content makes SCP superior to all the cereal proteins.

It would appear therefore that microbial cells grown on either liquid or gaseous hydrocarbons are potentially rich sources of amino acids. There is also a possibility that these fermentations may produce enough of certain amino acids as extracellular products to permit economical procedures to isolate and purify them as implied by several processes patented in this field (Humphrey, 1966; Shah et al., 1967; Ishii et al., 1967; Tanaka, 1968; Iguchi et al., 1965; Kyowa Ferment. Industry, 1968; 1969).

TABLE 8

## AMINO ACID COMPOSITION OF VARIOUS MICROBIAL PROTEINS

SOURCE OF SCP	FUNGUS (Graphium sp.)	FUNGUS (Graphium sp.)	BACTERIA	BACTERIA-Mixed Culture	YEAST - BP	YEAST - BP	YEASTS (JAP.)	YEASTS - IFP	ESSO-NESTLE YEAST	ESSO-NESTLE Bacteria	MICROCOCOCCUS CERIFICANS	PSEUDOMONAS SP.	F.A.O. STANDARD PROTEIN	
SUBSTRATE	natural gas	ethane	methane	methane	gas oil	n-paraffins	n-paraffins	gas oil	n-paraffins	n-paraffins	n-paraffins	gas oil		
% of crude protein														
<b>ESSENTIAL AMINO ACIDS</b>														
ARGININE	5.5	5.3			5.0	4.8	4.5		4.0			5.0		
HISTIDINE	2.5	3.3	1.3	1.9	2.1	2.0	2.2					1.2		
ISO-LEUCINE	5.1	4.0	5.0		5.3	4.5	5.4	4.8	3.6	3.6	4.1	3.8	4.3	
LEUCINE	8.1	6.0	8.4		7.8	7.0	6.9	8.2	5.9	5.6	6.6	6.6	4.9	
LYSINE	8.5	7.7	6.4	5.3	7.8	7.0	7.5	11.0	7.0	6.5	5.7	4.3	4.3	
METHIONINE	1.8	1.0	2.4	3.4	1.6	1.8	1.2	0.7	1.2	2.0	2.1	1.3	2.3	
PHENYLALANINE	4.6	3.6	5.2	6.2	4.8	4.4	4.4	4.3	3.7	2.9	3.4	3.3	2.9	
THREONINE	5.1	3.9	5.0		5.4	4.9	5.1	3.2	3.9	4.0	4.5	4.1	2.9	
TRYPTOPHAN	1.2	1.1	3.0		1.3	1.4	1.2	1.7	0.5	0.9	0.9	1.2	1.4	
VALINE	6.6	5.5	6.8		5.8	5.4	5.4	6.3	4.0	4.5	5.1	3.5	4.3	
<b>NONESSENTIAL AMINO ACIDS</b>														
ALANINE	6.0	6.2			5.8	7.4	6.1				6.6	6.4		
ASPARTIC ACID	9.8	7.1			10.0	9.2	9.4				8.9	7.2		
CYSTINE	0.4	.			0.9	1.1	1.7			0.6	1.1	0.4	2.0	
GLYCINE	4.9	5.8			4.5	4.8	4.8				3.4	3.8		
GLUTAMIC ACID	13.0	11.0	4.8	11	12.0	11.0	14.0				9.8	8.7		
PROLINE	4.1	4.0			3.7	4.4	4.4				3.0	2.9		
SERINE	4.6	3.5			5.1	4.8	4.7				3.3	3.0		
TYROSINE	4.3	3.6			4.0	3.5	3.3				2.8	2.7		
REFERENCE	Volesky and Zajic, 1970		Wolnak et al., 1967	Vary and Johnson, 1967	Bennett et al., 1969		Takata, 1969	Decerle et al., 1969	Anonymous 1967		Anonymous	Norris, 1968	KO and Yu, 1968	F.A.O., 1967

TABLE 9  
 AMINO ACID COMPOSITION OF SOME  
 FOODSTUFFS AND CARBOHYDRATE GROWN SCP'S.

ESSENTIAL AMINO ACIDS:	CASEIN	MAIZE	SOYBEAN	COTTONSEED	BEEF	MILK	EGG	(1) TORULA YEAST (sulfito)	(2) BREWER'S YEAST (grain)	(3) FRAGILIS YEAST (whey)	(4) PRIMARY DRIED yeast (molasses)
ARGININE	4.1						6.6	5.4	4.7	4.3	5.0
HISTIDINE	3.0						2.4	1.9	1.5	2.5	4.0
ISO-LEUCINE	6.6	4.6	5.4	3.7	5.2	6.5	6.6	5.3	5.7	5.5	5.5
LEUCINE	10.1	13.0	7.7	6.0	8.2	10.	8.8	7.0	6.3	9.9	7.9
LYSINE	8.1	2.9	6.3	4.1	8.7	7.9	6.4	6.7	7.3	8.8	8.2
METHIONINE	2.1	1.9	1.3	1.6	2.5	2.5	3.9	1.2	1.2	1.5	2.5
PHENYLALANINE	5.4	4.5	4.9	4.7	4.1	4.9	5.8	4.3	4.4	3.9	4.5
THREONINE	4.3	4.0	3.9	4.7	4.4	3.9	5.0	5.5	4.8	5.5	4.8
TRYPTOPHAN	1.3	0.6	1.4	1.2	1.2	1.4	1.7	1.2	1.1	1.5	1.2
VALINE	7.4	5.1	5.3	5.3	5.6	7.0	7.4	6.3	5.2	6.6	5.5

REFERENCE:

- Bressani, 1968
- Norris, 1968
- Norris, 1968
- Milner, 1962
- Norris, 1968
- Norris, 1968
- Bressani, 1968
- Peppler, 1968
- Standard Brands 1967
- Standard Brands 1967
- Universal Food Corporation, 1967

- (1) Candida utilis
- (2) Saccharomyces cerevisiae
- (3) Saccharomyces fragilis
- (4) Saccharomyces cerevisiae



## VITAMINS

Another class of compounds produced by hydrocarbon fermentation processes are vitamins found mainly as being intracellular, although there is some evidence (Wolnak, 1967) that they may be present as extracellular by-products in the fermentation liquid.

Table 10 shows the vitamin content of the dry biomass grown on hydrocarbon substrates as compared to some foodstuffs. The vitamin content of the SCP products is sufficiently high that it could make them a significant source of B vitamins (Fukui et al., 1968; 1969).

## NUTRITIONAL ASSESSMENT

On the basis of amino acid analyses and eventual "in vitro" digestibility determinations it is not possible to assess the true nutritional value of the SCP products. The factors which limit the usefulness of SCP as a food source are not entirely known. Bacterial cell walls are not easily digested and this may lead to a wastage up to 25% of dry weight of the biomass since the cell may pass intact through the intestinal tract. Since some of the amino acids of the bacterial cell wall are present in the form of the unnatural D-isomer non-utilization of the cell wall may be a beneficial feature to a certain extent.

Also nucleic acids present in microbial cell may have an adverse effect on digestion. Their purines are broken down to uric acid which could accumulate in the blood system to dangerously high levels leading to eventual kidney damage. Possibly the nucleic acids should be

TABLE 10

VITAMIN CONTENT OF MICROBIAL MASS  
AND SOME FOODSTUFFS

(mg/kg)

SOURCE	BACTERIA	YEAST ESSO- NESTLE	BACTERIA (PSEUDO- MONAS)	YEASTS	COTTON SEED MEAL	FISH	MILK
SUBSTRATE	methane	n-paraffins	gas oil	carbo- hydrate			
THIAMIN (B <sub>1</sub> )	19.7	11-13	2.88	95	4.0	0.66	3.3
RIBOFLAVIN (B <sub>2</sub> )	51	110-130	10.5	31	5.5	5.5	23.5
NIACIN	177	165-200	180	398	29	80	12.5
PANTOTHENIC ACID	24.5	14.23*	7.0*	108	9.7	5.5	35.5
CHOLIN	10650	150	.	4420	330	5500	6600
PYRIDOXIN (B <sub>6</sub> )	168	(4.8-7.6)	1.6	55.5	0.9	3.3	-
COBALAMIN (B <sub>12</sub> )	10.6	0.11-0.17		-	-	2.5	0.66
XANTHOPHYLL	0.8	.		.	.	.	.
BIOTIN		0.1-1.6					
p-AMINO BEN- ZOIC ACID		2.9-5.6					
FOLIC ACID		1.8-2.4					
INOSITOL		35-41					
REFERENCE	Wolnak et al., 1967	Anonymous, 1967	Ko and Yu, 1968	Wolnak et al. 1967	Wolnak et al. 1967	Wolnak et al. 1967	Wolnak et al. 1967

\* Ca - panthothenate

removed and converted to 5' nucleotides and certain food flavouring agents.

These are the reasons why detailed toxicological testing and short as well as long term feeding studies are required before any new SCP product can be considered safe for animal feed stuffs or eventually for incorporation into human food.

In a few reports which recently have been released by industry active in the field of SCP production from liquid hydrocarbons, the general accepted procedure for carrying out feeding test have been reported as well as characteristics of the protein (BP - Shacklady, 1967; BP - Llewelyn, 1967; BP - Bennett et al., 1969; Shell - Ribbons, 1967; Kanefaguchi - Takata, 1969).

Toxicity and carcinogenic property are normally tested using rats or mice for the latter and mice, rats, dogs, chicken, pigs were all used to test any possible toxic effects of the biomass. Neither toxic effects nor carcinogenic properties have been encountered during these short as well as long term tests. Special long-term trials needed to demonstrate the complete absence of long-term ill effects, using rats and mice are near completion at the present time without showing any sign of adverse results (Llewelyn, 1969).

Digestibility was evaluated by incorporating the SCP product as the only source of protein in the rations of rats on a special dietary regime, three ratios of significance can be determined:

- a) True digestibility: defined as the percentage of the nitrogen consumed not excreted in faeces,
- b) Net protein utilization: defined as the percentage of nitrogen consumed used for synthesis of body protein,
- c) Biological value: defined as the percentage of nitrogen absorbed and used by the animal in protein synthesis, this ratio is given as:

$$\frac{\text{net protein utilization}}{\text{true digestibility}} \times 100$$

The digestibility of SCP (particularly petroleum grown yeasts) has been measured in poultry, swine and fish. This material was shown to support a normal growth of animals even when over 20% was added to their feed. The nitrogen digestibility turned out to be 80-90% (Takata, 1969). Digestibility for nitrogen of BP-Protein Concentrate has been reported (Shacklady, 1967) to be 80 and 95% for ducks and pigs respectively using the biomass grown on gas oil.

"n-paraffin grown BP-Protein Concentrate" gave results of 77 and 86% for ducks and pigs respectively. The slightly different results obtained from feeding the two types of BP-PC's have been attributed to the different post-fermentation treatment of the biomass. Gas oil residues were extracted after the fermentation which is thought to have some effect on digestibility of this type of SCP.

In the course of the experiment with pigs when 40% of gas-oil grown yeast was added to the diet, no adverse effect was observed. In one case the addition of 60% yeast in the feed showed on post mortem examination that slight physiological changes occurred. This was hardly

surprising in view of the high protein intake. Daily growth rate of pigs using BP-PC replacement was quite comparable to the control group and there was no difference between the two treatments.

Long term experiments of feeding hydrocarbon grown SCP are still under way at present (BP, Kanefaguchi). Several generations of pigs and poultry are used to carry out these tests.

Applying different concentration levels of BP-PC from hydrocarbon yeasts in the rations the BP research team examined the viability and rate of growth of the chicks hatched in the course of completed laying/breeding trial. In addition to this a detailed macro and microscopic examination was made of these chicks at post-mortem.

As an indication of the absence of harmful residues in the products from animals fed BP material a sub-chronic (90 days) toxicity test with rats on eggs from hens which had been fed a 20% BP-PC in their diet for several months was performed. No abnormality on examination resulted.

In long term experiments with pigs in which the effect of feeding protein concentrate over three generations was examined, there was no difference observed between control and experimental groups of animals which could be considered as significant. The average number per litter and birth weights were recorded. Liveweight gain and food conversion ratios were examined over three generations. Post-mortem examination of the sows indicated no abnormalities (Shacklady, 1967).

## MICROBIAL GROWTH

### GROWTH IN BATCH CULTURE

It has been hypothesized that the decrease in surface area to volume ratio of the cell with increase in size leads to the necessity of segmentation of the cell, although evidence also exists in opposition to this hypothesis (Lamanna and Malette, 1965). With many species, the rate of growth of an individual cell may follow a sigmoid growth curve, the growth being least just prior to and after cell division (Lamanna and Malette, 1965; Knaysi, 1951; Monod, 1947). The generation time of organisms has been variously defined as the time interval between the fission of the nucleus of the mother and the daughter cell or the time period between the appearance of the daughter and granddaughter cells (Powell, 1958). Generation time, however, is not constant even for the organisms within a clone since the time of duplication of the genes within an organism varies as a chance event. The variability of the cell division rate (number of cell division / unit time) can be also modified by environmental conditions (Kubitschek, 1966). Therefore, organisms of identical hereditary characteristics are capable of exhibiting generation times varying within a range even when subjected to identical environments.

The growth of the population, i.e., the growth of the microbial culture, continues in smooth fashion until a characteristic maximum is reached when further growth is not possible due to limitation of food, accumulation of metabolic products or due to other factors.

Although exceptions have been noted, the time course of increase of numbers of cells or biomass frequently follows a sigmoid or S-shaped function which is repeated if a few microbes are transferred (subcultured) to a fresh medium. Analysis of the sigmoid curve or the so-called microbial growth curve reveals a complex and characteristic cycle of physiological and metabolic phenomena. The phases of the cycle are conveniently separated at points of the growth curve denoting changes of slope. The characteristics of the various phases of growth as defined by Monod (1942, 1949) are summarized below.

1. Lag phase: Adaptation to a new environment; very long generation time; growth rate null; duration longer for species with longer generation time in exponential phase; length of lag varies according to the phase from which inoculum is derived and decreases with increase in inoculum size; lag period does not affect course of subsequent phases; cell size and rate of metabolic activity maximum at lag phase; rate of multiplication lags much behind the rate of production of protoplasm; cell size is maximum.

2. Acceleration phase: Decreasing generation time and increasing growth rate.

3. Exponential phase: Minimal and constant generation time; maximal and constant growth rate; maximum rate of substrate conversion and product formation; achievement of steady state as indicated by nearly constant ratio of DNA/cell, RNA/cell, protein/cell, enzymes/cell, constant cell density and constant and minimum cell size; growth rate in this phase is a property of the particular species.

4. Retardation phase: Increasing generation time and decreasing growth rate due to gradual decrease in food concentration and increased accumulation of toxic metabolites; increasing death rate.
5. Stationary phase: Exhaustion of nutrients, high concentration of toxic metabolites, maximum physical crowding or M-concentration effect (Bail, 1929); multiplication rate balanced by death rate; growth rate null.
6. Phase of decline: Endogenous metabolism (Dawes and Ribbons, 1964), high death rate, lysis, regrowth or cryptic growth (Strange et al., 1961; Postgate and Hunter, 1962); multiplication over-balanced by death rate resulting in negative growth.

#### Mathematics of the Simple Growth Curve

Biologists have been inspired to develop mathematical expressions for growth ever since the presentation of the doctrine of human population growth by Malthus (Encyclopedia Americana, 1965; Encyclopedia Britannica, 1967). The sigmoidal curve, proposed empirically in the Malthusian doctrine, is described by a mathematical ("logistic") function proposed by Verhulst (1945) and Pearl and Reed (1920). The "logistic" function has been adopted by some (Keshevan et al., 1964; Edwards and Wilke, 1968) as the equation for microbial growth, the logic for acceptance being that the function represents all phenomena underlying population growth. It is known that the growth curve of a bacterium or of a bacterial population, of human or animal limbs, of population growth of rats, rabbits, swine, pigeons, horses, doves, flies, chicken embryos, pre-pubertal or post-pubertal growth in humans and rats (Brody, 1964), as well as the rate curves of numerous phenomena of physics, chemistry, biology, and human



society are sigmoid in nature. Thus the mere fact that a microbial growth curve is of sigmoid shape does not prove that growth is analogous to another phenomenon with the same type of development, although this assumption has sometimes been made.

While limited theoretical analyses have been performed for the lag phase (Monod, 1949; Dean and Hinshelwood, 1966), stationary phase and phase of decline (Postgate and Hunter, 1962; Harrison, 1962; Burleigh and Dawes, 1967), much more effort has been expended for the derivation of a theoretical expression for growth during the exponential phase. The exponential phase is of special importance to the process engineer by virtue of the associated maximal metabolic rate.

#### Mathematics of Exponential Growth

The growth of a culture during the exponential phase is said to be balanced (Campbell, 1957) to signify growth over a period of time during which all constituents of the organisms are, mathematically speaking, increased by the same factor. Moreover, balanced growth is said to be unrestricted balanced growth when medium constituents are not limiting for the rate of growth attained. Conversely, restricted balanced growth exists when the growth rate is limited by a constantly maintained limiting concentration of a single constituent of the medium.

Any one of the essential nutrients in the growth medium is a potential limiting factor. Composition of the medium may be such that concentrations of all essential nutrients are in large excess compared to

that of one of them which then becomes the limiting nutrient and remains so as long as its concentration is low enough to eliminate interference from other potential limiting factors (pH, oxygen concentration, etc.) or nutrients. Change of concentration of the limiting nutrient, by definition, should affect growth, substrate consumption, or product formation.

For balanced growth under restriction of a single limiting substrate or nutrient, the exponential growth rate tends to depend on variations in the concentrations of this limiting factor only in dilute media.

Growth rate during the exponential phase is the slope of the straight line in a plot of a logarithmic growth curve (Lamanna and Malette, 1965).

Slator (1916) first proposed the following mathematical formula for the determination of the specific growth rate constant or the velocity coefficient:

$$\mu = \frac{2.303}{t_2 - t_1} \log \frac{X_2}{X_1} \quad (1)$$

It is evident that  $\mu$  is the slope of the straight line plot of the logarithmic growth curve, and when computed from equation (1) it represents the average value of growth rates during the interval of time  $(t_2 - t_1)$ . By proposing equation (1), Slator in effect proposed the primitive of the following differential equation expressing the basic law of microbial growth:

$$\frac{dX}{dt} = \mu X \quad (2)$$

A mathematical model describing the effect of the concentration of the limiting nutrient on the specific growth rate of microorganisms has been proposed by Monod (1942, 1949). As stated by Monod (1949), this simple law expresses the growth rate in the exponential phase of growth as a function of the concentration of the limiting nutrient.

$$\mu = \mu_m \frac{S}{K_s + S} \quad (3)$$

The value of  $\mu_m$  is constant for the logarithmic phase and for binary fission it is defined as a ratio  $\frac{\ln 2}{g}$  where  $g$  is generation or doubling time. The doubling time is in turn reciprocal value of  $\mu$ . It is best determined by calculation based on experimental results, dividing the amount of biomass  $X$  at time  $t$  by the rate of biomass formation  $\frac{dX}{dt}$ . It should be added that the specific growth rate is differentiated from the "growth-rate" ( $\rho$ ) usually defined as the number of cell divisions per time (Monod, 1950). Therefore  $\rho = \frac{1}{g}$ .

In equation (3) it is important to note, that  $\mu_m$  and  $K_s$  are influenced by the degree of complexity of the medium and the physico-chemical nature of the environment.

Due to the empirical adoption of equation 3, often referred to as the Monod Growth Kinetic Model, numerous criticisms have appeared in the literature regarding its adequacy to describe microbial growth. The main objections are as follows (Ghosh, 1969):

1. The model is not expected to adequately describe growth in phases other than the exponential phase (Kono, 1968).

2. The model is too simple to describe complex metabolic processes giving rise to growth (Powell, 1965; Herbert, 1958).
3. Death rate during growth phase is neglected (Monod, 1949).
4. Implies constant generation time.
5. Interference of toxic metabolic products and modified environment towards the latter part of the exponential phase may become growth controlling instead of the limiting nutrient (Powell, 1965). Reduction of growth rate may be brought about by a variety of physico-chemical effects other than the concentration of limiting nutrient.
6. The model is not expected to describe growth when controlled by the rate of transport across the cell wall (Powell, 1959).
7. The model is inadequate to describe batch growth, since it does not correct for the effect of toxic metabolites, death, etc.
8. Equations for batch growth curves based on the Monod equation are not amenable to easy mathematical solution (Monod, 1942; Pearl and Reed, 1920; Downing and Wheatland, 1962).
9. Assumption of constant growth yield with respect to limiting nutrients is not valid (Kono, 1968; Powell, 1965; Herbert, 1958; Lipe, 1961; Schultze and Lipe, 1964; Schultze, 1964; Schultze, 1965; Marr et al., 1963; Pirt, 1965).
10. At large concentrations of limiting nutrients,  $\mu$  may decrease instead of approaching  $\mu_m$  due to inhibition by excess concentration or osmotic effects (Powell, 1965).

11. The model is incapable of describing growth and metabolism in completely mixed, continuous cultures (Kono, 1968; Herbert, 1958; Hempfling and Vishniak, 1967).

Owing to the aforementioned limitations of the Monod equation, various other growth kinetic hypotheses by Teissier (1936), Novick and Szilard (1950, 1959), Spicer (1955), Moser (1957, 1958), Ierusalimsky (1958), Contois (1959), Kono (1968), Powell (1959), Van Uden (1967), Saidei (1968), and Leudeking and Piret (1959) have been used in preference to equation 3. However most of the equations proposed to replace the Monod Growth Kinetic Equation have no more theoretical basis than the latter. Monod's equation is simple relative to the complex biochemical phenomena it describes and the constants of the equation have interpretable physiological significance. Generally it is acceptable because it is rational and firmly based on the law of growth by binary fission and the universally accepted Michaelis-Menten law of enzyme kinetics. The model is of proven value in analyses and prediction of the kinetics of fermentation (Aiba et al., 1965; Webb, 1964) and biochemical engineering processes. The equations derived by Monod for batch growth and nutrient assimilation are admittedly cumbersome and are not without tedium for mathematical manipulation. However, computer methods of solution by non-linear least squares analysis (Stratton and McCarty, 1967; Knowles et al., 1965; Edwards, 1967) or solution by graphical techniques (Gates and Marlar, 1968) are now available.

## GROWTH IN HETEROGENEOUS CULTURE

Since the classical works of Butterfield and co-workers (1929, 1931), the data of many researchers (Fair and Moore, 1932; Garret and Sawyer, 1952; Chynoweth, 1969) have conclusively shown that the growth curve in heterogeneous culture follows the same sigmoid function as observed with pure cultures. This is not very surprising since the dominant species surviving under the given environmental constraints express common physiological capabilities under the dictates of the physico-chemical factors of the growth medium; in this respect a heterogeneous culture is akin to a pure culture. Since the growth curves of heterogeneous (or dominant) and pure cultures are similar, it might be anticipated that the kinetics of growth in both cases could be described by a similar mathematical formulation. In fact, Garret and Sawyer (1952) have reported that the kinetics of substrate utilization by mixed cultures were the same as had been observed for pure cultures of bacteria.

## CONTINUOUS CULTURE TECHNIQUE

The advantages of the continuous culture technique are that precise manipulation and maintenance of the physical and chemical environment are possible and the cells can be held at steady state and constant physiological conditions for long periods. Thus the culture can be continuously maintained at a maximum growth rate with a maximum rate of product formation. The technique has proved to be a versatile research tool as it allows the investigator to control the environment at will. This continuous culture technique has been used for:

- a. study of kinetics of synthesis of DNA, RNA, protein (Herbert, 1961; Dean and Hinshelwood, 1960; Dean, 1962a, b), and glycogen (Holme, 1957);
- b. study of kinetics of respiration (Lipe, 1961; Schulze and Lipe, 1964; Schulze, 1964; Schulze 1965, Herbert, 1958; Pirt, 1957; Mor and Fiechter, 1968); and CO<sub>2</sub> production (Herbert, 1958; Mor and Fiechter, 1968);
- c. study of energy of maintenance (Lipe, 1961; Schulze and Lipe, 1964; Schulze, 1964; Schulze, 1965; Herbert, 1958; Marr et al., 1963; Hempfling and Vishniak, 1967);
- d. study of kinetics of inhibition (Van Uden, 1967);
- e. study of mutagenecity of compounds (Novick, 1959);
- f. study of induction of enzymes (Novick, 1959);
- g. study of diauxic phenomena (Baidya et al., 1967, Harte and Webb, 1967; Chian and Mateles, 1968; Mateles et al., 1967);
- h. study of spontaneous mutation rates (Novick and Szilard, 1951; 1950; Powell, 1958; Novick, 1950; Moser, 1957; 1958);
- i. selection of species (Bryson, 1952);
- j. simulation of aquatic ecosystems (Jannasch, 1965; 1967a, b) in order to study the nature of shifts of biotic composition of microbial population, nutritional requirements and microbial interaction;
- k. anabolic rates (Novick, 1959; Novick and Szilard, 1958); and
- l. study of kinetics of growth (Herbert, 1958; Lipe, 1961; Schulze and Lipe, 1964; Schulze, 1964; Schulze, 1965; Novick, 1959; Andrews and Pearson, 1965; Hetling et al., 1966; Baidya et al., 1967;

Harte and Webb, 1967; Jannasch, 1967a, b; Jannasch, 1965; Malek and FencI, 1961; Martin and Washington, 1966; Button and Garver, 1966).

Since the discovery of the principles of continuous culture and the apparatus (called Bactogene by Monod (1950) and Chemostat by Novick and Szilard (1950)), there has been a tremendous upsurge of interest in this research technique and several major symposia and literature reviews have been devoted to the subject (Maxon, 1955; Gerhardt and Bartlett, 1959; James, 1961; Malek and Hospodka, 1960; Malek and FencI, 1961; Malek and Beran, 1962; Beran, 1959; Ierusalimsky, 1958).

### Principles of Continuous Culture

The mathematical base of continuous culture fermentations is derived on the basis of studies of the growth obtained under the usual conditions of batch process.

If a bacterial suspension is removed from a reactor at a rate equal to the flow rate of fresh incoming nutrients in which the concentrations of all nutrients are in excess (except that of the growth controlling substrate), then the volume of the culture would remain constant and the rate of change of bacterial density is derived from the mass balance conditions for the microbial mass in the fermentor vessel (Powell, 1965; Novick, 1959; Herbert et al., 1956; FencI, 1966):

$$(\text{INCREASE}) = (\text{GROWTH}) - (\text{OUTPUT})$$

$$\frac{dx}{dt} = \mu X - D X \quad (4)$$



for steady state

$$\frac{dx}{dt} = 0 \quad (5)$$

Equation (4) then becomes:

$$\mu = D \quad (6)$$

The dilution rate  $D$  is defined as a ratio  $\frac{F}{V}$  expressing the condition for steady state. The system possesses a self-regulating property being able to establish a steady state corresponding to each dilution rate until  $D$  exceeds  $\mu_m$  when washout of the culture starts. Small accidental fluctuations from a steady state value may occur following a system disturbance. The continuous flow reactor permits the experimenter to work with a constant microbial density and steady physiological conditions.

Continuous culture may be homogeneous when the contents are completely mixed such that the concentrations of substrate and cells, as well as the physical environmental factors, are uniform throughout the culture. A reactor is heterogeneous if the flow is of ideal "plug" or a point in the flow path akin to a point in time in batch operation. The culture may be in single phase if grown in liquid media or multiphase as in biological processes in packed towers and trickling filters (Herbert, 1961). A system is closed when microbial cells are completely retained by recirculation of outgoing cells, or it may be open if cells are continuously lost. Continuous culture may be multistage when several reactors are operated in series or in a combination of series-parallel feeding (Herbert, 1961; Grieves and Kao, 1968).

A homogeneous or completely mixed single phase single stage

reactor may be operated by discontinuous flow which is actuated when a set culture density is reached. The device, called a turbidostat (Bryson, 1952, 1959), is characterized by a fluctuating culture volume and "saw-tooth" type of steady state concentration varying about a mean. At low dilution and growth rates, the frequency of discontinuous delivery of medium would be low approaching batch conditions and leading to unstable conditions and insensitive control (Herbert, 1958). A turbidostat is preferred at high dilution rates and for species having complex growth requirements (Bryson, 1959). The device involves the use of complicated electronic gear and a photocell, the operation of which is often hampered due to slime growth on the reactor walls.

The other homogeneous system is the chemostat or bactogene in which the bacterial density and the growth rate are controlled by keeping the culture volume and the flow rate constant for operation under a chosen growth rate. The chemostat is cheaper, more convenient, and simpler to operate and is also commercially available. It is preferred to a turbidostat particularly for operation at lower dilution rates (Herbert, 1958).

#### Mathematics of Chemostat Behaviour

Assuming growth rate to be described by the Monod equation it can be shown from equation (3) and (4) that steady state substrate concentration is given by

$$S = \frac{D K_s}{\mu_m - D} \quad (7)$$

Introducing culture yield  $Y$  defined as

$$Y = - \frac{\Delta X}{\Delta S} = \frac{dX}{dS} \quad (8)$$

the steady state microbial concentration obtained from chemostat mass balance is

$$X = Y (S_0 - S) \quad (9)$$

From Equations (7) and (9) it further follows that

$$X = Y \left[ S_0 - \frac{D K_s}{\mu_m - D} \right] \quad (10)$$

The dilution rate at which washout occurs is attained when there is no growth due to consumption of substrate, i.e., from Equation (9) when

$$S_0 = S \quad (11)$$

Designating dilution rate at washout as the critical dilution rate  $D_c$ , one obtains from Equations (7) and (11)

$$D_c = \frac{\mu_m S_0}{K_s + S_0} \quad (12)$$

Analogy of the microbial mass balance in chemostat can be written for substrate:

$$\begin{aligned} & \left[ \text{INCREASE OF SUBSTR.} \right] - \left[ \text{SUBSTRATE INPUT CONC.} \right] - \left[ \text{SUBSTRATE OUTPUT CONC.} \right] - \left[ \text{SUBSTRATE CONSUMPTION BY CULTURE} \right] \quad (13) \\ & - \frac{dS}{dt} = DS_0 - DS - \mu_m \frac{X}{Y} \frac{S}{K_s + S} \end{aligned}$$

for steady state

$$\frac{ds}{dt} = 0 \quad (14)$$

and Equation (13) becomes

$$D(S_0 - S) = \frac{\mu}{Y} \left[ \mu_m \frac{S}{K_s + S} \right] \quad (15)$$

Equations (7), (10), and (12), which were derived by Monod (1950), Novick and Szilard (1950, 1959), and Herbert et al. (1956), involve the following implicit or explicit assumptions:

- a. complete mixing and homogeneity of culture,
- b. growth controlled by a single nutrient,
- c. constant growth yield at all growth rates,
- d. pure culture and absence of genetic variants,
- e. absence of mixed metabolism,\*
- f. instantaneous mixing of influent particles,
- g. instantaneous conversion of influent substrate by reduction of its concentration from  $S_0$  to  $S$ ,
- h. constant dilution rate,
- i. capability of organisms to adjust to growth rates imposed by virtue of the applied dilution rate, and
- j. growth at exponential phase.

While some of the above assumptions are easily satisfied by the experimental conditions, others do not hold in many cases. Slime growth or "wall growth" on solid surfaces renders the culture hetero-

\*Mixed metabolism refers to the condition when different pathways leading to different products are operated simultaneously. Thus, under excessive aeration, yeast may produce more cells and  $CO_2$  as well as ethanol and  $CO_2$  by an alternate pathway (Maxon, 1955).

geneous and two phase (Bungay and Bungay, 1967). It has been shown by many researchers that the yield coefficient is not constant at all growth rates (Herbert, 1958; Lipe, 1961; Schulze and Lipe, 1964; Schulze, 1964; Schulze, 1965; Jannasch, 1967; Mor and Fiechter, 1968).

Mixed metabolism involving release of volatile acids at high growth rates has been observed in some cases (Chian and Mateles, 1968; Pirt, 1957; Maxon, 1955).

There are two schools of thought regarding instantaneous conversion of substrate and the adjustment of growth rate by the organisms. While Harte and Webb (1967), Kornberg, et al. (1958), Herbert (1964), and Martin and Washington (1966) have held onto the view that the assumption is valid, Mateles, et al. (1965) and Bungay and Bungay (1967) have disagreed with this viewpoint. Humphrey (1963) has mentioned that there is a definite lag before organisms respond to incoming nutrients. The problems of instability and shift of steady state to new values of substrate or organism concentrations due to spontaneous mutations or contamination by genetic variants have been discussed by Novick (1950), Powell (1958), and Moser (1957, 1958).

It remains a fact that the assumptions are many and the steady state Equations 7 and 10 seem to apply to idealized conditions hardly achieved in practice. Reported deviations of experimental data from the curves of steady state equations were so numerous that Herbert (1958) considered the agreements with theory to be only of qualitative nature.

Further complications are introduced in reactors with heterogeneous culture due to microbial interactions such as commensalism, mutualism, symbiosis, synergism, predation, antagonism, parasitism, neutralism, etc. Steady states are necessarily oscillatory due to microbial interactions and wall growth in heterogeneous culture (Bungay and Bungay, 1967). The same oscillatory pattern has been observed even in pure cultures by many researchers (Mor and Fiechter, 1968; Herbert, 1956; Malek and Hospodka, 1960; Malek and Beran, 1962).

Several types of divergencies from a typical relationship between microbial concentration and substrate concentration as functions of the dilution rate in a chemostat have been discussed, as reported by various authors. Ghosh (1969). Typical variations are seen in Figure 5 (after Ghosh, 1969).

#### POPULATION DYNAMICS AND SELECTION IN CONTINUOUS CULTURE

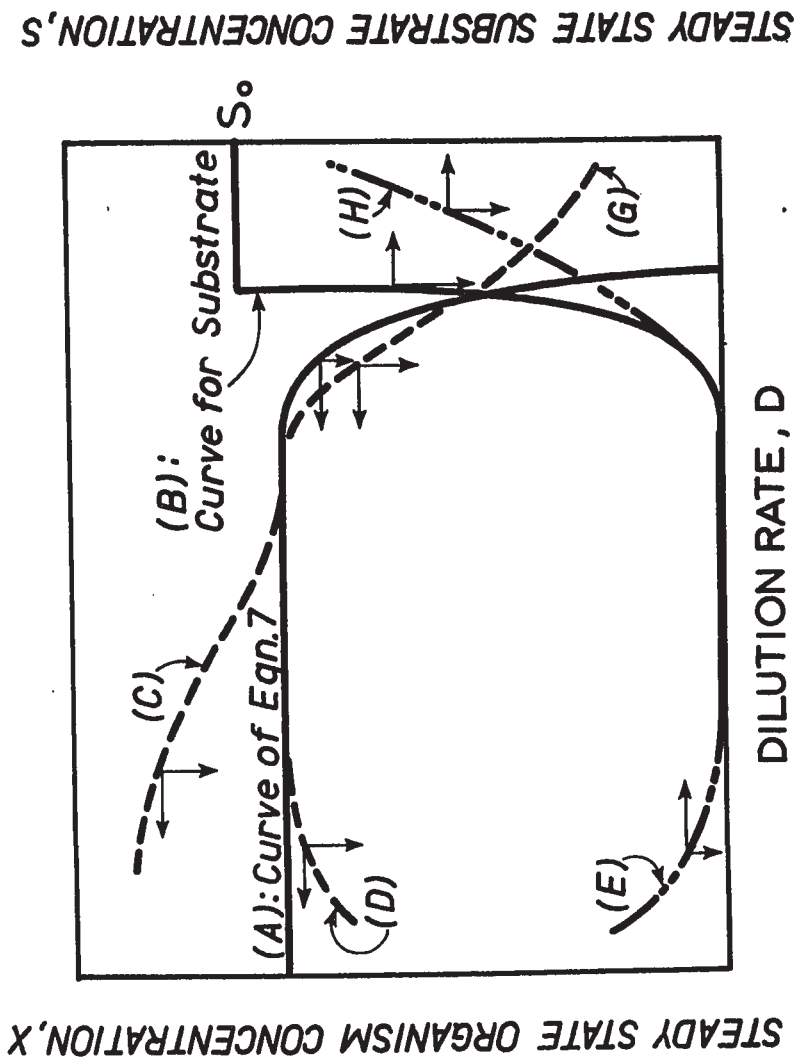
In natural ecosystems, growth of a heterogeneous population in an environment following inoculation with nutrients leads to a change in the environment which is conducive to the growth of a second group of organisms and further environmental change. Over a period of time the succession of various dominant populations is observed starting from bacterial and algal population of the lower trophic levels and higher productivity (by virtue of rapid growth and efficiency) to a population of progressively higher forms of life of higher trophic levels. The phenomena have been described by Bartsch and Ingram (1959) and at length more recently by Brock (1966).

## FIGURE 5

STEADY STATE CONCENTRATION OF MICROORGANISMS AND  
THE GROWTH CONTROLLING SUBSTRATE AS FUNCTIONS OF  
THE DILUTION RATE IN A CHEMOSTAT.

$S_0$  - Influent substrate concentration

- Curve (A) : Biomass concentration  $\underline{X}$
- Curve (B) : Substrate concentration  $\underline{S}$
- Curve (C) : Deviation of biomass concentration  
in case of nutrient deficiency
- Curve (D) : Deviation of biomass concentration  
due variation of observed growth  
yield with variation of specific  
growth rate
- Curve (E) : Deviation of substrate concentration  
due to inhibitory effect of popula-  
tion density
- Curve (G) : Deviation of biomass concentration  
attributed to "imperfect mixing"  
and wall-growth
- Curve (H) : Deviation of substrate concentration  
attributed to "imperfect mixing"  
and wall-growth.



STEADY STATE CONCENTRATION OF ORGANISMS AND THE GROWTH CONTROLLING SUBSTRATE AS FUNCTIONS OF THE DILUTION RATE IN A CHEMOSTAT.



In pure cultures, mutants with higher growth rates than parents, called prevalent mutants, ultimately displace the parent cells (Powell, 1965; Novick, 1969) and disrupt the steady state, shifting it to a new position. Mathematical treatment of the dynamics of mutant populations is presented by Powell (1958), Moser (1957, 1958), and Renneboog (1967).

Population dynamics or the selection of species by selection of dilution rates has been observed in heterogeneous cultures by many investigators (Downing and Wheatland, 1962, Andrews and Pearson, 1965; Chian and Mateles, 1968; Mateles, 1967; Jannasch, 1965, 1967; Dias and Bhat, 1964; Cassell, et al., 1966). Survival of a species in heterogeneous culture depends on a variety of less well defined factors, many of them not related to the elimination of the unsuccessful competitor by dilution which itself is only one of the important factors. Though the population remains heterogeneous in nature at all dilution rates due to mutual interactions (e.g., predation, symbiosis, neutralisms, etc.), the relative proportion of each species changes from one dilution rate to another.

Due to the mutual interactions, the population of each species is oscillatory at a given dilution rate. Such oscillations probably lead to the oscillations of the total steady state bacterial density (Bungay and Bungay, 1968; Cassell et al., 1966). The studies of Cassell, et al. (1966), Collard and Gossling (1967), Dias and Bhat (1964), Jannasch (1965, 1967), Chian and Mateles (1968), Mateles (1967), and Andrews and Pearson (1965) indicate that population change overs are rare during operation at high and low dilution rates and minimum at

highest dilution rates. A good rule to follow is that a species is selected against and may be displaced by another species which can grow more rapidly at the low concentrations of the controlling growth factor which occurs in the reactor (Novick, 1959).

In a continuous flow reactor, the environment is kept constant and only those species which are endowed with the genetic and physiological adaptive capacities to adjust to this controlled environment will persist. The experimenter can exert selective pressures at will by manipulating the chemical or physical factors of the environment. The survival and proliferation of a species confronted with increases in selective pressure depend on the genetic and adaptive potential of the species as expressed by continued growth in the modified environment. Bryson (1952, 1959) applied this principle for isolation of bacterial variants.

Since the dilution rate determines the mean growth rate, selection of variants may be effected by simply changing the hydraulic displacement rate while keeping other factors constant. The fate of a population is determined by the rate of washout such that if the latter exceeds a certain value (minimum generation time), the population eventually will be removed completely from the reactor (Powell, 1965; Novick, 1959; Powell, 1958).

Explanation of Symbols

The symbols employed in this chapter are based on the unified system of fundamental symbols suggested by the Second International Symposium on Continuous Cultivation in Prague, 1962 (Fenc1, 1963).

<u>Symbol</u>	<u>Dimension</u>	<u>Term</u>
D	$t^{-1}$	dilution rate
F	$lt^{-1}$	flow rate
g	t	doubling time
$K_s$	$gl^{-1}$	Michaelis-Menten constant
P	$gl^{-1}$	concentration of product
S	$gl^{-1}$	concentration of growth limiting substrate
V	l	working volume of the fermentor
Y	dimensionless	yield of biomass
X	$gl^{-1}$	concentration of microorganisms by mass
$\frac{dx}{dt}$	$gl^{-1}t^{-1}$	rate of biomass synthesis
$\mu$	$t^{-1}$	specific growth rate
$\rho$	$t^{-1}$	growth rate
<b>Indices</b>		
m		for maximum value
S		for substrate

## CHAPTER III

### ASPECTS OF GASEOUS HYDROCARBON SUBSTRATE UTILIZATION

The rate of growth of the microorganisms growing on gaseous hydrocarbons depends not only on its own genetic capability but also on:

- 1) Substrate composition,
- 2) Composition of gaseous mixture (air + substrate),
- 3) Aqueous supporting medium composition,
- 4) pH range,
- 5) Temperature range,
- 6) Mixing.

Yield of the fermentation process of this type is substantially affected by all of these parameters and it is essential to know their optimal values if the production is to be efficient and the process competitive.

The use of gaseous hydrocarbons present advantages and difficulties.

One of the main problems is to ensure a sufficient supply of the gaseous substrate to the organisms. The limiting solubility of both gaseous hydrocarbon substrate and oxygen under a given normal pressure and temperature is the primary one. Some stoichiometric relations have been developed describing the process of microbial tissue synthesis from hydrocarbons where  $\text{CO}_2$  and water are liberated (Darlington, 1964, Bennett et al., 1969). It is apparent that the presence of dissolved gases in stoichiometric proportions may be beneficial to the growing microbes (Klass et al., 1969). However, absolute concentrations of the gases remain to be determined for each particular fermentation. An excessively high concentration of oxygen may become inhibitory under certain circumstances. The mutual solubility ratio of the gases present in the feeding mixture is expected to play an important role.

From physical principles of the solubility of a gaseous phase in a liquid, there are apparently 2 possible ways to ensure the highest possible mass transfer. This mass transfer involves moving gases from gas phase through aqueous medium to the cell. At the stable temperature of the system which is given for the microbial culture, the 2 ways of ensuring increased mass transfer are:

- a) Increased pressure of gas phase, and
- b) Increased surface area for gas-liquid mass transfer by effective mixing,

or combination of both.

There have been reported some studies on the pressure effect upon microbial growth, but they have not been conducted from this point

of view. One recent report (Wayman and Wein, 1969) indicates negative results with yeast culture of Candida lipolytica grown under increased pressure. As seen from these studies the results of high pressure culture conditions have not been encouraging. It should also be kept in mind that an increase in operating pressure will bring a significant increase in capital and operating cost making the process less attractive.

Increased surface area together with efficient mixing in micro- as well as in macroscale is another approach to improve the culture yield. The solubility of the gaseous phase remains constant but the mass transfer in the system could be considerably improved, hopefully approaching theoretical estimates. This could be accomplished by means of extremely vigorous conventional mixing, which may have an adverse effect upon the culture due to the increased shear stress on the cells. Another possibility would be to use special fermentation equipment based on known principles for high rate mass transfer units used in the chemical engineering field (Mueller, 1969; Kitai et al., 1969; Humphrey, 1968). Vortexing fermentors, Waldhof type fermentors, both either mechanically agitated or without mechanical agitation, non-symmetrically shaped and aerated fermentors, air-lift fermentors and multiple plate type fermentors have been shown to achieve very high mass transfer rates (Greenhalgh et al., 1959; Wang and Humphrey, 1968).

Another basic approach is to improve the design of the reactor to foster gas exchange and release of heat from the reaction medium. Numerous designs used in trickling filters should be investigated.

Some compounds added to the fermentation broth may have a two-fold effect in increasing the mass transfer. First, an effect on the transfer between gaseous and liquid phases due to the increased surface area and second an effect on the liquid phase and cell associated with cell dispersion and cell wall permeability. It is also important from an operational point of view to prevent the microbial tissue from clumping and adhering to stationary parts of the reactor. The effects of any of such chemical agents upon specific culture remain to be studied and carefully evaluated.

The exact mode of gas uptake by the microbial cells has not been entirely elucidated. There are discussions as to whether the gas is taken up by the cell in dissolved form or from a solution or whether it can be utilized directly from the gaseous phase or from the gas-liquid interphase. Combination of all these means of gas uptake could take place simultaneously in submerged culture. Elucidation of the mechanism of gas transfer could enhance the successes of use of gaseous hydrocarbons for SCP.

#### FORMULATION OF THE PROPOSED RESEARCH

Most of the work with gaseous hydrocarbon-oxidizing microorganisms has been devoted to taxonomic and physiological type of studies on methane-utilizing bacteria. Further industrial interest in this field has suffered mainly from the lack of an efficient microbial culture which was capable of rapid growth on gaseous hydrocarbon substrates which give high yields of the desirable product. Engineering background in this

field has also been rather weak. In the past decade new cultures have been isolated and the possibility of production of SCP and other useful products from natural gas has sparked new interest in this field.

The research on the growth of microbial cells on gaseous hydrocarbon substrate reported in this thesis was initiated with the following objectives:

- 1) Isolation of a culture capable of stable growth in a simple inorganic supporting medium which utilizes natural gas or pure gaseous hydrocarbons as a sole source of carbon and energy.
- 2) To study the possibility of carrying out the fermentation under non-aseptic conditions in a mixed culture system.
- 3) Description and identification of the main cultures isolated from such a system.
- 4) To carry out studies on axenic cultures.
- 5) Production of biomass with good yields on gaseous hydrocarbon substrates and oxygen.
- 6) Investigation of some major factors which affect the cell production and yield. Determination of cell growth rate with varying conditions.
- 7) Investigation of growth parameters in batch culture in order to obtain basic data for continuous-flow culture.
- 8) To assess the possibility of application of continuous-flow culture techniques.
- 9) To obtain some preliminary data for scaling up the fermentation process.



10) To determine biomass composition and particularly its protein content.

11) To determine the spectrum of amino acids in the protein portion of the biomass in order to assess its value as a potential source of SCP.

12) To assess toxicity and nutritional value of the biomass in preliminary feeding trials with animals.

## CHAPTER IV

### MATERIALS AND METHODS

#### CULTURE MEDIUM

A synthetic supporting medium described by Johnson and Temple (1962) and Coty (1967) was used in most experiments. Its composition is shown in Figure 6 and the medium is further referred to as C-medium. The nitrogen source used was sometimes varied as specified for individual experiments. Also pH was adjusted as required. The medium did not contain any trace elements except those naturally present in tap water, which was used for its preparation. For pouring petri-dishes and slants 2% washed agar was added to the aqueous C-medium to solidify.

#### DOMESTIC NATURAL GAS COMPOSITION

Natural gas directly from domestic line was used in some experiments. It was filtered by passing through a loosely packed glass-wool filter bed for the mechanical removal of particulates and emulsions. The gaseous composition of natural gas in London, Ontario as specified

## *AQUEOUS SUPPORTING MEDIUM.*

*Single cell protein production  
from gaseous hydrocarbons*

*(Graphium)*

• $(\text{NH}_4)_2 \text{SO}_4$ .....	1.0	g
• $\text{K}_2\text{HPO}_4$ .....	1.0	g
• $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ .....	0.5	g
• $\text{NaCl}$ .....	0.1	g
• $\text{CaCl}_2$ .....	0.1	g
• $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$ .....	0.001	g
• Tap water .....	1,000	ml

*pH = 4.0 to 4.8*

by the Union Gas Company agreed very well with gas chromatographic analysis as carried out at certain time intervals during the course of the work. Its typical composition was as follows: 90.5% methane, 6.0% ethane, 3.0% nitrogen, 0.25% carbon dioxide, 0.21% propane, with traces of isobutane, n-butane, isopentane, and n-pentane. No mercaptanes or other sulphur compounds were detected.

#### NATURAL GAS-UTILIZING FUNGUS ISOLATION: CONTINUOUS-FLOW ENRICHMENT TECHNIQUE

Continuous-flow culture technique was used in connection with natural gas enrichment to select for the fungus and microorganisms capable of utilizing natural gas or its components as a sole source of carbon and energy. Only a few other microbial species also persisted in the mixed culture continuous enrichment system under given selective conditions. The technique was performed in a stirred tank-type fermentor. A five-gallon glass carboy was used for this purpose with no baffles inserted (Figure 7). In the first stage of operation mixing was accomplished by a magnetic rod rotating at 150 rpm. After 3 weeks of operation the agitation was changed. A disc-type impeller of 4.5 cm in diameter with 6 blades was rotated at 800 rpm. The gaseous mixture of air and natural gas (60:40  $V/V$ ) was sparged into the broth directly below the impeller using a single orifice sparger at a flow rate of 4 lpm.

A mean volume of 10 l broth was maintained in the reactor utilizing a harvesting syphon. The C-medium feed was metered in by kinetic clamp Sigma-motor pump using tygon tubing. The system was inoculated with 5% by volume of raw sewage taken from the Greenway Pollution Control Centre in London, Ontario. It was operated under entirely non-aseptic

conditions. Temperature was maintained at  $28 \pm 2^{\circ}\text{C}$ . Protozoal populations were determined microscopically whereas bacterial and fungal populations were determined both by microscopic examinations and by plating and isolating cultures.

The individual cultures present in the mixed culture system were isolated by streaking the effluent on agar plates of C-medium and incubating the plates in enclosed desiccators supplied with 40% natural gas and 60% air. Purification of cultures was achieved by subsequent restreaking of developing colonies. Hyphal tip isolations were applied for fungal subcultures. Liquid cultures of fungi were treated with different concentration levels of antibiotics (streptomycin, penicillin) in order to eliminate bacterial contamination.

All the yields of biomass from the continuously operated system are reported in milligrams dry weight of tissue in the effluent per hour. Production rate as based on dilution rate (retention time) is expressed in mg/l.hr. The biomass recovered from the effluent broth by filtration was dried in tared aluminum pans at  $70^{\circ}\text{C}$  overnight.

#### BATCH GROWTH OF GRAPHIUM SP. - SHAKE FLASKS

Studies were conducted in shake flask systems of two types:

(1) in an enclosed environmental shaker in which gaseous mixtures of natural gas (40% v/v) and air (60% v/v) were continuously passed through the system (Figure 8) under no head pressure; and (2) in closed ecological flasks. Five-hundred milliliter Erlenmeyer flasks fitted with cotton

## F I G U R E 7

CONTINUOUS-FLOW MIXED CULTURE NATURAL GAS  
ENRICHMENT SYSTEM  
EXPERIMENTAL ARRANGEMENT

Culture vessel with mixer and harvesting syphon.  
Flask collecting harvested broth.  
Fresh feed aqueous medium storage vessel and feed pump.  
Flowmeters for natural gas and air with inlet filter  
for the gaseous mixture which was sparged into the  
culture broth.

## F I G U R E 8

## ENCLOSED ENVIRONMENT BOX ON SHAKER

A mixture of natural gas and air was  
continuously fed in, diffusing into culture  
flasks. The culture was allowed to grow ad libitum.

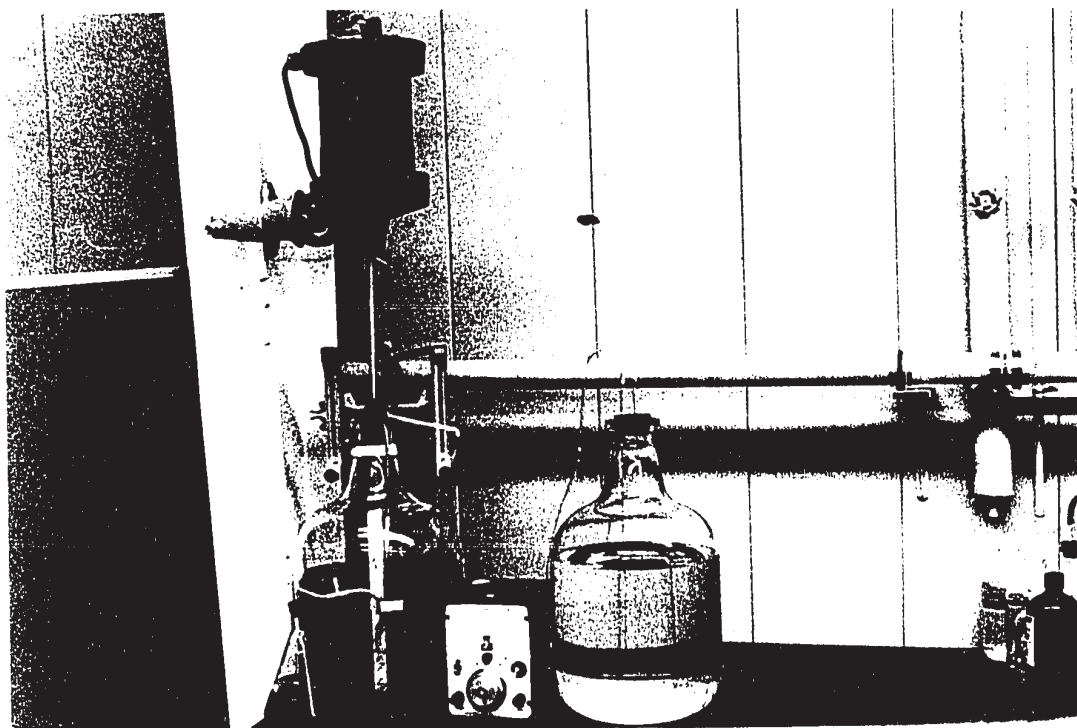


FIGURE 7

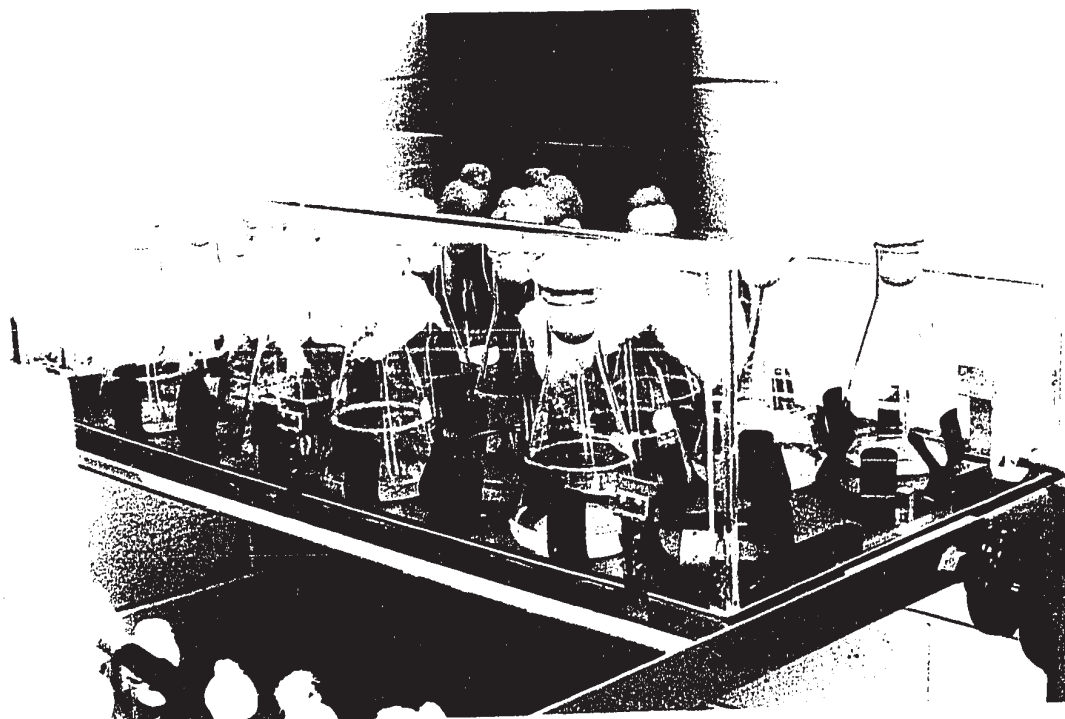


FIGURE 8

plugs were used in an enclosed Environmental Shaker. Five-hundred milliliter suction flasks fitted with a gassing port were used for closed fermentation flasks. Flasks were filled with 100 ml of C-medium and, unless otherwise indicated, were inoculated with 2.0% by volume of the Graphium sp. culture. The rotary shaker was operated at 200 rpm and incubation was at 28°C ( $\pm$  2°C). The incubation period varied from 4 to 20 days, depending on the type of experiment conducted. The composition of gases used are shown with the individual experiments. A typical composition was 40% natural gas and 60% air (V/v).

Gas analyses were conducted on an F & M Scientific Hewlett-Packard (model 5750) gas chromatograph, using columns of silica gel and/or molecular sieve 13X. Silica gel gives good separation of methane, ethane, and carbon dioxide. However, the air peak contains both oxygen and nitrogen. Helium was employed as a carrier gas for the iso-thermal or programmed temperature analyses used. Dry weight analysis for fungal tissue was carried out by filtering a measured volume of fermentation broth through Millipore filters, drying overnight at 105 C, and determining the amount of tissue synthesized.

Methane (98%), ethane (99%), propane (99.5%), and butane (99.5%) of instrument grade purity were supplied by Matheson of Canada Ltd.; oxygen and nitrogen were supplied by Canadian Liquid Air Ltd.; and carbon dioxide and helium were supplied by Liquid Carbonic, Canadian Corp. Ltd.



## UNCONVENTIONAL TECHNIQUES FOR GROWTH OF GRAPHIUM

Three different techniques have been employed to develop information on growth of Graphium. These were:

- a) packed bed column fermentor
- b) air lift fermentor
- c) fermentation under increased pressure

### a) PACKED BED COLUMN FERMENTOR

A glass column of 6 cm in diameter 2m long was packed with glass beads 6 mm in diameter to cca 2/3 of its height. In the bottom part of the column there was cca 400 ml space for collecting the liquid broth which was permeated through the column from above. The harvesting port was located at the very bottom. The packed beads were supported by a grid. Air in the mixture with natural gas was passed through the packed bed from a sparger located at the bottom at the rate of 3 lpm. Spent gases were exhausted from the upper part of the column. Liquid broth was continuously recycled with fresh C-medium being added in the upper part of the column. Harvesting was accomplished by kinetic-clamp pump. A counter-current mode of contact was provided in respect to gaseous and liquid phases.

The culture of Graphium sp. was used as inoculum for the non-aseptic fermentation system.

### b) AIR-LIFT FERMENTOR

A vertical glass column of 6 cm in diameter and 2 m long was

used with another slightly shorter glass pipe of 3 cm in diameter located co-axially inside. Annular space between the two pipes served to recycle the broth. A stream of 4 lpm of air-natural gas mixture was dispersed by a sintered glass sparger into the bottom part of the inner tube. Movement of gaseous mixture caused consequent upward movement of the liquid broth through the inner tube. The broth was recycled by overflowing into the annular space between outer and inner tubes where downward flow was accomplished. No additional mixing was provided except for this natural movement of both liquid and gaseous phases. Below the sparger there was ~400 ml volume for biomass collection and withdrawal. Fresh C-medium was introduced at the top of the column where the exhaust port for gases was also located.

The system was operated under non-aseptic conditions. The culture of Graphium was applied as inoculum.

### c) FERMENTATION UNDER INCREASED PRESSURE

For the studies of growth under increased pressure special stainless steel 500 ml cylinders were used (HOKE Comp., Ltd.). The cylinders of 2" in diameter and 14" long were provided with necessary fittings, needle valve and pressure gauge (Figure 9). These were easily detachable for inoculation. The whole system together with 100 ml of C-medium was sterilized in autoclave at 120°C (15 psi) for 30 minutes. After inoculation with 5% by volume of Graphium sp. culture a gaseous mixture of 20% oxygen, 20% ethane ( $V/v$ ), with the balance of the gaseous phase as nitrogen was introduced into the cylinder under desired pressure. Glass

wool packed sterile filters were used to sterilize the gases.

The pressurized culture system was incubated at a 45° angle on a rotating shaker at 180 rpm. After incubation period the pressure was released slowly and the amount of tissue synthesized measured.

### BATCH FERMENTATION-STIRRED TANK

#### 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).

#### Medium

A synthetic aqueous mineral salt medium previously described was used. pH was adjusted to the 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 l/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. Domestic natural gas was used. 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 used for special analyses was freeze-dried.

## F I G U R E 9

## STAINLESS STEEL CULTURE PRESSURE CYLINDER

500 ml cylinder with gasing port  
and pressure gauge.

## F I G U R E 10

NEW BRUNSWICK SCIENTIFIC MODEL MF-14 LITER  
STIRRED TANK-TYPE FERMENTOR

Heated inlet and exhaust filters inside;

Variable mixing speed;

Adjustable flow rate for 2 gases;

Automatically controlled temperature;

Automatic pH control (above).

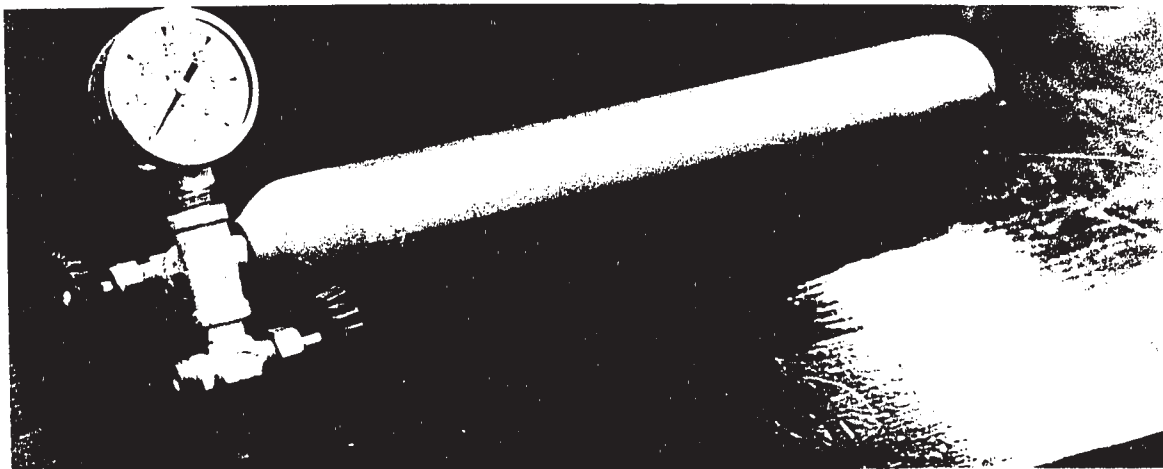


FIGURE 9

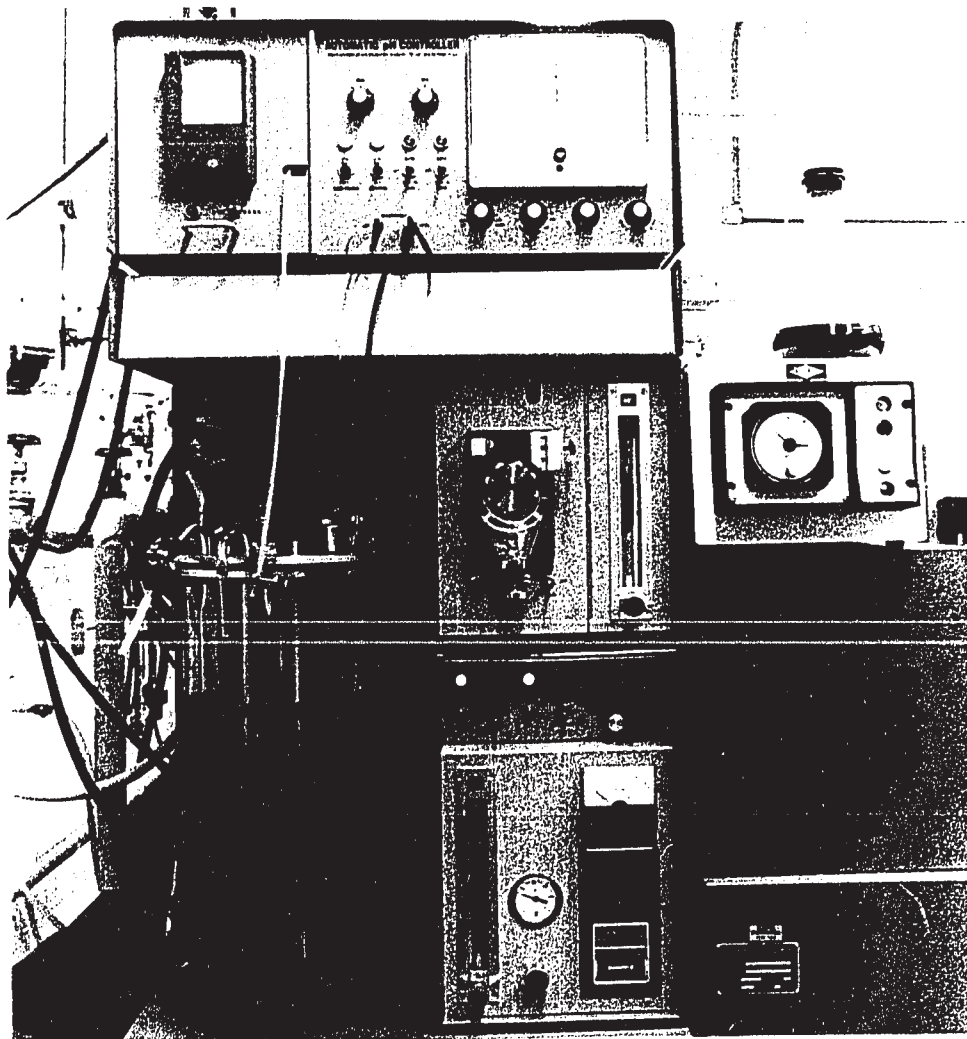


FIGURE 10

## Equipment

For studies on the recycle of gases, a Virtis magnetic drive fermentor was utilized. In all other experiments a New Brunswick Scientific Microferm model MF 14 stirred-tank type 14 l fermentor was used (Figure 10). The fermentor was equipped with four baffles of standard geometry. A single orifice air sparger was located under the 4-bladed disc type impeller. The operating volume of the fermentor was 10 l; it was agitated at 400 rpm.

Temperature was maintained automatically  $\pm 31^{\circ}\text{C} \pm 0.5$ . pH was controlled with an accuracy  $\pm 0.1$  of a unit by means of automatic addition of either 3N 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 minutes.

The gas recycle fermentation system was slightly more complex. Figure 11\* shows schematically the experimental arrangement of equipment used in gas recycle. A magnetically bottom driven Virtis fermentor with 10 l glass jar operated at 6 l 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 rpm. The gaseous phase was introduced with a diaphragm pump at the rate of 2 lpm and dispersed into the broth by sintered glass sparger located under

*\*) Figure 11 - page 81*

the lower impeller.

The recycle gas mixture consisted of pure ethane (Matheson, C.P. Grade, 99.0%) and air slightly enriched with oxygen. As the fermentation proceeded the  $\text{CO}_2$  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  $\text{O}_2 + \text{C}_2\text{H}_6$  as described.

Analysis of recycle gases for oxygen was accomplished by a Servomex Controls Ltd. Model OA. 150 paramagnetic oxygen analyser. An in-line gas-liquid chromatograph manufactured by F & M Scientific, Hewlett-Packard Model 5750 was used for analysis of  $\text{CH}_3\text{CH}_3$  and  $\text{CO}_2$ . This unit was operated using a TC detector and 15 ft. Porapak "Q"  $\frac{1}{4}$ " I.D. column. Quantitative separation of these components was conducted at  $115^\circ\text{C}$ ., 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 l. Excess  $\text{CO}_2$  was removed as required by passing gases through a column of Baralyme (National Cylinder Gas, Div. of Chemetron Corp.). The temperature of the fermentation medium was maintained at  $31^\circ\text{C}$ . pH control was not utilized in the gas recycle experiments. C-medium with sodium nitrate as a nitrogen source was used in these experiments.

#### CONTINUOUS CULTURE

Three experiments using continuous culture system were completed.

A different size of fermentor vessel was used for each experiment i.e., 14 l; 28 l and 12 l of working volume respectively. The first two experiments were carried out using natural gas as a sole source of carbon and energy for growth of Graphium sp. culture. The natural gas used was of the composition described. The mixture of 60% natural gas and 40% by volume of air was passed through the fermentation broth continuously and was exhausted without being recycled.

In the third system a complete recycle of the gaseous phase consisting of pure ethane (Matheson, 99% purity), oxygen, nitrogen and CO<sub>2</sub> was accomplished. The spent portion of gases was continuously replenished by oxygen and ethane (2:1 by volume) mixture. Adjustment of the concentration of all components of recycled gaseous mixture was possible.

In all three types of continuous culture system temperature was maintained at  $30^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ . pH of the liquid broth was adjusted initially to pH 4.3 and controlled at steady level throughout the entire fermentation period by adding either 3N NaOH or 3N HCl. An automatic pH controller, New Brunswick Scientific-Model pH 152, was used.

#### Continuous Culture I - in 14 l stirred-tank fermentor

In this system the 14 l New Brunswick Scientific-Model MF 14 Microferm fermentation unit was used in similar arrangement as for the batch culture experiments (page 76 ). 2 disc-type four-bladed impellers were rotated at 400 rpm. The filter-sterilized mixture of natural gas



and air was continuously fed into the fermentor at the rate of 4 lpm. No gas recycle was done. The fermentation vessel was kept under slight pressure to prevent outside contamination.

The assembled fermentation vessel, with attached inlet and exhaust filters, containing 10 l of fresh C-medium ( $\text{NaNO}_3$  was the nitrogen source) was sterilized in autoclave at  $120^\circ\text{C}$ , 15 psi, for 40 minutes. A 5% by volume of broth culture of Graphium sp. was used as inoculum. Continuous feed and harvest was accomplished by using two Sigma-motor pumps. Harvested tissue was recovered by filtration and freeze-dried.

The dilution rate was adjusted to desired level by synchronizing both feed and harvest pumps. An electronic level controller failed to work because of tissue build-up on the electrode inside the fermentor.

#### Continuous Culture II - in 28 l stirred-tank fermentor

A 28 l New Brunswick Scientific in-place sterilizable fermentor Model CMF-128 S was utilized. It was equipped with 4 baffles of standard geometry and was operated at volume of 18 l. A single orifice sparger was located under a single disc-type impeller with 6 blades. A filter packed with glass wool (5-15  $\mu$  ) was used to sterilize the mixture of natural gas and air. The gas mixture was pumped through the medium at a rate of 10 lpm and exhausted. Sigma-motor pumps were used for feeding and harvesting. Additional kinetic-clamp low-rate pump was used for addition of concentrated yeast extract.

Fresh C-medium (with ammonium sulphate) was prepared and sterilized batchwise in a separate 80 l mixing tank. The whole sterile system was kept under slight overpressure to prevent outside contamination. Harvested broth was collected, tissue periodically recovered by vacuum filtration and freeze dried. The experimental arrangement is presented in Figure 12.

### Continuous Culture III - with recycled gaseous phase

An experimental arrangement was used which is schematically shown on Figure 11. Recycle gaseous mixture was prepared by mixing pure ethane (Matheson, Instrument Grade-99%) as the only source of carbon and energy, with oxygen and nitrogen added to balance out the gaseous phase. Concentration of each gas in the mixture was adjusted to the desired level. A paramagnetic oxygen analyzer, Servomex Model OA.150, was used in connection with recorder-controller to maintain oxygen concentration in the recycle. A mixture of oxygen and ethane (2:1 by volume) was prepared and used to replenish the gases used in order to maintain their concentration in the system. The volume of the mixture admitted was measured by wet-test volume meter and recorded on time basis.

Ethane, CO<sub>2</sub> and also methane concentrations when required were measured by research gas-chromatograph Hewlett-Packard Model 5750 operated as specified previously (pg 77). A correction for the ethane concentration off-set was accomplished periodically by manually metering the required amount of ethane into the system. The CO<sub>2</sub> was removed from the recycle by passing gases through a column of Baralyme granules

## FIGURE 11

SCHEMATIC FLOW DIAGRAM OF THE FERMENTATION SYSTEM  
WITH RECYCLE AND CONTROL OF GASEOUS PHASE

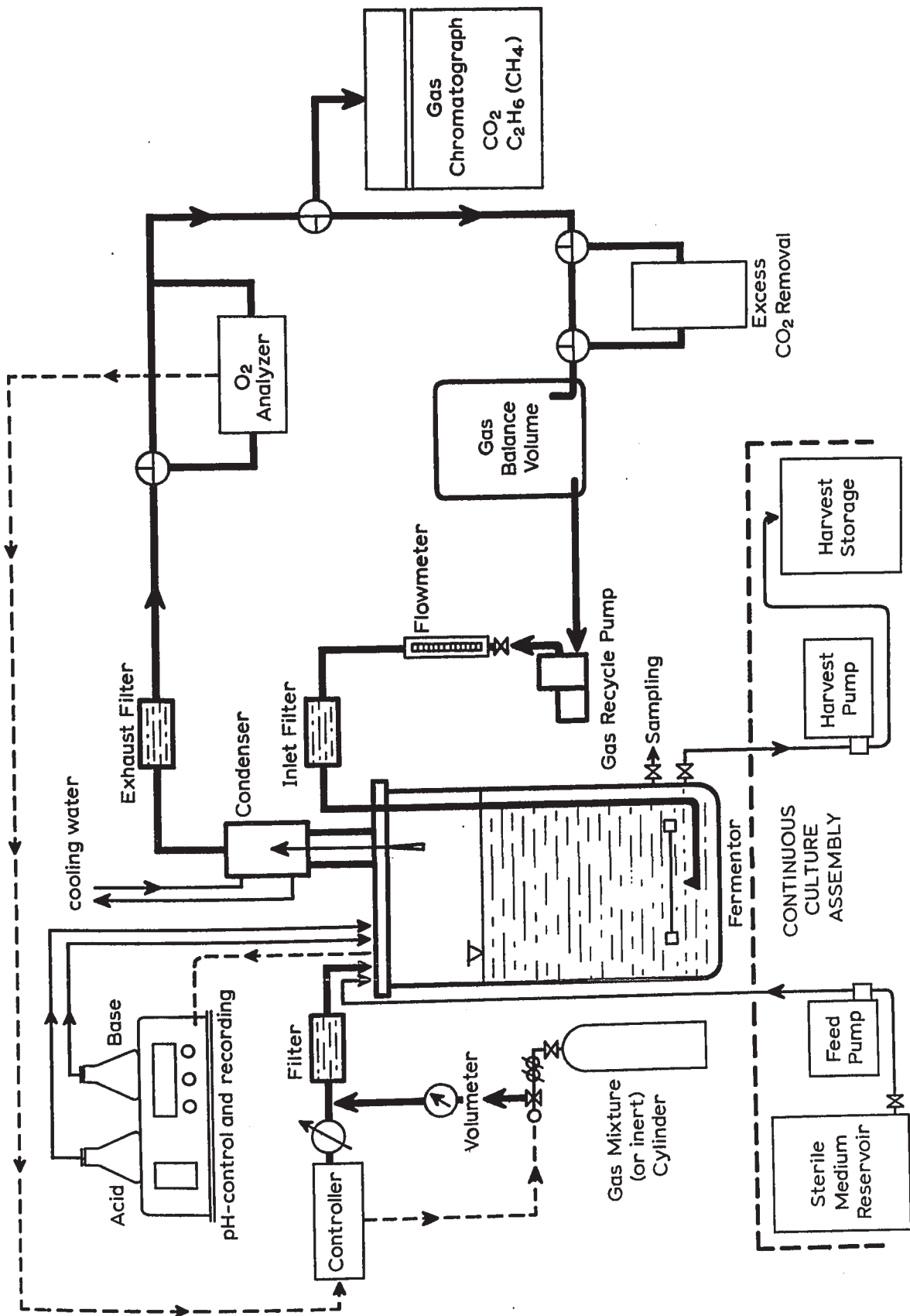
This fermentation system was used for batch (Chapter X) as well as for continuous culture experiments (Chapter XI - Continuous Culture III).

Oxygen concentration was measured by a paramagnetic oxygen analyzer. Ethane, methane and CO<sub>2</sub> concentrations were measured by gas chromatograph.

The pressure controller was used for the experiment where oxygen was allowed to become limiting (Chapter X - Experiment 1). Nitrogen was added in this experiment to balance the decreasing pressure in the system.

Premixed oxygen-ethane mixture (2:1) was added to the system (Chapter X - Experiment 2) as the gases were utilized in order to maintain their concentrations.

FERMENTATION SYSTEM WITH GASEOUS PHASE, RECYCLE, ANALYSIS AND CONTROL



## F I G U R E 1 2

## CONTINUOUS CULTURE - EXPERIMENT II ARRANGEMENT

28 l New Brunswick Scientific model CMF-128S fermentor in continuous culture arrangement with feeding and harvesting pumps.

Fresh aqueous supporting medium was prepared, sterilized and aseptically stored in the 25gal. tank on the right. Harvested broth was collected in a glass vessel and periodically filtered to recover the biomass.

NBS model pH-152 controller (left) was employed.

## F I G U R E 1 3

CONTINUOUS CULTURE - EXPERIMENT III ARRANGEMENT  
WITH RECYCLE OF GASEOUS PHASE

VIRTIS 12 l glass magnetically bottom-driven fermentor is shown with continuous culture accessories.

NBS pH-151 automatic pH controller and oxygen meter with recorder-controller are behind.

Gas chromatograph, a volumetric gas mixture addition meter with a gas storage cylinder are shown (right).

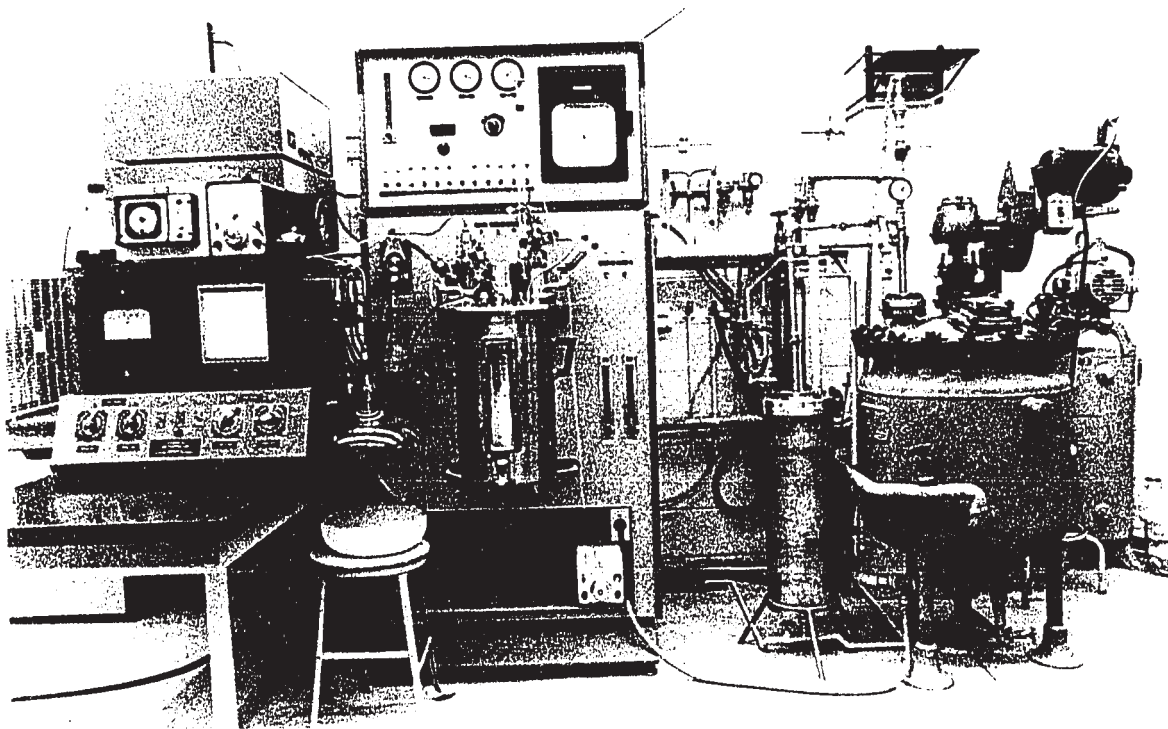


FIGURE 12

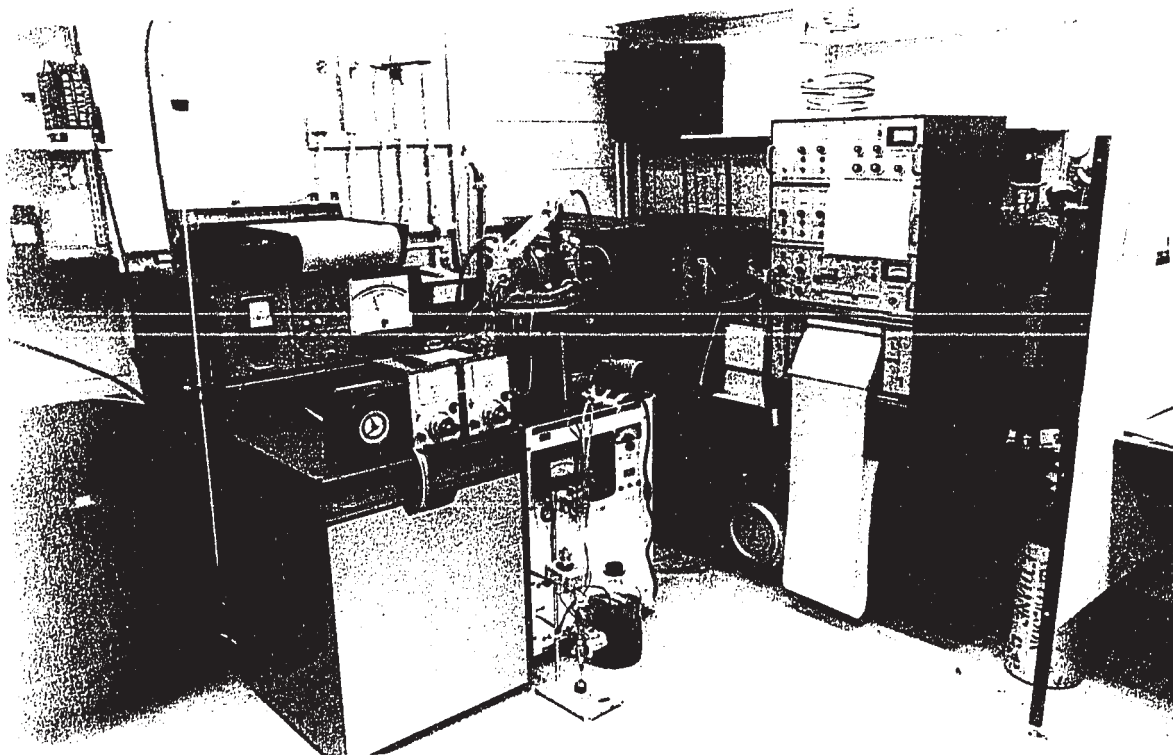


FIGURE 13

(National Cylinder Company).

The total volume of recycled gases was 25 l. The average flow rate of 1.5 lpm could be varied by adjusting a by-pass valve on a diaphragm gas pump. Gases were passed through a sterile filter before being sparged into the liquid broth in the fermentor by a single orifice sparger. A 12 l magnetic bottom driven glass fermentor (VIRTIS Co.) was used. All connections were gas-tight and the pressure within the system measured. There were no baffles in the vessel and the 2 paddle-type 6 blade impellers were rotated at ~600 rpm. Fermentor was operated at a volume of 8 l. The pH was controlled by means of New Brunswick Scientific-Model pH 152 controller. The fermentation vessel with electrodes, inlet and outlet filters and with fresh C-medium  $[(\text{NH}_4)_2\text{SO}_4]$  was sterilized with steam in autoclave under standard conditions.

A few drops of antifoam Hodag-F28 were added to the medium to prevent foaming and to give dispersed growth of Graphium.

Two Sigma-motor kinetic-clamp pumps were used for feeding and harvesting. In order to also prevent gaseous bubbles to be withdrawn by the harvest stream a bubble separator was incorporated returning gases back to the system. The dilution rate was kept constant throughout the continuous culture period, minor adjustments being completed by adjusting both feeding and harvesting pumps. Experimental arrangement is shown in Figures 13, 14, and 15.

The mineral salt medium was sterilized batchwise using steam

## F I G U R E 1 4

DETAIL OF CONTINUOUS CULTURE - EXPERIMENT III  
ARRANGEMENT

Continuous culture console with fresh medium storage vessel. Feeding and harvesting pumps and timer.

pH controller is behind with oxygen meter (right) and oxygen concentration recorder-controller (above).

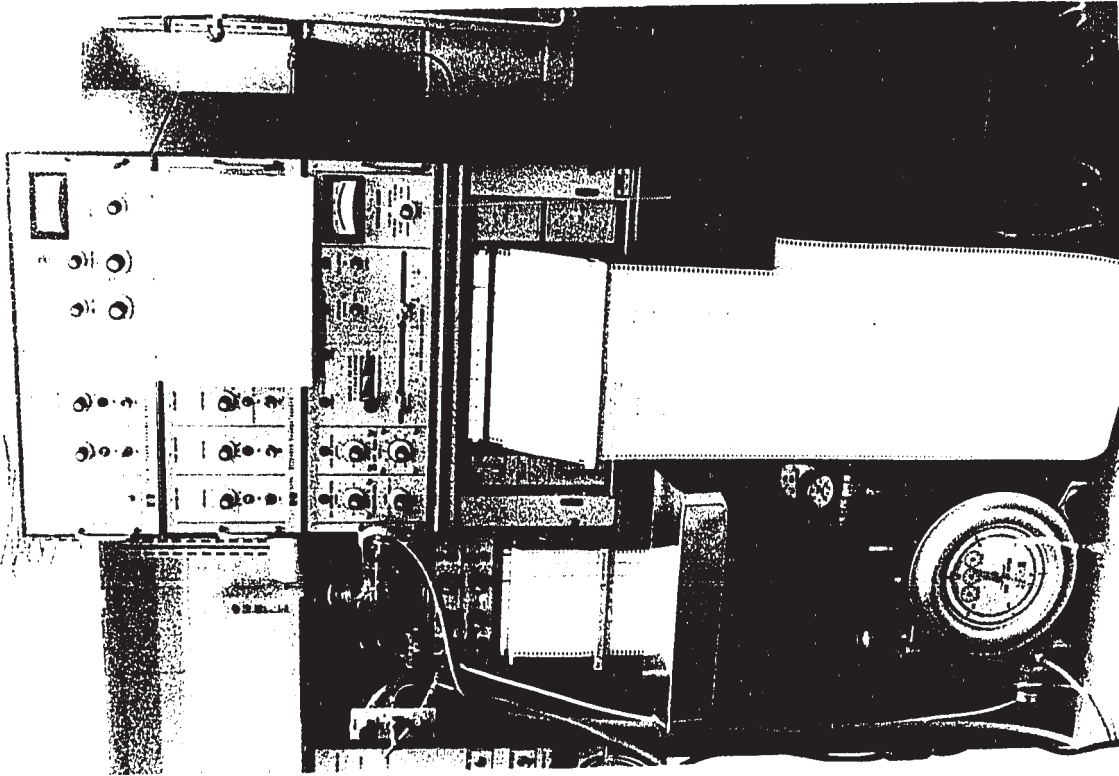
## F I G U R E 1 5

DETAIL OF CONTINUOUS CULTURE - EXPERIMENT III  
ARRANGEMENT

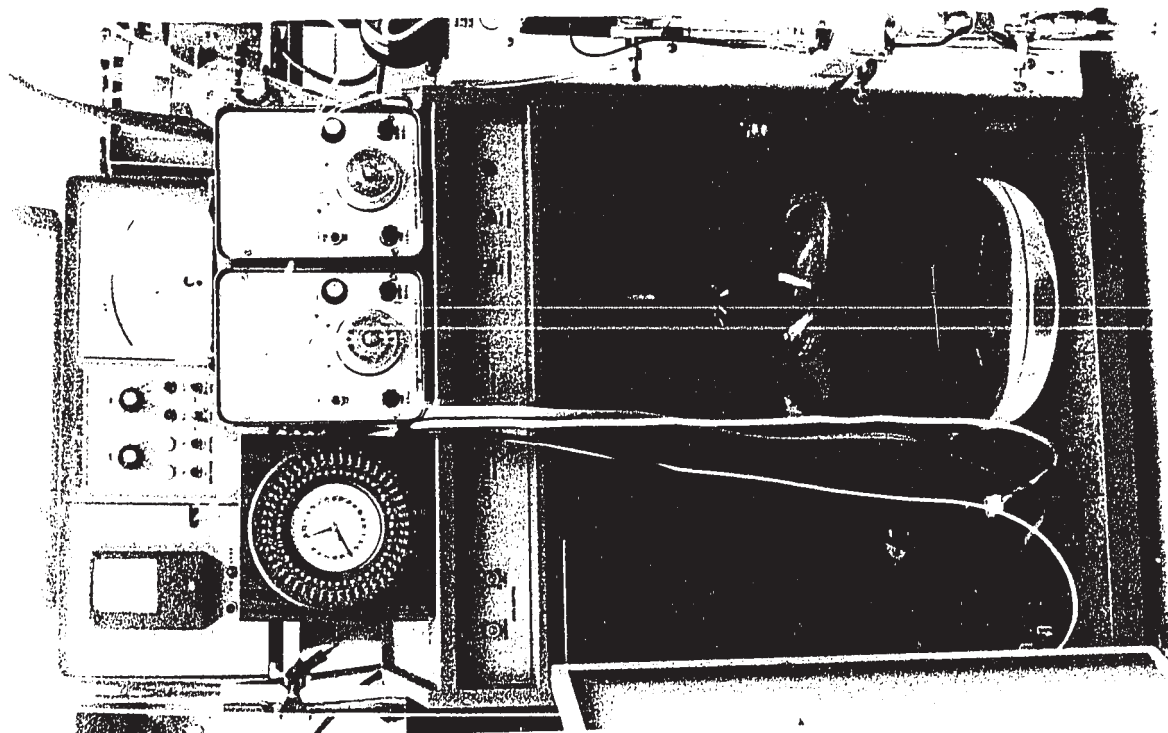
Concentrations of ethane, methane and carbon dioxide were measured with an in-line gas chromatograph. Below is the cylinder with premixed oxygen and ethane (2:1) mixture which was metered into the system. Wet-test meter measured the volume added on time basis.



F I G U R E 4 5



F I G U R E 4 6



heat. Antifoam was stirred into it in small amounts (100 ppm) prior to the sterilization. The harvested broth was collected, the tissue recovered by vacuum filtration and freeze-dried.

## ANALYSES OF GRAPHIUM BIOMASS

### CULTURE AND CULTURE CONDITIONS

The culture described herein and identified as a certain species of Graphium was grown under many nutritive conditions. It was cultivated under conditions using classical exacting aseptic conditions unless a mixed culture system was used. Under these circumstances a pure culture of Graphium sp. was used as inoculum but the culture and supporting systems were not maintained under sterile conditions. This resulted in a mixed culture system in which Graphium sp. predominated and in which only very low levels of other bacteria and fungi grew.

The low pH values used in the fermentation and the fact that not a great number of microbes can utilize gaseous hydrocarbon substrates helps reduce secondary contamination.

Secondary contaminants in the mixed culture system include a few strains of acid-tolerant bacteria and fungus Trichoderma viride.

Several different fermentations were conducted under various conditions and the microbial mass was collected. The biomass was recovered by filtration and freeze-dried. Samples of the biomass were analyzed in detail. Fermentation conditions used are described in Table 11.

TABLE 11

## DESCRIPTION OF DIFFERENT CULTURE EXPERIMENTS

EXPERIMENT NUMBER (SAMPLE)	SUBSTRATE	FERMENT. TIME [days]	TERMINAL CELL CONCN. [g/l]	CULTURE	NITROGEN SOURCE	MEDIUM SUPPLEMENTS	pH	pH CONTROL WITH	BROTH VOLUME [l]	FERMENTOR TYPE	DETAILS COMMENTS
1	NG	4	2.5	batch pure	amm. sulph.		4.3	NH <sub>4</sub> OH	10	NBS MF-14	pg 76, 171
2	NG	3	4.2	batch mixed	"		4.3	NH <sub>4</sub> OH	10	NBS MF-14	pg 76, 171
3	NG	4	5.5	batch pure	"	0.4% yeast ext. 80 ppm F28	5.5	NaOH	10	NBS MF-14	pg 76, 171
4	4% EtOH	3 7	1.4	batch pure	"		4.5 6.4	NH <sub>4</sub> OH	10	NBS MF-14	pellet-type growth pH changed after 3 days 260 rpm
5	NG	4	3.1	batch mixed	"		4.0	NaOH	300	400 l PILOT PLANT	marine-type impeller ø 3" at 1300 rpm ring sparger; pm 173
6	C <sub>2</sub> H <sub>6</sub>	7	0.8	batch pure	NaNO <sub>3</sub>		4.5 + 6.4	none	6	VIRTIS GAS RECYCLE	pg 77, 171 GASES LIMITING
7	NG	CONT. 27		contin. I pure	NaNO <sub>3</sub>		4.0	HC 1	10	NBS MF-14	pg 78, 185
8	NG	CONT. 11		contin. II pure	amm. sulph.		4.5	NaOH	18	NBS CMF 128S	pg 79, 184

C-medium was used for all fermentations

## NITROGEN DETERMINATIONS OF BIOMASS

Standard micro-Kjeldahl procedure (Furman; 1962) was carried out in Fischer Nitrogen Distillation Apparatus to determine the content of nitrogen in the tissue of Graphium. The formula for calculating the amount of nitrogen in the sample was as follows:

$$\text{mg N organic/g of sample} = \frac{(\text{ml HCl} - \text{ml HCl blank}) \times 0.14}{\text{mg of sample}} \times 1000$$

One ml of standard solution of 0.01 N HCl is equivalent to 0.14 mg nitrogen. The protein content of each sample was calculated approximately by multiplying the amount of nitrogen determined by micro-Kjeldahl method x 6.25.

## AMINO ACID ANALYSES OF GRAPHIUM BIOMASS PROTEIN

Fungal tissue was recovered from various fermentation experiments by filtration and then freeze-dried. Nitrogen content of each sample was determined by micro-Kjeldahl method.

10 mg of sample (containing approximately 50% protein) was weighed into a hydrolysis tube. After adding 10 ml of 6 N HCl the tube was flushed with nitrogen gas and tightly capped. Hydrolysis was carried out at 105°C for specified length of time from 24 to 140 hours. When hydrolysis was completed the solution was cooled to 80°C and evaporated to dryness under the stream of nitrogen. The hydrolyzed material was stored under nitrogen atmosphere in a refrigerator before further being analyzed. Prior to chromatographic analyses on automatic TECHNICON T.S.M. AMINO ACID ANALYZER the hydrolyzate was redissolved in 10 ml of

buffer solution.

In one evaluation citrate buffer was used (pH 3.25) giving the sequence of amino acids as eluted from the chromatographic column:

Aspartic Acid	Iso-leucine
Threonine	Leucine
Serine	Nor-leucine
Glutamic Acid	Tyrosine
Proline	Phenylalanine
Glycine	Histidine
Alanine	Lysine
Cystine	Tryptophan
Valine	Ammonia
Methionine	Arginine

50 nanomoles of Nor-Leucine was added to each sample as an internal standard.

To enable quantitative analysis of amino acids in the sample a standard chromatogram was obtained by applying solution containing 50 nanomoles of each amino acid and ammonium sulphate.

In another evaluation lithium buffer was used which gives the following sequence of amino acids:

Aspartic Acid	Iso-leucine
Threonine	Leucine
Serine	Nor-leucine
Asparagine	Tyrosine
Glutamic Acid	$\gamma$ -Amino Butyric Acid
Glutamine	Phenylalanine
Proline	Histidine

Glycine  
Alanine  
Cystine  
Valine  
Methionine

Tryptophan  
Ornithine  
Lysine  
Ammonia  
Arginine

Ninhydrin colour reactions were read at 570 m $\mu$  wavelength. 75 nanomoles of Nor-leucine was again added to each sample as an internal standard for recovery efficiency estimation. Standard chromatograms were prepared by applying a solution containing 75 nanomoles of each amino acid. These were compared to hydrolyzed samples of Graphium biomass.

The data calculated from chromatograms had to be corrected for some amino acids with regards to the loss during hydrolysis. Corresponding correction factors for recovery of aspartic acid, threonine, serine, glutamic acid, lysine and ammonia are dependent upon the time of hydrolysis as reported by Tristram (1966).

#### TRYPTOPHAN CONTENT DETERMINATION

Tryptophan is destroyed during acid hydrolysis of protein, particularly in the presence of carbohydrate. Methods of analysis that have been used either involve analysis of the intact unhydrolyzed protein, or the tryptophan is released by enzymic or alkaline hydrolysis. The method described by Miller (1967) based on hydrolysis with barium hydroxide, followed by precipitation of barium sulphate from an acid solution and colorimetric analysis using p-dimethylaminobenzaldehyde (DMAB) has proved

useful for the routine analysis for tryptophan in feedingstuffs and microbial tissue. The recovery of tryptophan by this method (Miller; 1967) ranges between 80 to 95%.

Hydrolysis was carried out in 125 ml glass Erlenmeyer flasks according to the method of Greene and Black (1944). Every hydrolysate was analysed at least twice in separate runs.

Neutralization with hydrochloric acid or precipitation of barium sulphate from a solution already neutralized and made slightly acidic with HCl, caused almost no loss of tryptophan. The value of recovery taken for calculations was 87% as extrapolated from values reported by Miller (1967).

#### EXTRACTION AND ANALYSIS OF LIPID FRACTION

Freeze-dried biomass of Graphium was extracted with a solvent mixture consisting of chloroform and methanol (2:1 by volume). Two types of extraction procedures were employed; both giving similar results on further analyses of extracted material.

In the first method, the tissue of Graphium was disintegrated either by sonication (2g of dry cells in the solvent mixture) for ~10 minutes followed by 12 hour extraction or by shaking (5g of dry tissue) for 72 hours with glass beads, glass powder and the solvent mixture in a tightly stoppered round bottom flask. The mixture was filtered through coarse filter paper and then through Whatman M-1 paper. The residue was re-extracted several times with  $\text{CHCl}_3$ -methanol solvent mixture in order

to free it completely from any lipid material. The extraction was repeated three times and the solvent was left each time for half an hour in contact with the residue.

In the second extraction method used, no disintegration of tissue was needed. Instead, freeze-dried finely powdered biomass (3g) was weighed into extraction thimble and extracted with the same type of solvent in Soxhlett extraction apparatus for 40 hours. In this type of an extraction unit solvent is recycled by evaporation and condensation so that fresh solvent is always in contact with biomass material in the thimble. Extracted lipid material is concentrated in boiling flask where temperature was maintained  $\sim 70^{\circ}\text{C}$ . This elevated temperature may in some cases affect the stability of the extracted lipid material.

The solid residue was either discarded or used for amino acid analysis of its dry proteinaceous fraction. Combined  $\text{CHCl}_3$ -MeOH extracts were evaporated to dryness and redissolved in  $\text{CHCl}_3$ . This latter procedure was done to remove most of the water soluble material present in the extract. This was not considered serious and no attempt was made to eliminate it completely at this stage.

The  $\text{CHCl}_3$  insoluble residue was filtered off by passing the solution through a millipore filter ( $0.45\mu$ ) and the  $\text{CHCl}_3$ -soluble fraction of the extract was used for further analyses. Quantitative estimation was completed by weighing the lipid portion after evaporating the chloroform solvent under nitrogen in vacuo.



When redissolved in chloroform again, it was further fractionated by acid-treated Florisil chromatography as reported by Carroll, Cutts and Murray (1968). Florisil was previously treated with hot HCl in order to eliminate Mg from the Si-O-Mg-OH complex (Carroll, 1963). The resulting material is chemically a silicic acid polymer, similar to commercial silicic acid. The advantage of acid-treated Florisil, compared to most commercially available silicic acid is its coarse mesh size which simplifies the technical operation and permits faster flow rates.

Individual fractions were eluted with three different solvents as follows:

Fraction:     A - chloroform  
                  B - chloroform-methanol (1:1)  
                  C - methanol-chloroform (3:1)

The individual fractions were evaporated to dryness under vacuum, weighed and analyzed by thin-layer chromatography (TLC).

#### Analytical thin-layer chromatography (TLC)

Glass plates (20 x 20 cm, 20 x 10 cm or 20 x 5 cm) were washed in sodium dichromate-sulphuric acid, rinsed with water followed by methanol and coated with Silica gel H (acc. to Stahl, E. Merck, AG, Darmstadt, Germany) using a Desaga TLC applicator. The coated plates (ca 75  $\mu$  thick) were allowed to dry for 0.5 - 1 hour at room temperature and were then activated for two hours in an oven at 120°C. The activated plates were stored in a dust-free, dry atmosphere before use. In some

cases a strip application and division of the plate by vertical lines improved the chromatogram, allowed more detailed analysis, and eliminated a concave shape of the front. When the plate was divided in this way, more material could be applied and a more accurate analysis of the individual spots obtained.

The plates were developed, either by a one- or two-dimensional technique, in chromatography jars whose interior sides were lined with a qualitative filter paper. The solvent was added to the jar 15-30 min. before the plate, to allow for equilibration. The solvents used were:

a. For one-dimensional TLC:

Solvent A:  $\text{CHCl}_3$ -MeOH- $\text{H}_2\text{O}$  (65:20:3)

Solvent B:  $\text{CHCl}_3$ -MeOH-28% ammonia (65:35:5)

Solvent C: petr. ether - ethyl ether - acetic acid (60:40:1)

b. For two-dimensional TLC:

The solvent systems used for these experiments were described by Rouser, Kritchevsky and Yamamoto (1967).

Solvent for the first dimension:

$\text{CHCl}_3$ -MeOH-28% ammonia (65:35:5)

Solvent for the second dimension:

$\text{CHCl}_3$ - acetone - MeOH - acetic acid -  $\text{H}_2\text{O}$  (5:2:1:1:0.5)

In the case of two-dimensional TLC, the plate was air dried for 10-15 min. until no ammonia could be detected by smell. Only then was the plate placed into the second solvent. After developing, the plates

were air dried, sprayed with the appropriate spray reagent and heated at 200°C if necessary.

The following specific spray was used for detection of lipid spots on thin-layer plates:

Sulphuric acid spray for carbon-containing compounds

55% (by weight) sulphuric acid in water containing 0.6% potassium dichromate was used as spray. After being sprayed, the plates were heated in an oven at 200°C for 15-30 min. Black spots appeared due to charring of carbon-containing organic material.

Gas-liquid chromatography of fatty acid methyl esters

The fatty acids in the original extracted lipid material from the tissue of Graphium, as well as in the individual fractions (neutral lipids, phospholipids) as eluted from the florisil column by corresponding solvent, were analyzed by gas liquid chromatography.

Before chromatography, the individual fatty acids were converted into the corresponding methyl esters in the following way (Carroll et al., 1968): to a dry sample, weighing about 15 mg, 5 ml of 10% acetyl chloride solution in methanol was added and refluxed for 2 hours. Methyl esters were then extracted with petroleum ether. The extraction was repeated twice and the ether extracts were combined and washed with distilled water until neutral. The sample was evaporated to dryness under vacuum, dissolved in petroleum ether and injected into the gas chromatograph.

The chromatograph used was a Beckman gas chromatograph, Model GC-45 with a flame ionization detector. The following temperatures were maintained during chromatography: injection port 220°C, column 184°C and a detector 255°C. A 10 foot long 3/16" I.D. stainless steel column was used with 5% EGSS-X on Chromosorb W as a packing material. The carrier gas was helium at a rate 60 ccm/min.

The quantitation of the individual acids from the chromatograms was done by multiplying retention time and peak height according to Carroll (1961). Fatty acid methyl ester standards (National Heart Institute, Bethesda, Md., USA) were used for testing the linearity of response in this chromatography.

## RESULTS

### CHAPTER V

#### ISOLATION PROCEDURE CONTINUOUS-FLOW ENRICHMENT SYSTEM

##### Population Changes during Enrichment Process

Ten liters of aqueous C-medium was added to a 5-gallon carboy and inoculated with 5% by volume of raw sewage taken from Greenway Pollution Control Centre, London, Ontario. A gaseous mixture of 40% natural gas and 60% air ( $v/v$ ) was fed at the rate of 4 l/min. The system was agitated at 150 rpm for 3 weeks and then the agitation was increased to 800 rpm. No fresh C-medium was fed into the system during the first 3 days. On the fourth day a nutrient input consisting of C-medium (pH 7.0) was begun. Initially the dilution rate was adjusted to replace 10 liters every 4 days (Figure 16).

During this period a mixed population of bacteria, protozoa, and fungi was observed. Bacteria and protozoa predominated in the early phases of the enrichment process. At the end of 25 days, the original sewage had been diluted 5120 times. The pH of the effluent decreased to

## FIGURE 16

CONTINUOUS MIXED CULTURE NATURAL GAS  
ENRICHMENT SYSTEM

- The biomass concentration of mixed culture of micro-organisms measured on dry weight basis,
  - pH changes in the system, and
  - the dilution rate,
- versus time during the initial 20-week period.

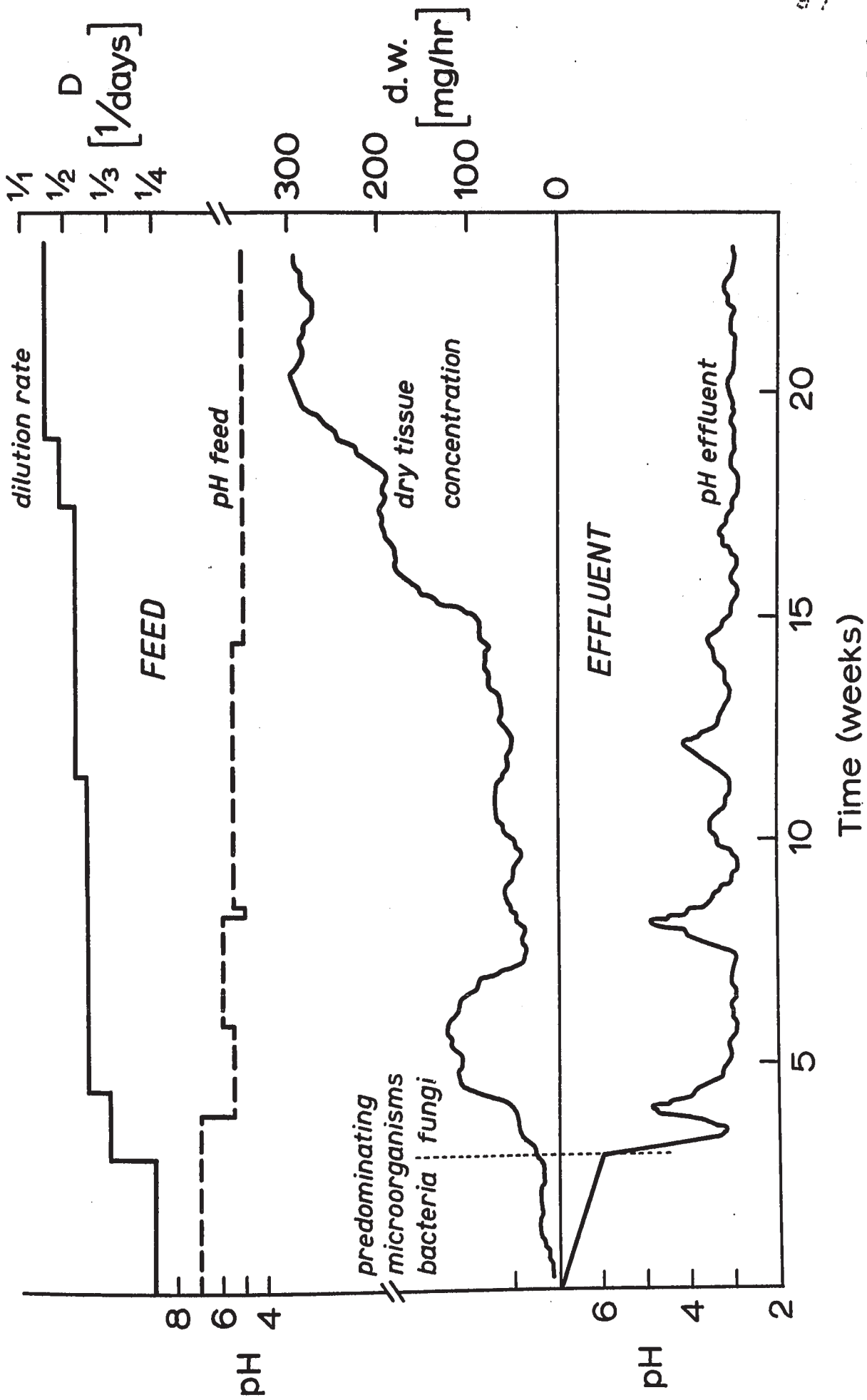


FIGURE 16

6.0 and the dry weight of the tissue in the effluent averaged 11 mg/h.

After 3 weeks, the dilution rate was adjusted to replace 10 liters volume every 3 days and at the end of 4 weeks, the pH of the input medium was decreased to 5.5. Both these changes affected the microbial population dramatically resulting in a decrease in the pH of the effluent fermentation broth to approximately 3.3. During this time period, the bacterial population decreased, the protozoa were washed out, and the fungi became predominant.

A slight increase in the effluent pH during the fourth week indicated a need for decreasing the pH of the input medium to 5.0 or lower if fungi oxidizing natural gas were to be encouraged to grow. This proved quite effective, although the pH of the effluent broth increased above 4.0 at times and even 5.0 on one occasion, it later stabilized between 2.7 and 3.5. The pH of the input medium was maintained at 5.0 for the next 4 months. The formation of fungal tissue increased to 50-70 mg/h between the 4th and 13th weeks. The bacterial population continued to decrease and two fungi increased to high levels. During the 5th to the 14th week the dilution rate was adjusted to 10-liter volume replacement every 2.5 days. The dilution rate during the 14th to 30th weeks was maintained at 10 liters per 1.7 days. The dry weight of tissue in the effluent varied from 100 to 275 mg/h. The mixed culture has been under continuous cultivation for 30 months.



## ISOLATION AND DESCRIPTION OF CULTURES OCCURRING IN THE MIXED CULTURE SYSTEM

The continuous-flow enrichment mixed culture system was operated and biomass produced for over a period of 30 months. The nutrient composition was changed several times during this period in order to study the response. Feeding nitrates as a source of nitrogen caused the pH to increase reaching values 7.5. This resulted in a more complex mixed microbial population.

When ammonium salts were used as a nitrogen source the pH decreased to values 2.7 - 3.5. This low pH range together with the use of natural gas as the only source of carbon and energy resulted in selecting for microbes which can reproduce under these selective conditions.

The type of population observed in the mixed culture system depended mainly upon the operating pH of the culture broth which was in turn dependent upon the source of inorganic nitrogen.

Two different fungi and one bacterial culture of importance were isolated by streaking the effluent on agar plates of C-medium and incubating the plates in enclosed desiccators supplied with 40% natural gas and 60% air.

Hyphal tip isolations were taken from developing colonies. Pure cultures were obtained and stock cultures of these strains were maintained on C-medium, natural gas, and air. Sporulating stages of fungi were studied both in liquid media and on solid media in petri dishes and in slide cultures. Of the two fungi isolated, one grows on C-medium and natural gas. This culture has been provisionally identified as a species of Graphium (Zajic et al., 1969; Appendix)

However, Graphium sp., Trichoderma, Fusarium, Epicoccum, Cladosporium, Candida and other yeast could be isolated on glucose agar. Except for Graphium sp., Trichoderma and natural gas oxidizing bacteria, isolations of other cultures were made in conjunction with a study by J. Traquair (1970).

#### Characteristics of Graphium sp.

A vigorous aerial mycelium formed on C-medium incubated in a gaseous atmosphere of 40% natural gas and 60% air for 5 days (Figure 17, Petri dish 2) and 10 days (Figure 17, Petri dish 1), thus demonstrating the use of natural gas as a carbon source. In the absence of gaseous hydrocarbons only slight growth of Graphium occurred on C-medium incubated in air (Figure 17, Petri dish 3 (control)). The agar does contain sufficient carbohydrate to support weak growth. The isolate is not dependent on natural gas for growth and several carbohydrates can be substituted as a carbon source.

On solid C-medium to which a carbohydrate was added, sucrose gave the best growth, followed by maltose and dextrose; colony diameters after 10 days were 4 cm, 2.5 cm, and 2 cm respectively. Colonies were white, becoming faintly grey with age, and growth was relatively sparse. Similar colony growth was observed on Difco malt agar, potato dextrose agar, corn agar, and Czaneck-Dox agar. On oatmeal agar a luxurious growth of aerial hyphae was produced, white at first, turning grey as sporulation increased. The margin and underside of the colony became black as the culture aged. Scattered droplets of clear exudate may appear on the surface of the colony. Cultures grown upon carbohydrate still retain the ability to use natural gas for growth on subculture.

## FIGURE 17

THE GROWTH OF GRAPHIUM SP. ON SOLIDIFIED  
C-MEDIUM AND NATURAL GAS - AIR MIXTURE

Petri dish 1 : Incubated for 10 days

Petri dish 2 : Incubated for 5 days

Petri dish 3 : Control - incubated for 10 days  
on air only.

Initial pH of the agar plates was 4.5

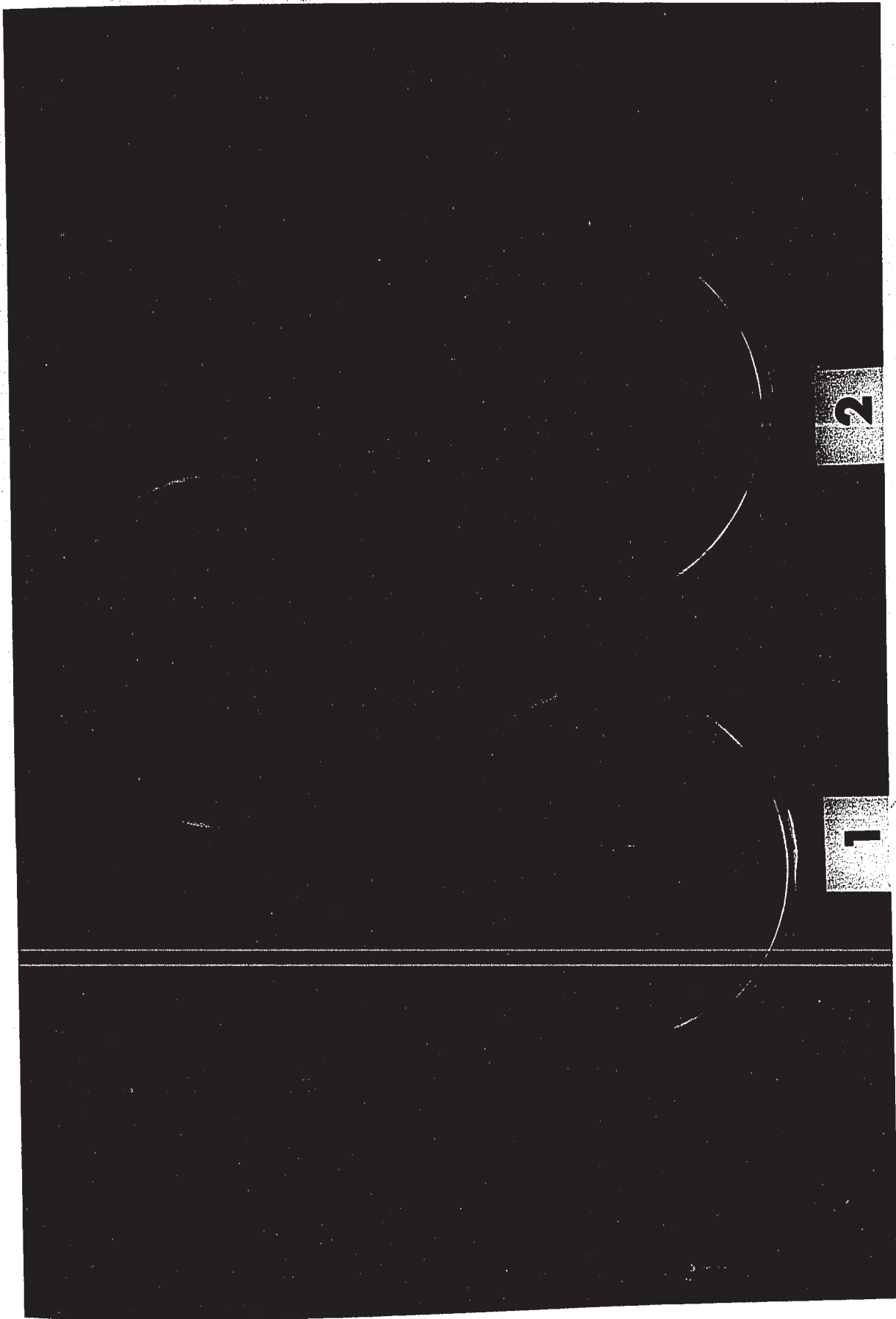
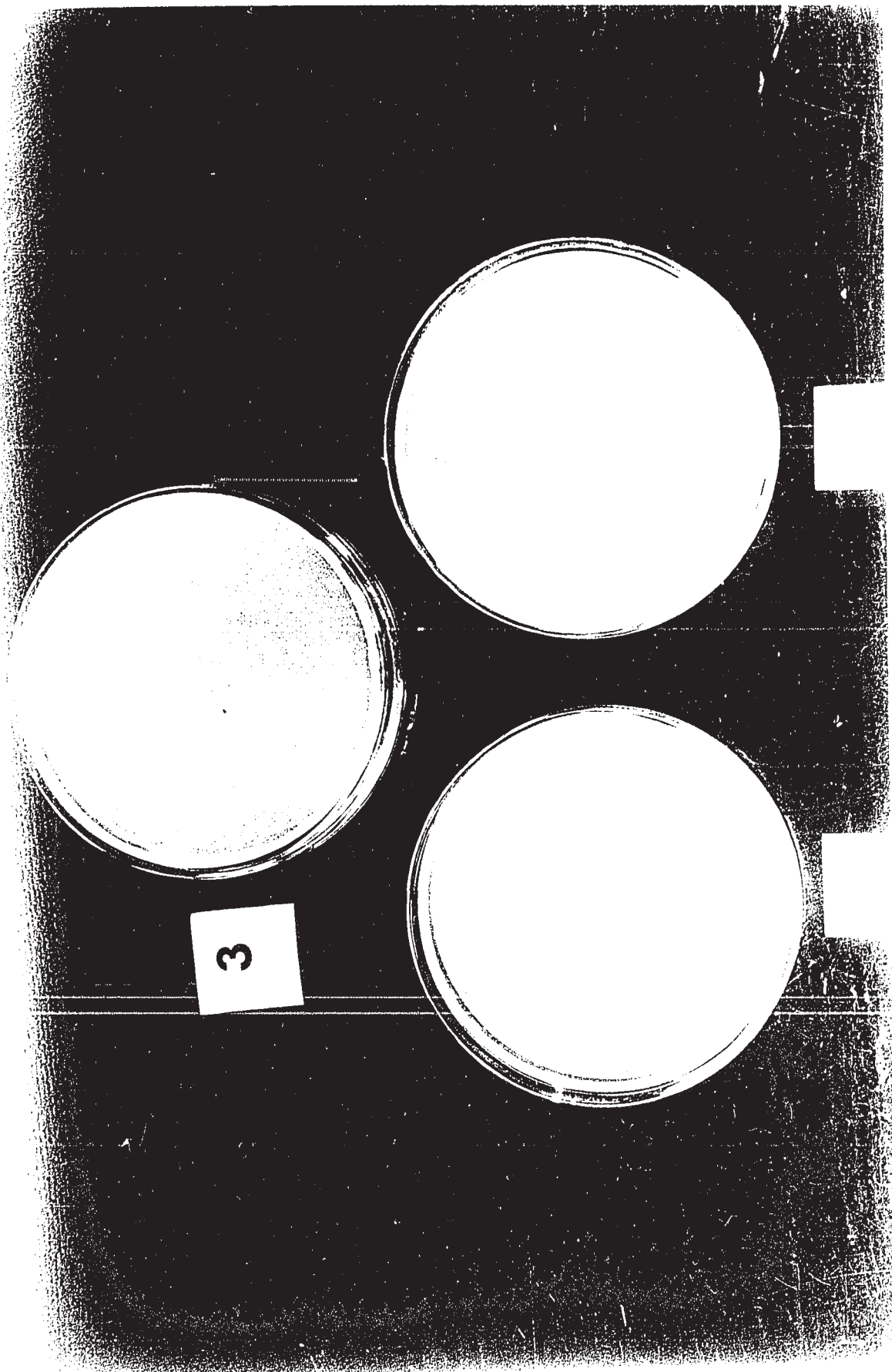


FIGURE 17





3

In submerged culture (shake flasks) with natural gas as a sole source of carbon *Graphium* sp. forms little light grey-greenish pellets ~ 1-3 mm in diameter. On microscopic observation hyphae appear to be septate and highly vacuolated. Great numbers of conidia are produced which break off from the parent hyphae very easily in this type of culture due to mixing shear forces.

The morphology of this fungus and its taxonomic relationships have been described in greater detail by Dr. A. M. Wellman (Appendix; Zajic et al., 1969).

#### Trichoderma

The second fungus isolated and identified as Trichoderma viride Link ex Fries did not grow on natural gas under the test conditions used. It probably used metabolites produced by Graphium or the methane oxidizing bacterium. The relationship may be either symbiotic or commensal. This second fungus grew well upon nutrient agar and Czapek-Dox media but only sparsely on C-medium agar without an added carbon source. Addition of a gaseous atmosphere of 40% natural gas and 60% air did not affect growth on C-medium agar in any way.

The Trichoderma strain and an acid-tolerant bacterium have co-existed in the continuous natural gas enrichment system with Graphium for over 30 months. In liquid culture under these conditions the mycelium of Trichoderma is quite small in diameter and is atypical. Its morphology changes upon cultivation on standard carbohydrate media. The hyphal diameter increases and typical conidiophores and conidia are formed. The relation-

ship of the three microorganisms discussed is probably commensal rather than symbiotic. They comprise a mixed microbial system and the relationship of these microorganisms was not studied in detail. A number of intermediates such as methanol, ethanol, formaldehyde, acetaldehyde, formic acid, and acetic acid are formed during the oxidation of natural gas compounds ( $\text{CH}_4$  and  $\text{CH}_3\text{CH}_3$ ) and it is not known which of the oxidative intermediates of natural gas is used by the Trichoderma.

#### Protozoa and Rotifers

In the initial population of the system some protozoans were recorded. These ciliates were identified as Colpoda and Paramecium (Dr. C. F. Robinow - personal communication). They were assumed to be feeding on bacteria and ceased to be detected when the pH in the system decreased to pH 3.

A rotifer resembling Hydatina could also be observed in the system when pH was higher than 5.5. Bacteria and fungal spores served as a food source for this metazoan.

Presence of these organisms could result in marked decrease in population of primary microorganisms. Lower optimal pH maintained in the system prevented this from happening.

#### Bacteria

It was observed that certain bacteria persisted in the system over a long time period. Most of these isolates could not be subcultured on C-medium with glucose. All bacterial cultures were examined for their ability to use natural gas, ethane or methane. Three isolates were found to grow well on ethane.

The acid tolerant bacterium isolated from the culture grows well on natural gas, methanol and ethanol.

#### Brief Taxonomic Description of Acid-tolerant Bacterium

The organism is a gram negative rod, sometimes slightly bent, in older cultures nearly spherical. Dimensions of single cells are approximately 0.5 to 0.8 by 1 to 3 microns, although pairs or even chains of cells occur.

The cells are highly motile in young as well as in old cultures, possessing a single polar flagellum. They can be cultivated in liquid culture or on the surface of supporting agar medium in presence of methane, natural gas or higher gaseous hydrocarbons.

Growth in liquid mineral salt medium which is shaken during the cultivation period is either dispersed or clumpy. A tendency towards dispersed growth was noted. The culture gives a light yellow to pink turbidity throughout the medium with accumulation of sedimented cells or clumps if allowed to settle. Colourless cultures are found. Polysaccharide synthesis varies with the isolate.

Colonies on agar media are usually very light yellow to orange in colour. They are raised, whole, circular, convex, smooth and range from 0.5 to 3 mm in diameter. Colonies have a mucoid consistency.

These features resemble very closely the description of Pseudomonas methanica (Methanomonas methanica) as given in Bergey's Manual of Determinative Bacteriology (1948) and in Index Bergeyana (1966).

From typical strains of Pseudomonas methanica this particular culture described above differs by persisting even under highly acidic conditions at pH 2.5 for long periods of time. It grows extremely well in mixed culture in conjunction with Graphium sp. and Trichoderma sp.



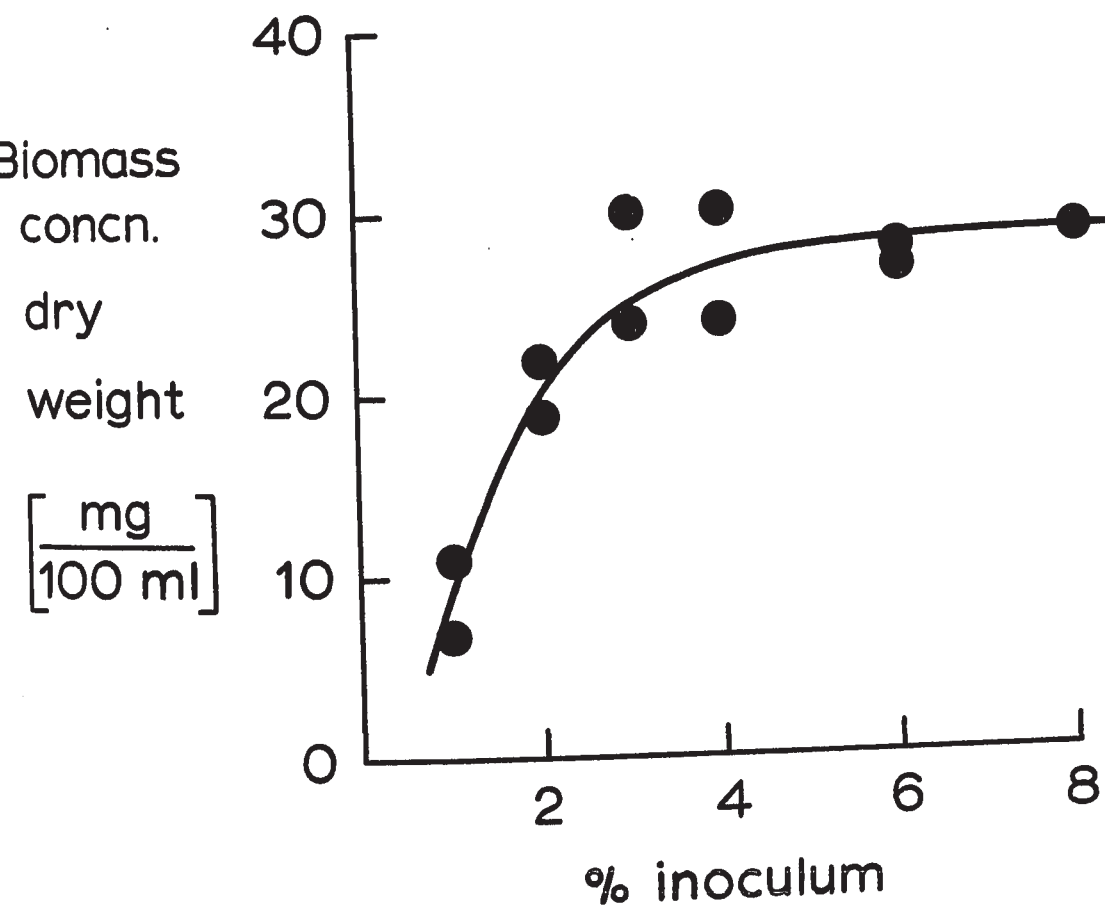
## CHAPTER VII

### BATCH GROWTH OF GRAPHIUM SP. - SHAKE FLASKS

The fungal culture, identified as a certain species of Graphium, has been purified and transferred from petri-dish agar culture into shake flasks with liquid C-medium. It was found to grow well in submerged culture and in order to obtain more information on its growth characteristics a series of shake flask studies were completed in which many important growth parameters were investigated.

#### Inoculum

The inoculum levels tested were 1, 2, 3, 4, 6, and 8% (v/v). Flasks were inoculated in duplicate and incubated in an enclosed shaker with a gaseous atmosphere of 40% natural gas and 60% air. This gas mixture was fed continuously into the closed system at 2 l/min. and the Graphium sp. was permitted to grow on the gas as it entered the shake flasks and medium. Flasks were incubated for 5 days and the dry weight of mycelium was determined. One and 2% inoculum gave 9.5 and 20.5 mg dry weight of mycelium per 100 ml, respectively (Fig.18). At 3.0% inoculum or above, a maximum of 27-29 mg dry weight of mycelium was obtained per 100 ml broth.



*Inoculum Effect.*

FIGURE 18

### Inorganic nitrogen source and pH

Several sources of inorganic nitrogen were tested. The basic composition of C-medium was held constant except for the nitrogen containing salt which was altered. The incubation period was 9 days. Three types of nitrates, potassium nitrate, sodium nitrate and ammonium nitrate were tested, as well as three types of ammonium compounds i.e. ammonium sulphate, ammonium chloride and ammonium phosphate. Initial pH of the medium was adjusted to pH 4.5. Since no pH control was applied in these shake-flask experiments a dramatic change in pH was observed during the fermentation with different terminal pH values for each of the two groups of inorganic nitrogen containing salts applied. This pH change affected in turn the concn. of mycelial tissue.

When ammonium salts are used as nitrogen sources, there is a decrease in pH during growth because of the assimilation of ammonium ion and the attendant effects of acid anions, such as sulphate, chloride or phosphate, remaining in the medium.

On the other hand assimilation of nitrates gives increase in pH due to the cationic nature of ions released. An exception is  $\text{NH}_4\text{NO}_3$  where ammonium group is preferentially utilized by the growing culture and pH consequently decreases.

Table 12 shows terminal pH values and mycelial yields obtained for various sources of inorganic nitrogen.

TABLE 12

The effect of inorganic nitrogen on growth of Graphium sp.  
on natural gas

Nitrogen source	Terminal pH	Cell concn. [mg/100ml]
NaNO <sub>3</sub>	7.0	147
	7.2	181
KNO <sub>3</sub>	6.8	130
	7.1	157
NH <sub>4</sub> NO <sub>3</sub>	2.7	31
	2.8	35
NH <sub>4</sub> Cl	2.9	30
	3.0	28
NH <sub>4</sub> PO <sub>4</sub>	3.0	27
	3.1	29
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	2.8	35
	2.8	33

Initial pH: 4.5

As seen from Table 12 all tested sources of inorganic nitrogen were utilized by Graphium sp. culture. The low concentrations of mycelial tissue when ammonium was applied were attributed to low culture pH.

The pH change could be controlled to some extent by the buffering action of the medium or if possible, by adjusting the carbon and nitrogen sources. The most effective means of controlling pH is to apply automatic pH control which was done in fermentor experiments. Results are reported in another chapter (VIII).

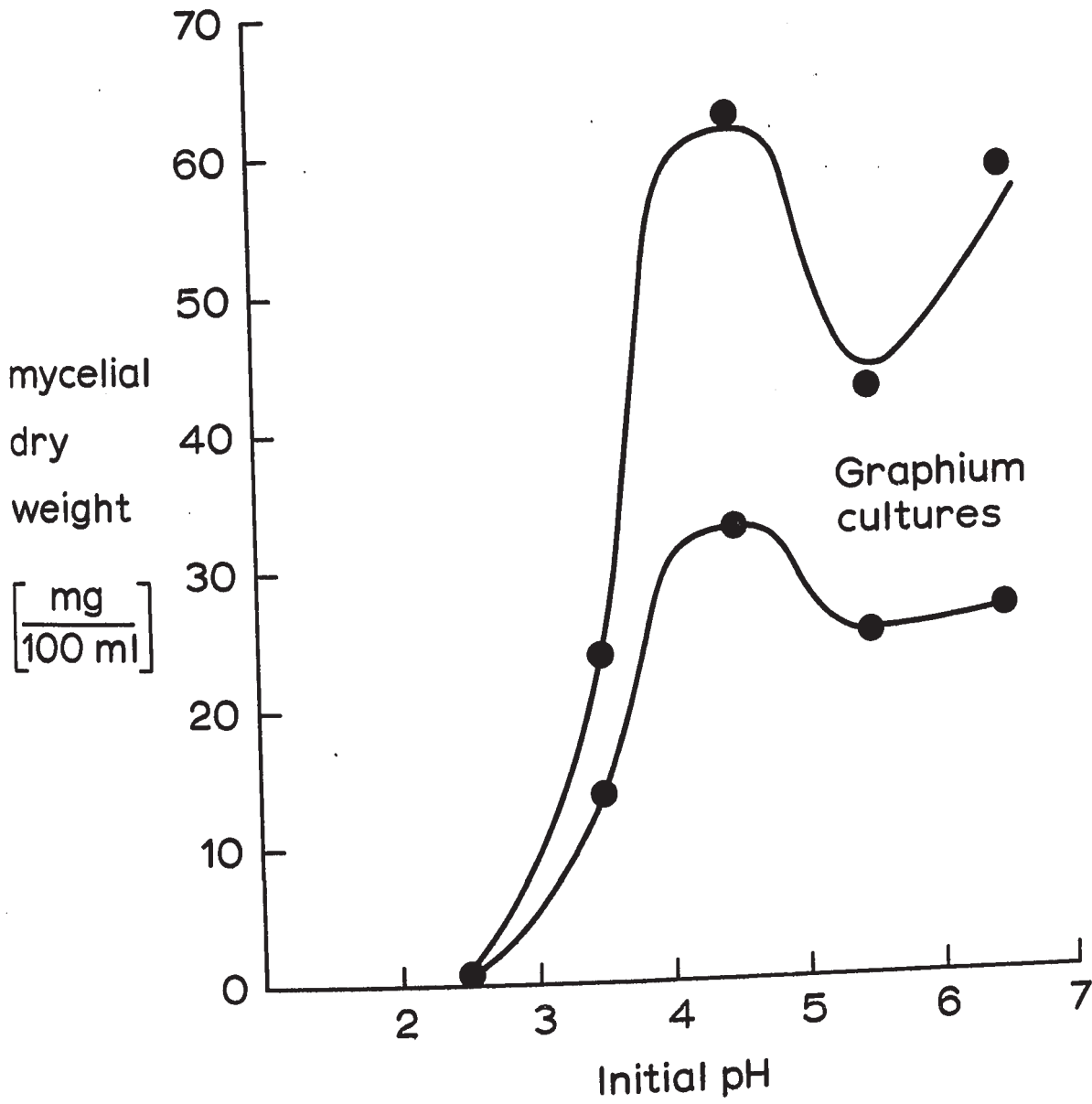
In further shake-flask experiments ammonium sulphate was used as nitrogen source since it gave good dispersed growth and a low culture pH which is restrictive to growth of secondary contaminants.

#### Optimal pH in shake flasks

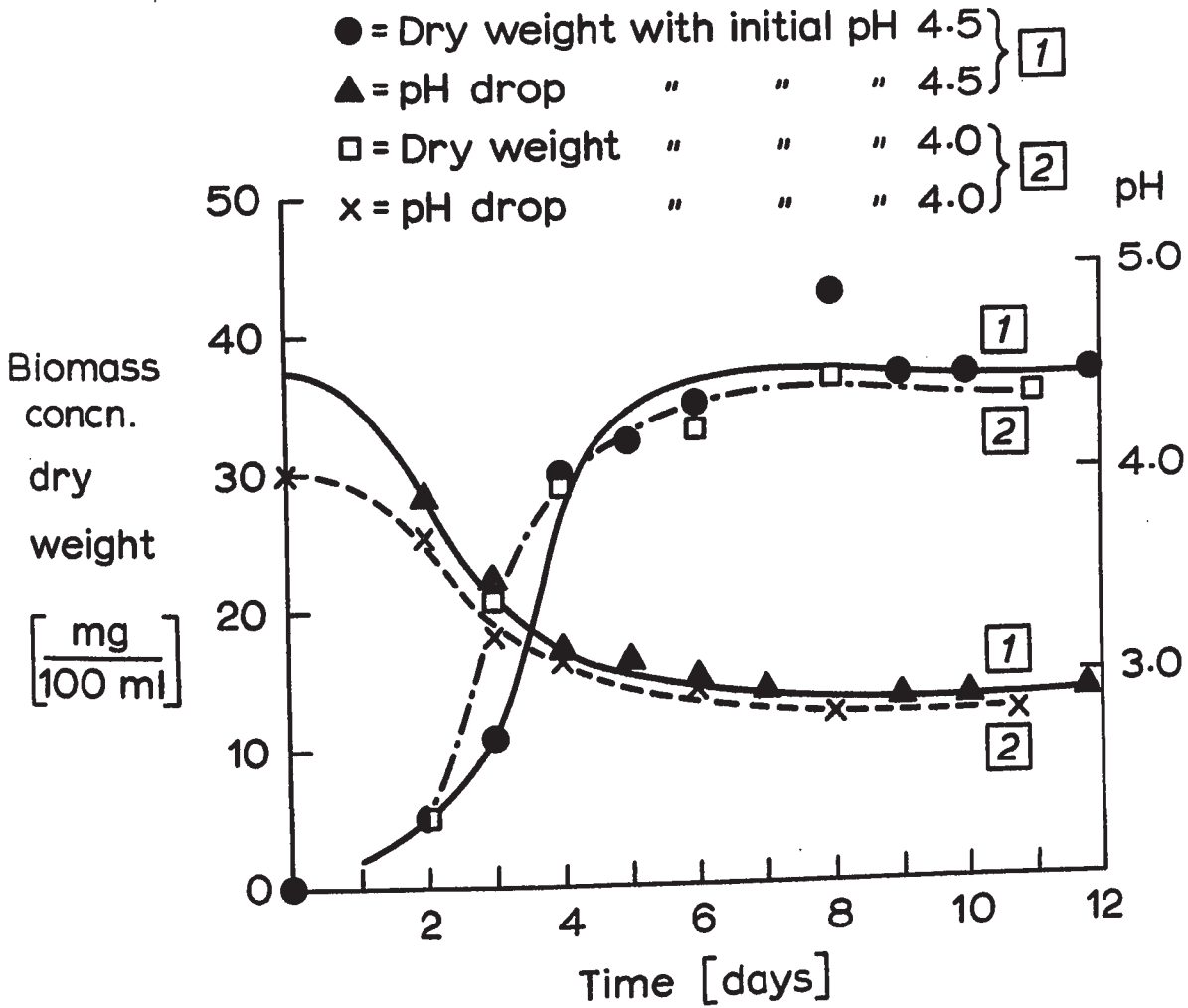
The optimal pH for oxidation of ethane and/or natural gas by the Graphium sp. on C-medium in shake flask culture was determined. The pH of the mineral salts broth of C-medium was adjusted to 2.5, 3.5, 4.5, 5.5, and 6.5. The results in Figure 19 show clearly that the optimal initial pH is 4.5. At this pH, a concn. of 33 mg dry weight of mycelium was obtained per 100 ml of medium. The terminal pH of the broth in this flask was 3.1. No growth occurred at pH 2.5. The pH range for growth on natural gas of the Graphium sp. under the condition tested varies from 3.5 to 6.5. The pH optimum and range can be extended upward by using nitrate rather than ammonium salts as a source of nitrogen.

#### Growth curve

The rate of mycelial synthesis on C-medium has been determined in shake flasks fed a mixture of 40% natural gas and 60% air. Two series of flasks were utilized. In one series, the pH was adjusted to 4.5, and in the second, to pH 4.0. The rate of mycelial synthesis was followed over a period of 8 days (Fig. 20). A standard plot of mycelial synthesis vs time gives a typical sigmoid curve. The lag phase is close to 2 days, followed by accelerated or exponential growth between 2 and 4 days. The stationary phase commences at the end of 4 days. During logarithmic



*Initial pH Effect on Growth.*



*Growth Curve and pH drop.*

FIGURE 20

growth in batch systems, the rate of mycelium synthesis is 7.5 mg/1 hr. The pH decreased from 4.5 to 3.2 in one series and from 4.0 to 3.1 in the second series at the end of 4 days. Further decreases in pH are minimal as the growth phase enters and proceeds through the stationary phase. Better growth was obtained at an initial pH of 4.5 than at 4.0. However, the same general growth response was observed under both conditions.

### Carbon Dioxide effect

To test the effect of CO<sub>2</sub> on the Graphium sp. culture, closed ecological flasks were adjusted on a volume basis to 20% ethane, 20% O<sub>2</sub>, and to concentrations of 0.0, 2.0, 6.0, 12.0, 20.0, and 30.0% CO<sub>2</sub> with the balance of the gaseous atmosphere added as He. In flasks where no CO<sub>2</sub> was added, little growth was observed (Fig.21.). It has been observed that the lack of CO<sub>2</sub> in the early stages of the fermentation prolongs the lag phase.

At all other levels of CO<sub>2</sub> tested, the concn. of mycelium increased to greater than 7.5 mg/1 hr., with the highest tissue concn. observed in flasks adjusted initially to 2.0 and 6.0% CO<sub>2</sub>.

### Methane and Ethane oxidation

The oxidation of pure methane and ethane by the species of Graphium was tested. Closed ecological flasks containing C-medium were evacuated and 50% air and either (A) 50% ethane, (B) 50% methane, or (C) 25% ethane and 25% methane were metered into the vessels. Flasks were inoculated and gas chromatographic analysis completed at 0, 5, 9, 15,

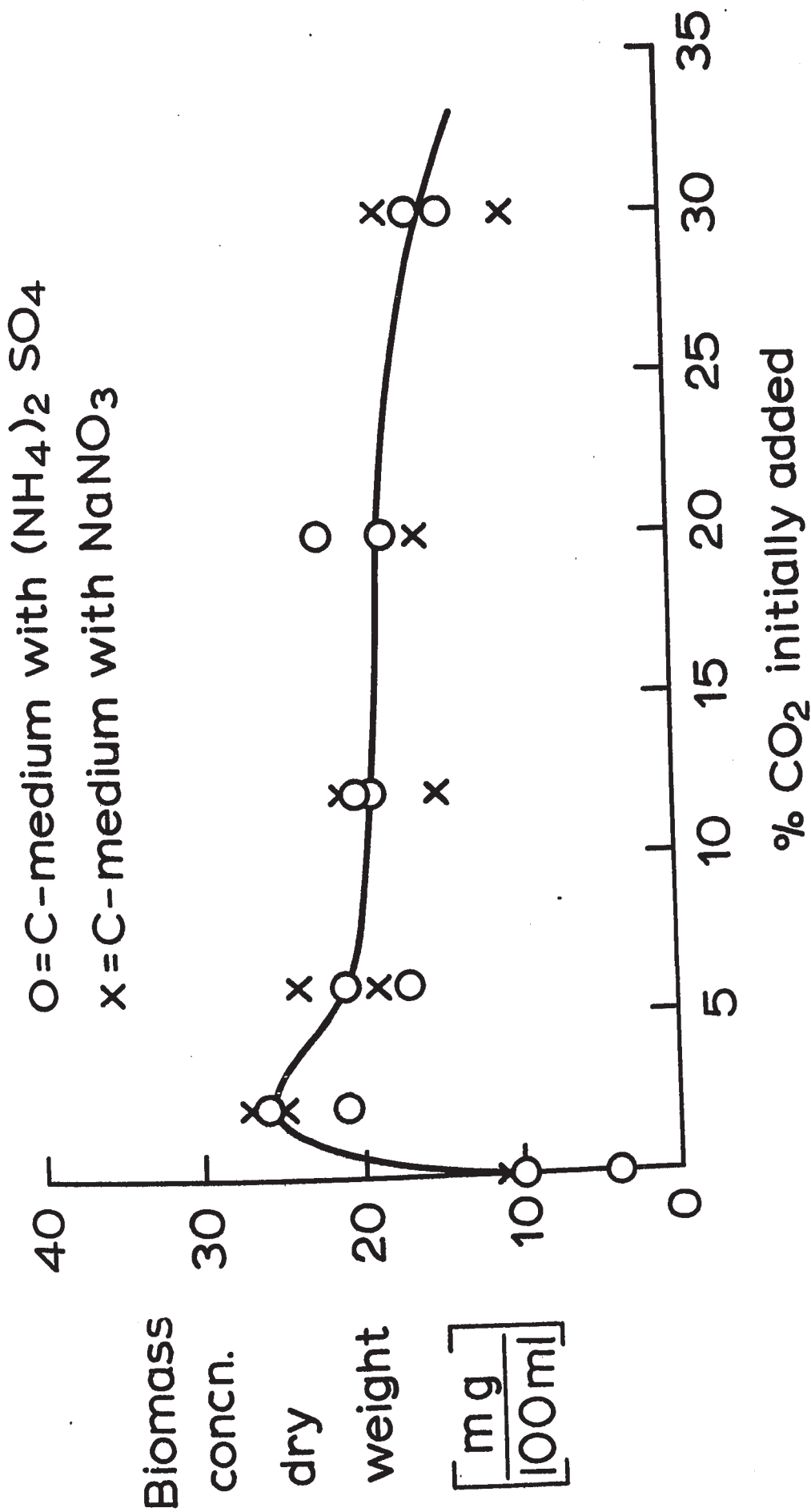


## F I G U R E 2 1

THE EFFECT OF INITIAL ADDITION OF CO<sub>2</sub>  
ON THE GROWTH OF GRAPHIUM SP. CULTURE

Graphium sp. was incubated in submerged culture on 20% ethane and 20% oxygen. Balance of the gaseous phase was added as helium.

The culture was tested in C-medium with either (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> or NaNO<sub>3</sub> as a source of nitrogen.



*$\text{CO}_2$  - Effect on Growth.*

FIGURE 21

TABLE 13  
Utilization of methane and ethane by the Graphium sp.

	Gaseous Mixture % by Vol.	days	Peak Area (Dimensionless Units)				Terminal pH	Mycelium Dry wt. mg/100 ml
			0	5	9	15		
A <sup>a</sup>								
CH <sub>3</sub> CH <sub>3</sub>	50	126	128	123	109	105		
CO <sub>2</sub>	0	0	3	5	53	60	3.4	18
B <sup>a</sup>								
CH <sub>4</sub>	50	40	41	39	39	39		
CO <sub>2</sub>	0	0	0	0	0	0	4.5	2.0
C <sup>a</sup>								
CH <sub>4</sub>	25	75	75	72	71	70		
CH <sub>3</sub> CH <sub>3</sub>	25	110	105	89	80	76		
CO <sub>2</sub>	0	0	19	68	80	64	3.2	25

<sup>a</sup>All flasks in A, B, C received 50% air to balance out the gas phase.

C-medium, initial pH 4.5.

Experiment continued for 20 days.

and 20 days on the gaseous phase of each flask. In test A, in which ethane and air were added, ethane utilization (Table 13) was easily demonstrated. The ethane in terms of peak area (dimensionless units) decreased from 126 to 105 in 20 days. During this period, 18 mg of mycelium were synthesized.

In the group of B flasks, pure methane was tested alone with air and no oxidation was observed. Concurrent with this observation, no increase in mycelial synthesis occurred. This is not true under all conditions, e.g., in group C flasks, where mixtures of methane, ethane, and air were added. In the presence of methane, greater amounts of ethane were utilized as was shown by the decrease from 110 to 76 units of peak area. The methane level also decreased from 75 to 70. How ethane influences methane oxidation is not known. It could be an additional example of co-oxidation in which the culture only oxidizes methane in the presence of an exogenous substance such as ethane. Whether the methane oxidized in the presence of ethane is utilized as a source of energy for growth or whether it is co-oxidized will have to be ascertained. In group C, a high concn. of mycelium was obtained along with high yields of  $\text{CO}_2$ .

#### Oxygen and ethane proportions

Various mixtures of  $\text{O}_2$  and ethane were used to determine the gaseous mixtures that gave the highest utilization of ethane. In one experiment, the ethane was adjusted to either 10 or 20% volume and the  $\text{O}_2$  was adjusted to 50, 40, and 30%, respectively at each level. Nitrogen

was added to balance out the gaseous mixture to 100% by volume. A relatively high per cent of  $O_2$  is required for good oxidation of ethane (Table 14). The highest per cent of ethane oxidized was 91% in flasks where the initial volumes of  $O_2$  and ethane were 50% and 10%, respectively. Under these latter conditions, ethane was completely depleted. As oxygen becomes more limiting, ethane oxidation becomes slower. This was observed at 10% ethane and 30%  $O_2$ . At 20% ethane and 50%  $O_2$ , a 44% decrease in ethane oxidation was observed.

TABLE 14

Ratio of  $O_2$  to  $CH_3CH_3$  required for growth of the Graphium sp.

% $O_2$ by Vol.	% $CH_3CH_3$ by Vol.	% $CO_2$ Produced	% Utilization of Ethane
50	10	10	91
40	10	6.4	72
30	10	4.3	45
50	20	4	44
40	20	3	19
30	20	1.0	5

Balance of gas in ecological flask was nitrogen. Experiment continued for 16 days. C-medium, initial pH 4.5.

The ratio of oxygen and ethane in the fermentation is a little more complex than the simple presentation shown in Table 14. Under certain conditions, high amounts of oxygen are inhibitory to hydrocarbon oxidation.

### Air and natural gas proportions

Closed ecological flasks were used to test the proportions of air and natural gas and their effect upon mycelial tissue synthesis. The experiment was run in duplicate. Metered amounts of both air and natural gas were pumped into 500 ml Erlenmeyer flasks with 100 ml of C-medium with an initial pH 4.5. The incubation period was 8 days. Table 15 shows the amounts of mycelial tissue synthesized during this period and the terminal pH for each group of flasks. The pH decrease during growth as shown in terms of the terminal pH is proportional to the amount of tissue synthesized.

TABLE 15

Air and Natural Gas Utilization by Graphium sp.

Group	1	2	3	4	5	6*
Air	25	40	50	60	75	40
Natural Gas	75	60	50	40	25	60
Terminal pH	3.18	3.21	3.28	3.55	3.61	2.8
Tissue concn. [mg/100 ml]	15	13	11	9	6	43

\*control group with gases fed continuously in excess.

It is obvious that in each group of flasks some component of the gaseous atmosphere became limiting. The lower the natural gas, the lower the biomass. These data indicated that methane might not be the primary substrate in natural gas and that ethane probably is the primary substrate. Notably its concentration in domestic natural gas from London Ont. amounts ~6%. Where ethane was in excess, oxygen became limiting for growth, this

being the case for group No. 1 and 2.

If both gases are fed continuously and the culture is allowed to grow ad libitum the amount of mycelial tissue synthesized is considerably higher which is seen in the control group, where a gaseous mixture consisting of 60% natural gas and 40% air (~3.6% ethane and 8.2% oxygen) was made available. The proportion of oxygen to ethane utilized for growth is more accurately determined in later experiment described in Chapter X on batch culture with recycle of the gaseous phase.

#### Methane co-oxidation

As observed in one of the previous experiments (Table 13) methane is probably co-oxidized or even co-utilized in the presence of ethane. To test the effect of methane upon the growth of Graphium sp. a set of shake-flasks was inoculated with Graphium sp. A 5% inoculum was used and the initial pH was 4.5. The gaseous atmosphere metered into one series of flasks (A) consisted of 20% oxygen, 10% ethane and 0, 5, 10, 20, 30, 50, and 70% methane. Another series (B) was filled with 20% oxygen, 20% ethane and 0, 5, 10, 20, 30, 40, and 60% methane. Helium was used to balance the mixture to 100% by volume. C-medium with  $\text{NaNO}_3$  was used in all tests.

Table 16 shows the terminal pH values and average weight of mycelial tissue recovered after 14 days of culture. A calculation was also made which expresses the increase in the tissue concentration over the control.

TABLE 16  
METHANE CO-UTILIZATION

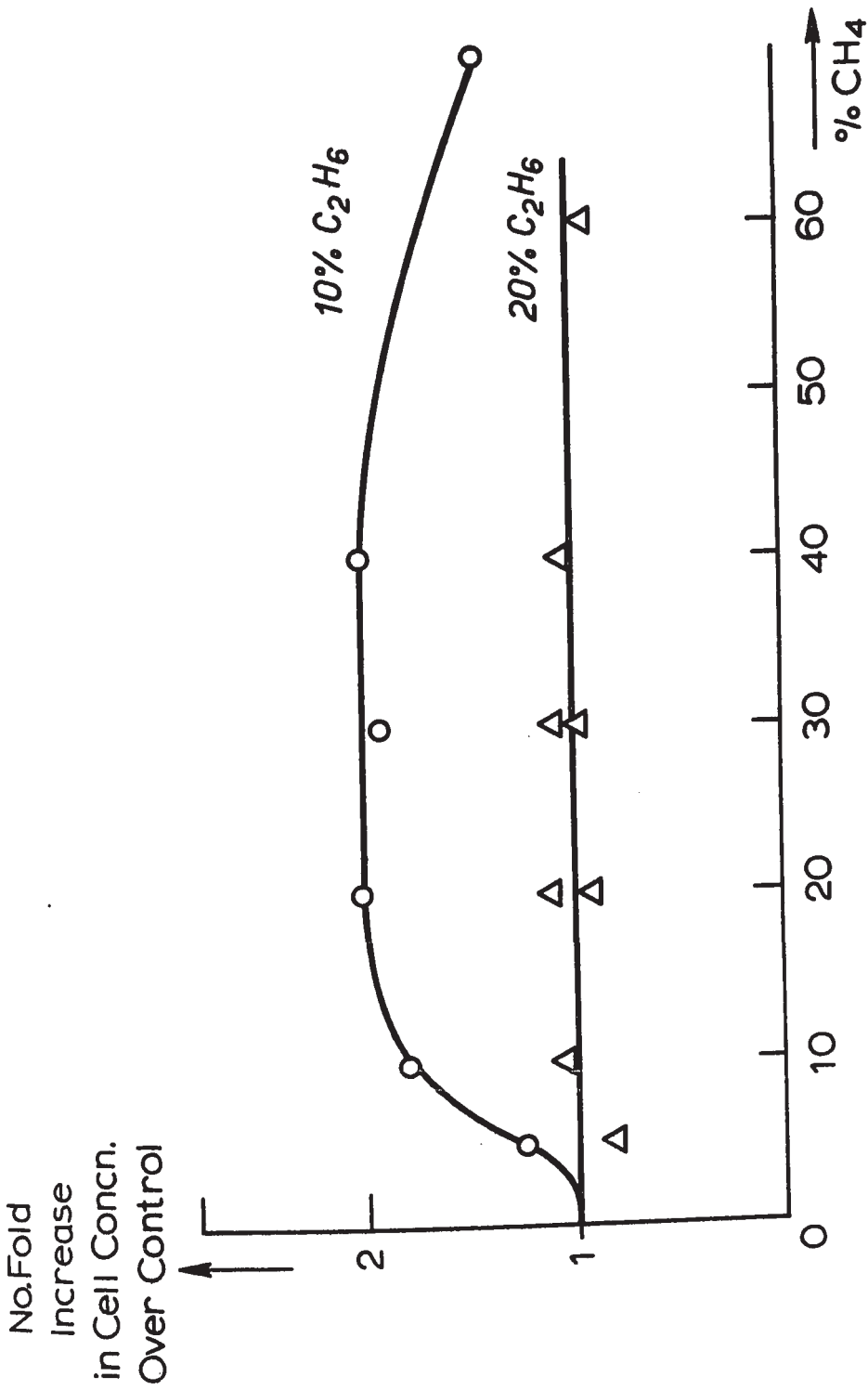
(A) *BASIC ATMOSPHERE:		20% oxygen + 10% ethane						
% CH <sub>4</sub> added	θ (control)	5	10	20	30	50	70	
Terminal pH	5.7	5.8	6.7	6.6	6.7	6.8	6.0	
Tissue d.w. [mg/100 ml]	10	12.5	18	17	17.5	19	14	
Tissue increase [fold]	1	1.25	1.8	1.7	1.75	1.9	1.4	
(B) *BASIC ATMOSPHERE:		20% oxygen + 20% ethane						
% CH <sub>4</sub> added	θ (control)	5	10	20	30	40	60	
Terminal pH	6.8	6.9	7.1	7.0	7.15	7.1	6.9	
Tissue d.w. [mg/100 ml]	22	23	27	25	28	27	24	
Tissue increase [fold]	1	1.05	1.23	1.18	1.28	1.23	1.09	

\*Gas concentrations are % by volume  
Purity of CH<sub>4</sub> is 99.5% (Matheson C.P. grade)

It was observed that the concentration of mycelial tissue was slightly higher in flasks where methane was present. The increase in concn. over the control in series (A) where ethane was expected to be limiting would imply that methane was co-utilized by Graphium for mycelium synthesis. This is more pronounced under conditions where the primary substrate eventually becomes limiting. Where the primary substrate i.e. ethane, is increased as in series B, the increase in tissue synthesis attributed to co-utilization of methane is much decreased.

Figure 22 shows a plot of tissue concentration increase i.e. number of fold over the control, as plotted against the amount of methane added. The most pronounced effect of methane addition was observed between 10 - 50% CH<sub>4</sub>.





**METHANE CO-OXIDATION**  
*(Graphium sp. on ethane + 20% oxygen)*

FIGURE 22

This data explains why tissue concentrations obtained on natural gas are higher than those obtained on pure ethane. Whether the presence of methane enhances ethane utilization or if methane is truly co-utilized remains to be proved. A conclusive experiment conducted using  $C^{14}$  labelled  $CH_4$  and following the metabolic fate of labelled radioactive carbon has not been made. True co-utilization would be predicted if  $C^{14}$  were found incorporated into the cellular material.

#### Other gaseous hydrocarbons

Growth of the Graphium species on higher molecular weight gaseous hydrocarbons has been tested. Propane and butane were added in concentrations of 25, 50, 75% by volume to closed ecological flasks. The C-medium was used as the mineral salts supporting medium. The balance of the gas volume was made up with air. Both propane and butane are utilized as a source of carbon and energy by the culture (Table 17). At levels of 50% of propane or of butane, the concn. of mycelium was 22 and 24 mg/100 ml, respectively. This is not as high a concn. as was observed with ethane, but it certainly exceeds the controls. Higher molecular weight hydrocarbons have been tested and no growth was observed.

TABLE 17

Growth of the Graphium sp. culture on either propane or butane

Gas Mixture	% Composition (V/v)			
Propane:	25%	50%	75%	0
Air:	75	50	25	100
Mycelium $\frac{\text{mg}}{100 \text{ ml}}$	31	22	13	3(inoculum)
Butane:	25%	50%	75%	0
Air:	75	50	25	100
Mycelium $\frac{\text{mg}}{100 \text{ ml}}$	3	24	16	3(inoculum)

Conditions: C-medium, pH 4.5.  
 5% inoculum. Incubation period: 10 days  
 Mycelial weight: mg dry weight/100 ml.

### Growth of Graphium sp. on alcohols

The culture has been tested for growth on alcohols. 2% and 4% concentrations were used of methanol, ethanol, propanol and butanol respectively in C-medium. A higher initial pH seemed to be preferred by the culture for growth on alcohols so that initial pH was adjusted to 6.5.

TABLE 18

#### Growth of Graphium Sp. on Some Alcohols

Substrate	Concentration	Terminal pH	Tissue concentration [mg/100 ml]
METHANOL	2%	(6.5)	negligible
	4%	(6.5)	negligible
ETHANOL	2%	2.9	88
	4%	2.9	104
PROPANOL	2%	(6.5)	negligible
	4%	(6.5)	negligible
BUTANOL	2%	2.9	90
	4%	3.7	37

Table 18 summarizes results obtained with certain primary alcohols as growth substrates. Ethanol and butanol are readily utilized by Graphium sp. while methanol and propanol gave almost no growth. The culture apparently has some difficulty in assimilating odd carbon number low molecular weight hydrocarbons, as well as corresponding alcohols. Lower concentrations of alcohols (2%) gave greater biomass than concentrations of 4% alcohol or better.

### Growth of Graphium sp. on carbohydrates

Some carbohydrates were also tested as a growth substrate for Graphium sp. 2% by weight of glucose and sucrose respectively was mixed

into C-medium with sodium nitrate added as a source of nitrogen. The initial pH was adjusted to 4.5 and the broth was inoculated with 2% by volume of a culture of Graphium sp. The incubation period was 5 days.

Table 19 summarizes results obtained.

TABLE 19

Growth of Graphium sp. on Carbohydrates

Substrate	Air Cultivated		Air + Natural Gas Mixture	
	terminal pH	tissue concn. [mg/100 ml]	term. pH	tissue concn. [mg/100 ml]
Control (no carbohydrates)	4.5	inoculum only	6.7	70
Glucose	7.4	760	7.3	314
Sucrose	6.8	93	6.6	103

As seen in the Table 19 there was very good growth observed on glucose while sucrose gave lower mycelial tissue concentrations. When natural gas in mixture with air was present in conjunction with carbohydrates the results were different. A mixture of natural gas and air (60:40) was added continuously and ad libitum growth of Graphium permitted. Lower tissue concentrations obtained on glucose with natural gas present in the incubation atmosphere could imply that consequently lowered partial pressure of oxygen resulted in less growth. However growth on sucrose in presence of natural gas was greater than on sucrose alone with air atmosphere. In this case probably some natural gas was utilized together with the carbohydrate, while glucose is probably utilized preferentially if present as pure substrate.

Some methane oxidizing cultures have been reported to lose the ability to grow on hydrocarbon substrate when cultivated for certain time on carbohydrate substrate (Davis, 1957).

Graphium sp. was tested for growth on natural gas substrate after being cultivated on sucrose and glucose for two subsequent culture period. It grew in a normal manner. The subculture on natural gas grown in C-medium with  $(\text{NH}_4)_2\text{SO}_4$  gave biomass concentration of 69 and 50 mg/100 ml for the cultures previously grown on sucrose and glucose respectively. The use of C-medium with  $\text{NaNO}_3$  for Graphium sp. subculture on natural gas resulted in even higher biomass concentrations of 131 and 141 mg/100 ml for sucrose and glucose grown cultures respectively.

#### Growth Factors

Beef extract and yeast extract can be added to the mineral salts medium with a resultant increase in both natural gas oxidation and mycelial growth. To evaluate results of beef extract and yeast extract, a series of experiments was conducted with and without the extracts and with and without natural gas. Natural gas (40%) in a mixture with 60% air was continuously fed into the system, and fungal cells were permitted to grow. Mycelial growth in mg/100 ml broth in the presence of natural gas and air is designated  $G_{\text{NG+A}}^{\text{Y.E.}}$  and in the presence of air alone  $G_{\text{A}}^{\text{Y.E.}}$ . In both cases equal amount of yeast extract is present.  $G_{\text{NG}}$  designates growth in the presence of natural gas and air without yeast extract. The number fold increase of mycelial concentration is expressed by:

$$I = \frac{G_{NG+A}^{Y.E.} - G_A^{Y.E.}}{G_{NG+A}^{Y.E.}} \quad (16)$$

$G_{NG+A}$  averages about 37 mg/100 ml broth.

Addition of 0.1% beef extract and no natural gas gave a yield of 16 mg/100 ml of mycelium of the Graphium sp. culture. Addition of natural gas increased the mycelial mass to 83 mg/100 ml, which is approximately a twofold increase in cell synthesis.

Yeast extract is far more effective than beef extract in supporting growth of the culture either with or without natural gas. The highest yield of mycelium as judged by the difference of growth in natural gas ( $G_{NG+A}^{Y.E.}$ ) and air alone ( $G_A^{Y.E.}$ ) or ( $G_{NG+A}^{Y.E.} - G_A^{Y.E.}$ ) was observed at 0.4% yeast extract. At this level of yeast extract,  $G_{NG+A}^{Y.E.} - G_A^{Y.E.}$  was 196 mg/100 ml. This was a 5.95-fold increase of mycelial synthesis over the control. Upon addition of 0.2-0.4% yeast extract, the number fold increase (I) was always greater than 4.5, but with more than 0.8% yeast extract, the "I" value began to decrease.

The plot of relationship between yeast extract addition and tissue concentration increase is shown in Figure 23. The two resultant curves are for relative number fold increase (I) and absolute mycelial tissue concentration increase over standard control cultivated on natural gas respectively. As seen from the diagram the most efficient addition is between 0.4 to 0.8% yeast extract where is the peak for relative yield increase (I) and where the slope of the curve for absolute increase becomes less.

# EFFECT OF YEAST EXTRACT ADDITION UPON THE GROWTH OF GRAPHIUM ON NATURAL GAS

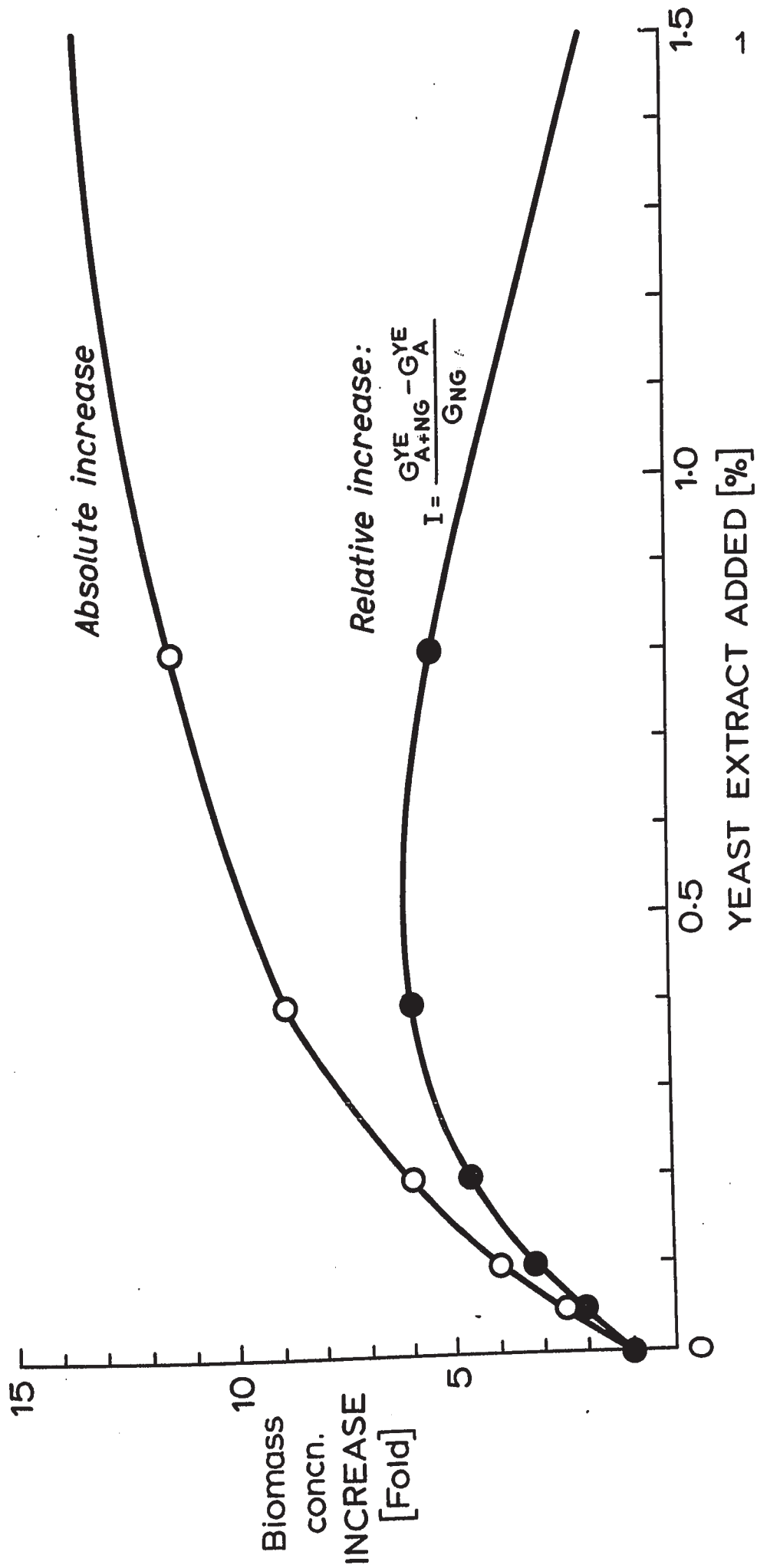


FIGURE 23



## CHAPTER VII

### UNCONVENTIONAL TECHNIQUES FOR GROWTH OF GRAPHIUM

Using gaseous hydrocarbon as a substrate of course presents numerous difficulties, which do not occur with the conventional substrates. One problem is to ensure a sufficient supply of the gaseous substrate to the organism. From physical principles of solubility of gaseous phase in liquid, there are apparently two possible ways to ensure the highest possible contact of microorganisms with the substrate at given temperature. These two are:

- increased gas-liquid mass transfer in the system;
- increased concentration of dissolved gaseous substrate and oxygen by increasing their partial pressures (or introducing pure  $O_2$  into the system)

Three different techniques were tested to study their effect upon the growth of Graphium. These were:

- a) packed bed column fermentor,
  - b) air-lift fermentor,
  - c) fermentation under increased pressure
- a) A packed bed column fermentor was designed as described in Chapter IV

in the Materials and Methods. The purpose of this experiment was to test a design and type of culture vessel and determine its effect on growth of Graphium sp. Liquid broth was pumped to the top of the fermentor and permitted to descend by gravity through the bed packed with glass beads while gaseous phase was pumped counter-currently to the liquid flow from the bottom. Broth was collected in the bottom part of the column and a certain fraction harvested, the remaining broth was recycled through the inlet port located at the top of the column. Fresh medium was continuously added at the top at rate equal in amount to that being withdrawn. The packed bed served to increase surface area for gas-liquid mass transfer. Conditions of flow had to be adjusted to avoid flooding the column.

The system was operated under non-aseptic conditions, the medium was not sterilized and the inoculum consisted of a mixed culture which had been cultivated on natural gas. Graphium sp. was the predominant micro-organism in the system. After inoculation with 5% of this mixed culture the recycle of liquid phase in the column fermentor was started. No fresh medium was added for initial 36 hours of cultivation. During this period certain deposits of biomass within the packed bed were observed. These clumps of biomass could not be washed out and continued to grow within the bed. After four days, even though fresh medium was being added, a definite filtering effect of the packed bed was observed in which biomass was trapped in the bed.

After one week operation, the packed bed became overgrown with the biomass to such an extent that no liquid would pass through. The

column was effectively plugged, the biomass and packing material creating a solid impermeable cake. This is illustrated on Figure 24. The operation was therefore terminated. Packed bed column fermentor was shown not to be effective for this type of culture which tends to grow in clumps and filaments and eventually plugs the whole system. Carefully designed three phase fluidized bed system could solve this problem but further detailed studies would be required.

b) An air-lift fermentor designed as described in Chapter IV in Materials and Methods was tested as to the effect upon the growth of Graphium sp. mixed culture. A mixture of air and natural gas was sparged into the liquid broth through a sintered glass disc located at the bottom of the column. Ascending air bubbles travelling through the inner tube caused the bulk of liquid to move in upward direction. No mechanical means of mixing were employed. Overflowing liquid broth was recycled in downward direction by descending through an annular space between inner and outer tubes. Liquid entered the inner tube again through four slots located at the bottom and directly below the sparger. The whole system was operated as non-aseptically with non-sterile medium which was inoculated with 5% of the mixed culture of Graphium sp. Approximately 12 hours after initial inoculation it was observed that some of the tissue was being trapped around the sparger, and was growing in filamentous form. This phenomenon became more pronounced as the fermentation proceeded. Growing filaments served as a base for tissue deposits which were observed in the entire bottom part of the fermentor around the supporting structure

Packed bed fermentor plugged with biomass

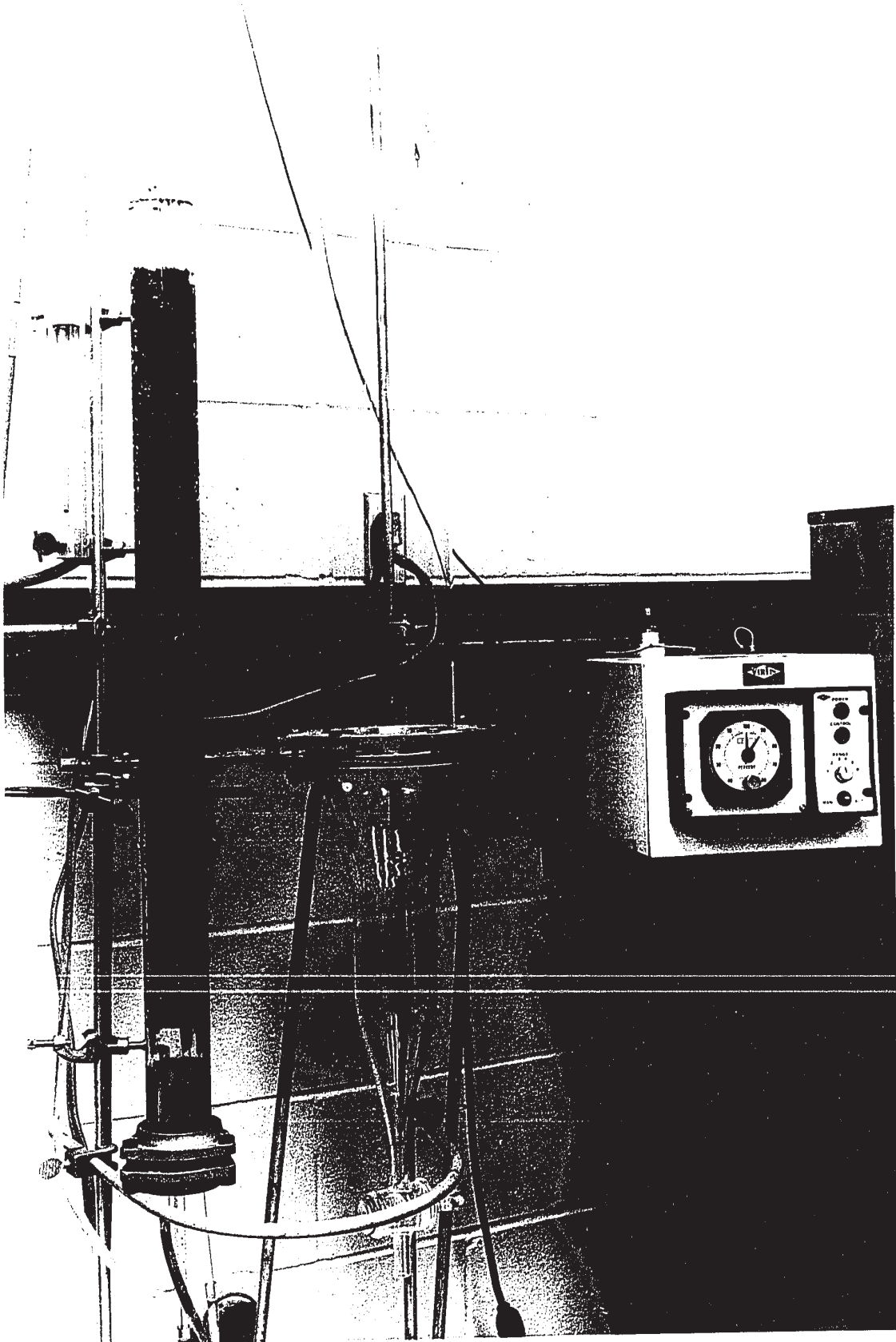


FIGURE 24

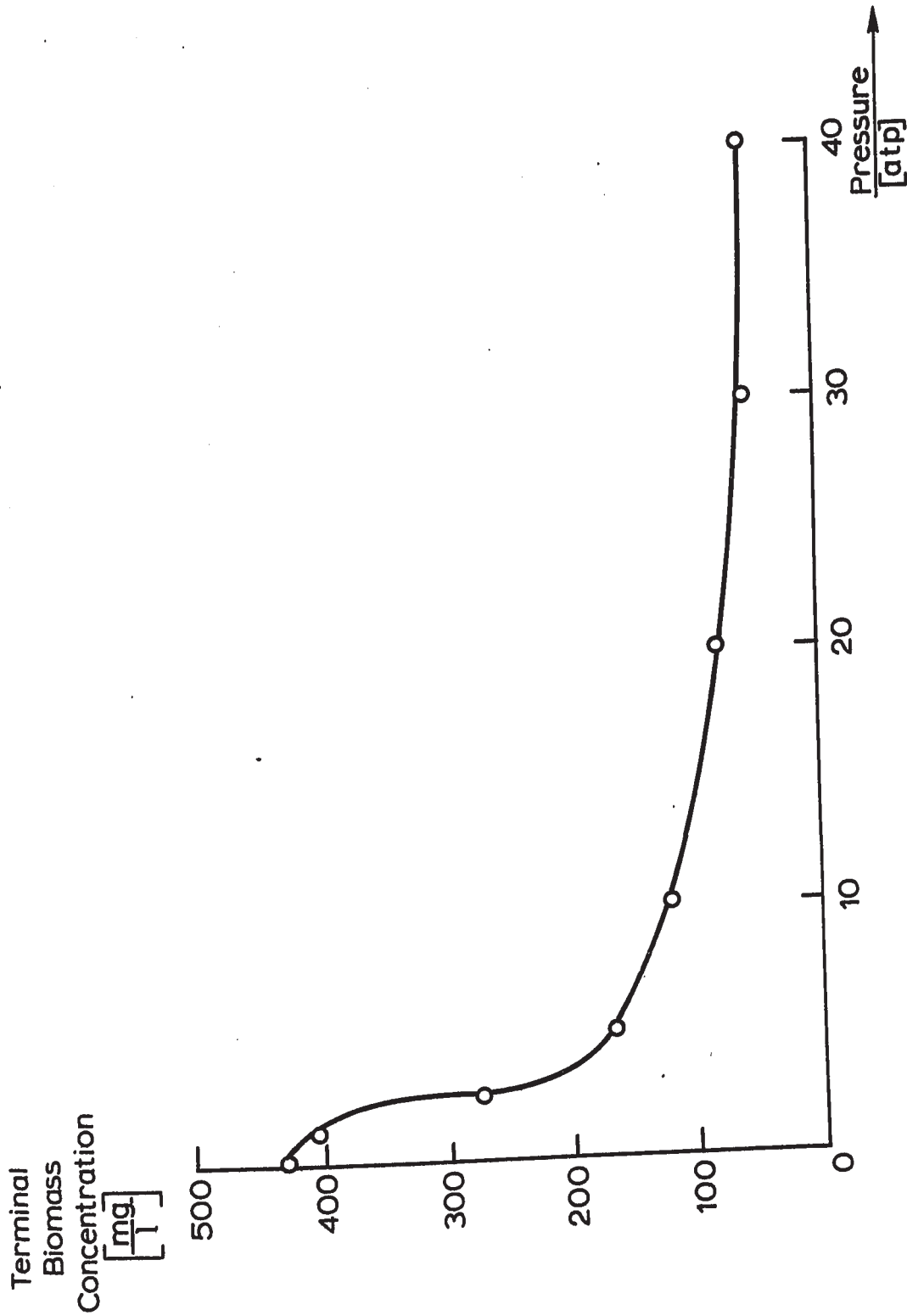
for the inner tube and sparger. After four days of operation when continuous harvesting was to have commenced, it was impossible to proceed. Most of the biomass was growing in filamentous form adhering to surfaces and the liquid broth did not contain sufficient biomass to continue. Heavy growth was observed also on the surfaces of the sparger.

The fermentation extended over a few additional days. Since it did not show any improvement it was terminated. The experiment showed that air lift fermentor was not suitable for this type of culture which requires vigorous mixing to prevent adherence of biomass to stationary surfaces. The slow liquid flow and insufficient mixing in air-lift fermentors tend to support a filamentous type of growth which is uncontrollable and undesirable for biomass production.

c) Fermentation under increased pressure

Stainless steel pressure tested cylinders of 0.5l capacity were used for this type of experiment as described in Chapter IV on Materials and Methods. A pure culture of Graphium sp. was used to inoculate 100 ml quantities of C-medium containing ammonium sulphate as a source of nitrogen. The culture was incubated for 10 days and the dry weight of biomass produced measured. The composition of the gaseous phase used was identical for all the experiments while pressure was varied from atmospheric to 40 atp (588 psi). All experiments were run in duplicate.

The effect of pressure upon the biomass concentration is shown by plotting cell concentration against the pressure of incubation as it is seen on Figure 25. There was no decrease in tissue concentration when the



GROWTH OF *Graphium* sp. UNDER PRESSURE

FIGURE 25

culture was grown at 1 atp pressure. The biomass decreased to 275 mg/l at 2 atp pressure which was approximately two thirds of that observed for the control grown at atmospheric pressure (430 mg/l). Further increases in pressure showed inhibitory effect on biomass synthesis. At pressures above 30 atp there was no growth of Graphium sp. observed. The value of increased pressure in increasing the solubility of gases was of no advantage. The culture of Graphium sp. is obviously sensitive to pressure increases.

#### THE EFFECT OF SURFACE-ACTIVE AGENTS

During batch growth in stirred-tank type fermentor sometimes heavy foaming is a problem. In some instances it was so great that completion of fermentation process was impossible. Foam contaminates exhaust ports, condensers, and the exhaust filters. Heavy foam covers control elements and electrodes and has a high nuisance value in fermentations when it is encountered.

Before Graphium sp. was cultivated in stirred-tank type fermentor, which is the most widely used type of culture vessel, a number of antifoam agents were tested as to their eventual toxic effects towards Graphium sp. These tests were carried out in 500 ml shake flasks inoculated with 5% of Graphium and incubated (32°C) for 7 days in enclosed environmental box placed on shaker rotating at 180 rpm. A mixture of air and natural gas (60:40 by volume) was passed through the box. The experimental arrangement corresponded to that described in Chapter IV on Materials and Methods for shake-flask experiments. To 100 ml C-medium used (with NaNO<sub>3</sub>), 1 ml

of surface-active agent was added. Antifoaming agents were chosen mainly according to their ability to withstand sterilization in autoclave and with respect to their properties for pumping.

The experiment was run in duplicate and the results were averaged and summarized in Table 20. Most of the antifoams applied gave biomass concentrations slightly higher than those measured in the control. Some of them supported dispersed pattern of growth while others did not prevent tissue from adhering to the vessel. It was not possible in this system to observe any inhibition of mass transfer and this side-effect of antifoam required actual testing in the fermentor vessel under proper mixing conditions.

On these tests antifoam HODAG-F 28 gave the best results measured in terms of terminal biomass concentration. Some other antifoams, even though they were very good in shake-flasks, proved to inhibit mass transfer to such an extent that biomass concentration of Graphium sp. was very low as compared to control fermentation. Only preliminary tests showing this effect were completed and no in-depth studies on actual effect of antifoam agents on mass transfer coefficient were conducted.

The addition of a trace amount (80 ppm) of HODAG-F 28 into the culture broth in the fermentor resulting in a well dispersed gaseous phase within the liquid, prevented the biomass from clumping and adhering to the fermentor vessel and in turn positively affected biomass concentrations.



TABLE 20

Effect of Selected Defoamers on Growth of Graphium sp. in Shake-Flasks

Surface Active Agent	Growth after 4 days*	Terminal		Observations
		Cell concn. [mg/100 ml]	pH	
NALCO 127	++	205	7.2	light tissue adherence
ADVANCE HDA		128	7.1	oily surface light tissue adherence
HALLCO C-369	+	52	5.7	milky appearance poor growth
DF-91	+	128	7.0	no adherence
RX-10499	+	173	7.2	light tissue adherence
HODAG K-20	++	175	7.3	dispersed growth
K-21	+	131	6.9	light ring above surface
K-24	+	102	7.2	- " - " - " - " - " -
K-58	+	105	6.9	no adherence
K-67	+++	194	7.3	- " - " - "
M-8	+++	193	7.3	light adherence to submerged surfaces
F-28	++	183	7.3	dispersed growth
Control - no antifoam	+	143	7.25	small pellets

Experiment conducted for 7 days

\* + light growth

++ good growth

+++ very good growth

## CHAPTER VIII

### BATCH FERMENTATION PROCESS STIRRED-TANK TYPE FERMENTOR

#### Culture pH Requirements

A series of experiments was conducted in order to determine the pH giving the highest mycelium concentrations as well as the highest rate of tissue formation. Experiments were conducted using C-medium containing either  $\text{NaNO}_3$  or  $(\text{NH}_4)_2\text{SO}_4$  as a source of nitrogen. Figure 26 shows the rate of tissue synthesis 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  $(\text{NH}_4)_2\text{SO}_4$  caused the pH to decrease to 3.0 as the fermentation proceeded, whereas addition of  $\text{NaNO}_3$  caused the pH to increase to 7.5. A medium containing  $(\text{NH}_4)_2\text{SO}_4$  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

**BATCH FERMENTATION WITHOUT pH CONTROL**  
*(Graphium growing on natural gas)*

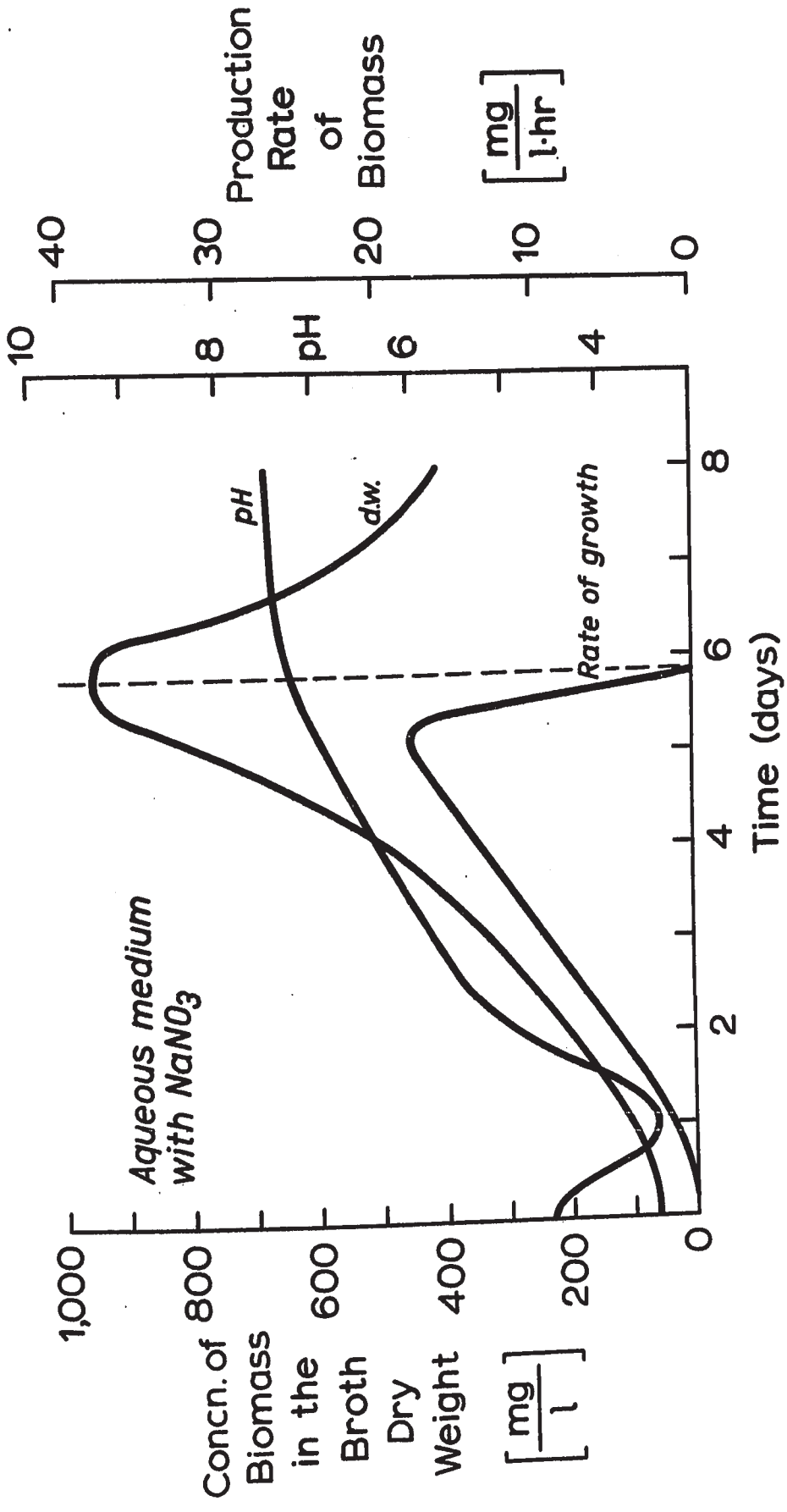


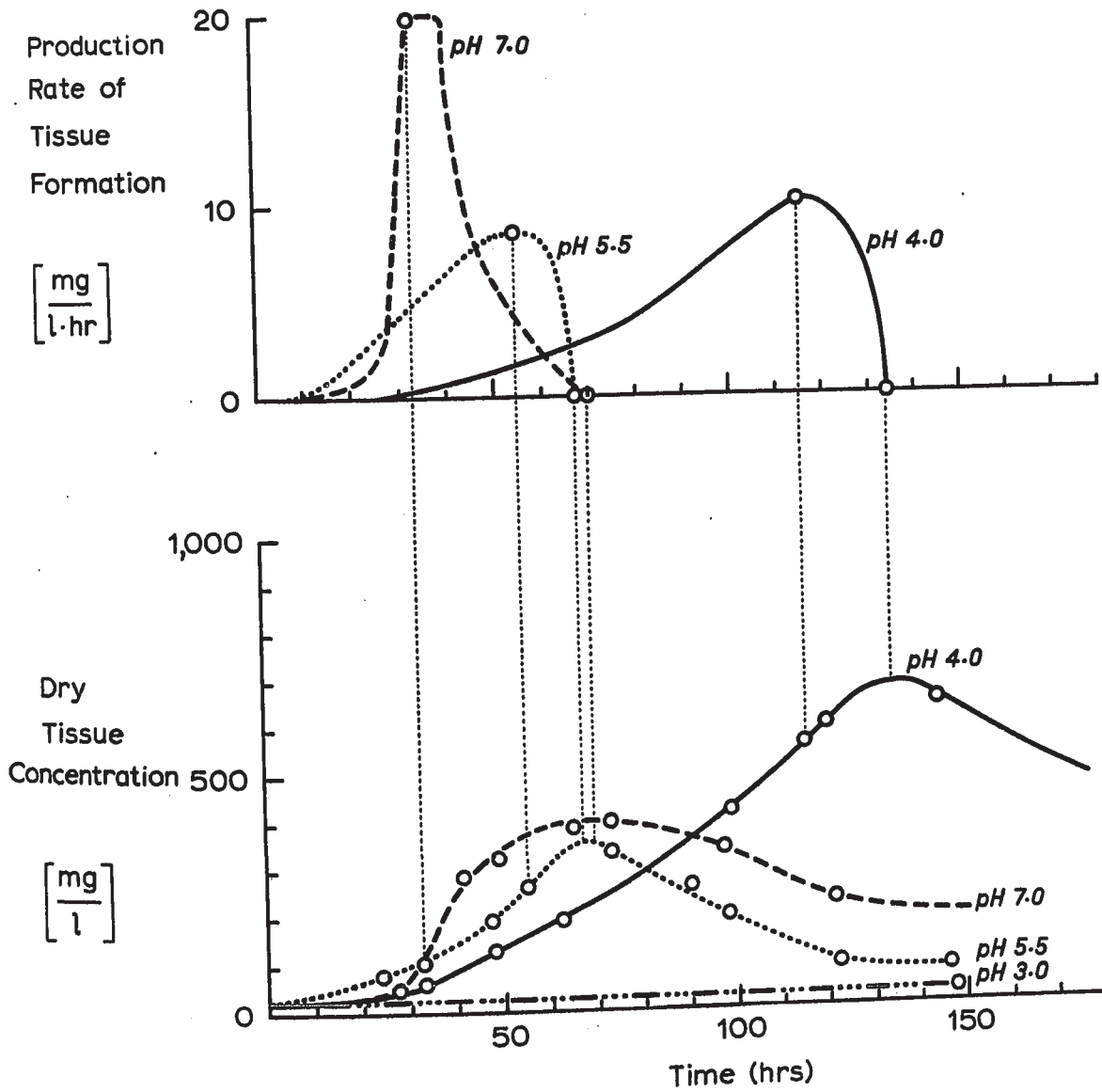
FIGURE 26

proceeded into stationary phase of growth, the tissue adhered to all the stationary parts of the fermentor. The  $\text{NaNO}_3$  medium gave a yield of 96 mg of tissue per 100 ml medium when no pH control was applied. The pH during the fermentation decreases sharply from initial pH 4.5 to pH 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.

pH is a very important parameter for the culture of Graphium growing on natural gas since the concn. 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 Figures 27 and 28, 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  $\text{NaNO}_3$ . Natural gas was the only source of carbon and energy. Biomass concentrations at pH levels of 3.9, 4.9, 5.5, and 7.0 are shown in the lower plot of Figure 27. 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 time and unit volume. The upper plot (Figure 27) obtained this way is showing biomass production rates as a function of time for the corresponding pH levels. The highest concentration of tissue was obtained at pH 4.0. The minimal doubling time of 38.3 hr. was observed at 100 hrs. The corresponding specific growth rate was  $0.0181 \text{ hr}^{-1}$ . The doubling time decreased to 16.5 hr (specific growth rate:  $0.042 \text{ hr}^{-1}$ ) at tests conducted at pH 5.5. A further decrease in doubling time to 5.5 hr (specific growth rate:  $0.125 \text{ hr}^{-1}$ ) was observed at pH 7.0. However, cell concentrations decrease at the highest pH levels tested. The biomass production rate was  $10 \frac{\text{mg}}{\text{l.hr}}$  after 120 hours 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 sulphate rather than  $\text{NaNO}_3$ , pH 5.5 did not give satisfactory results. Clumping was severe after 70 hours. On nitrate media basically no growth was observed at pH 3.0. In terms of the highest production rate the pH

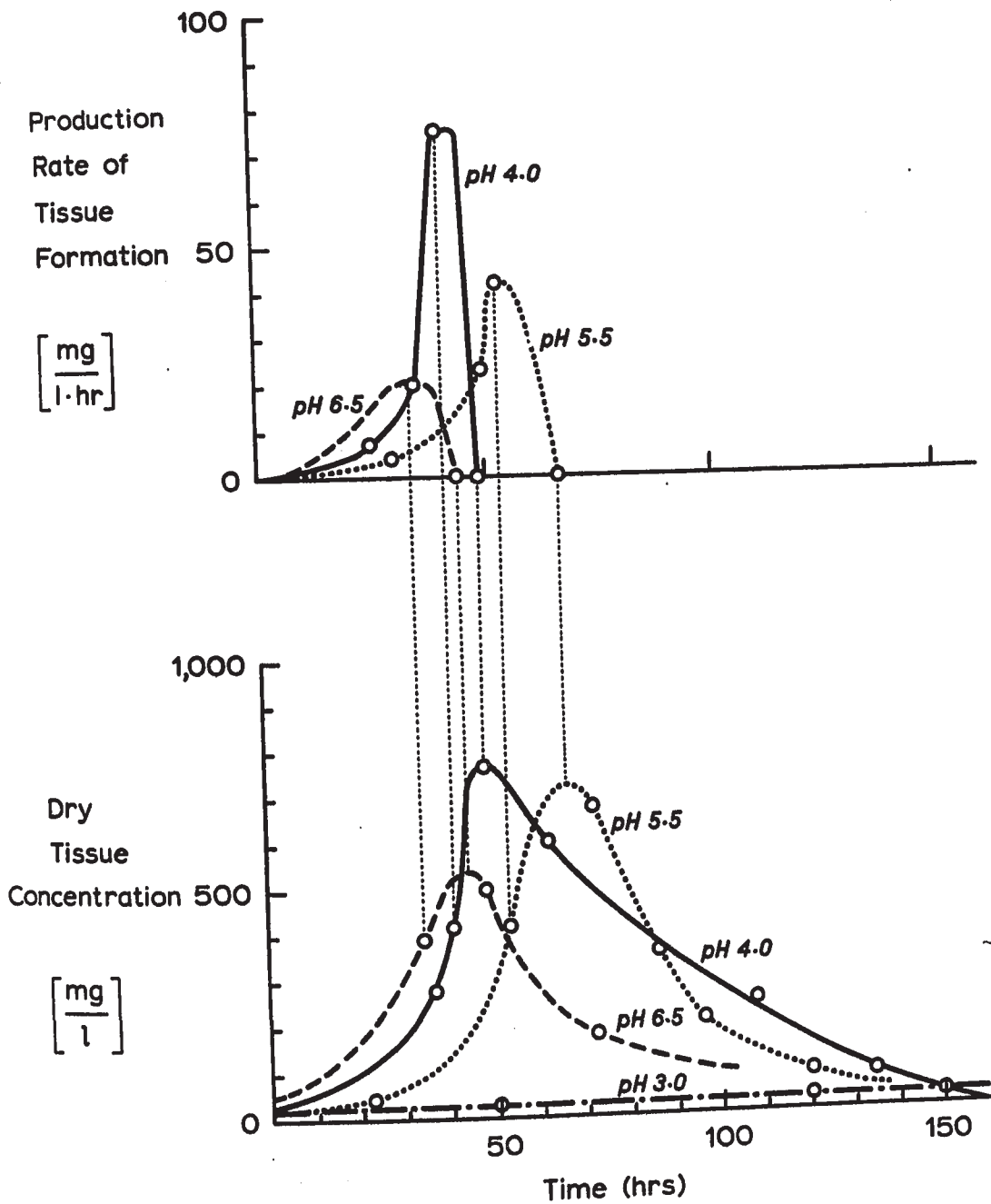


*Graphium* sp. on natural gas.  
 Tissue concn. and rate of growth under various  
 pH of cultivation. (Medium with  $\text{NaNO}_3$ ).

7.0 was optimal giving  $20 \frac{\text{mg}}{\text{l.hr}}$  in 40 hours. After 40 hours, tissue concentrations ( $400 \frac{\text{mg}}{\text{l}}$ ) 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 hours.

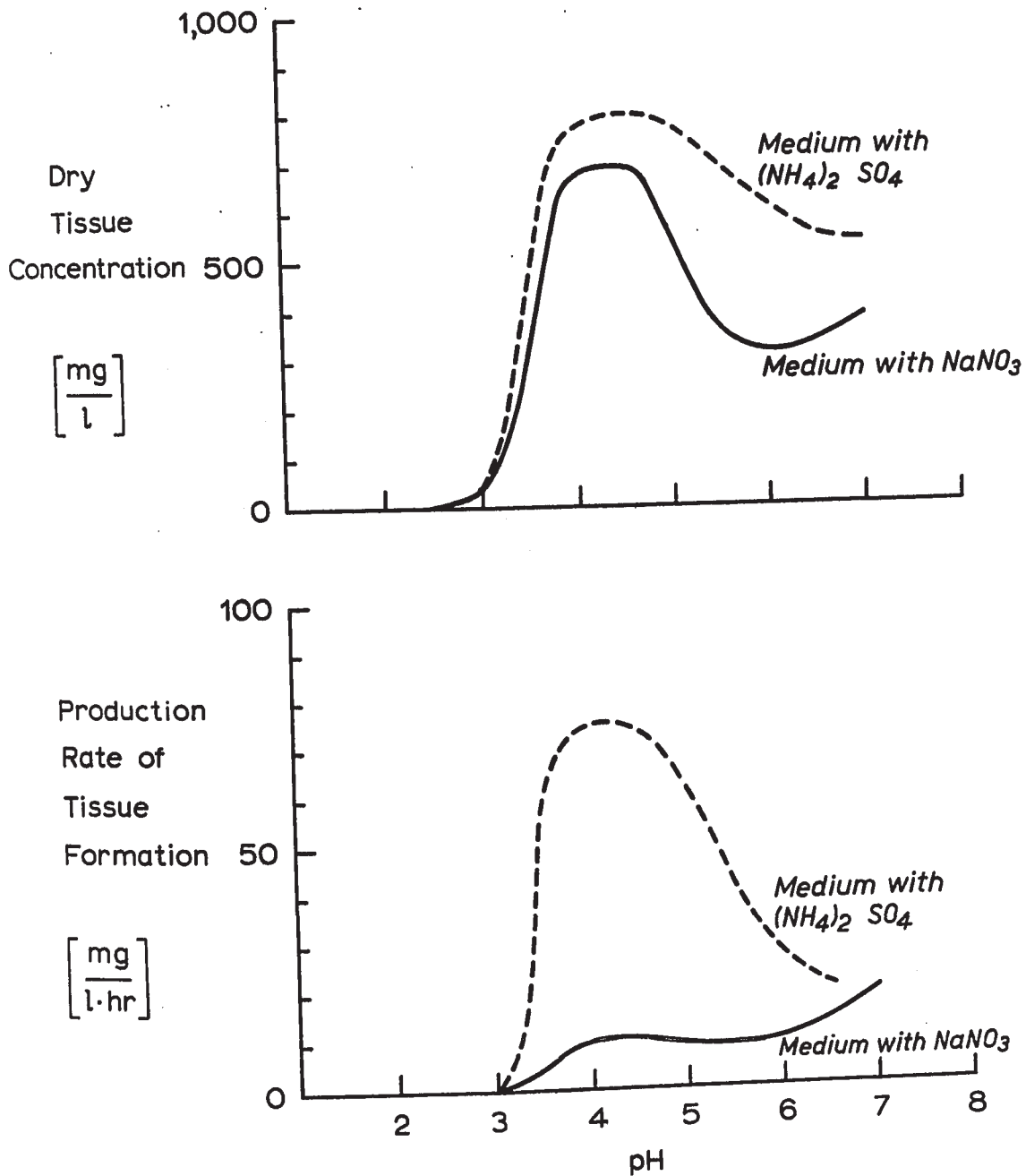
More encouraging results were obtained in experiments employing C-medium with  $(\text{NH}_4)_2\text{SO}_4$  (Figure 28) as a source of nitrogen. In experiments in which the pH was controlled over the entire fermentation period, the biomass concentration was higher and the production rate of biomass increased. Figure 28 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 Figure 4 and derivative functions representing production rates in the upper. The highest concentration of tissue was observed at pH 4.0 the maximum being  $780 \frac{\text{mg}}{\text{l}}$ . This pH also gave the highest rate of tissue formation of  $75 \frac{\text{mg}}{\text{l.hr}}$  in 40 hours. The doubling times and specific growth rates were calculated at 35, 40 and 43 hours and they were respectively: doubling times: 8.4, 3.7 and 5.5 hr; specific growth rates: 0.0825, 0.187 and  $0.125 \text{ hr}^{-1}$ . They were lower at other time intervals tested. They also decreased at pH 5.5 and pH 6.5. In comparison with pH values of pH 5.5 and pH 6.5, pH 4.0 is the optimum. Basically no growth was observed at pH 3.0. Using a medium with ammonium sulphate as the nitrogen source also results in less adherence of the tissue to the stationary parts of the fermentor.

The results of the experiments with controlled pH are summarized in Figure 29 where biomass concentration measured and calculated corresponding production rates are plotted against pH of fermentation. It shows



*Graphium* sp. on natural gas.  
 Tissue concn. and rate of growth under various  
 pH of cultivation [Medium with  $(\text{NH}_4)_2 \text{SO}_4$ ]





*Graphium* – Rate of tissue formation  
 in relation to pH.  
*Graphium* sp. on natural gas.

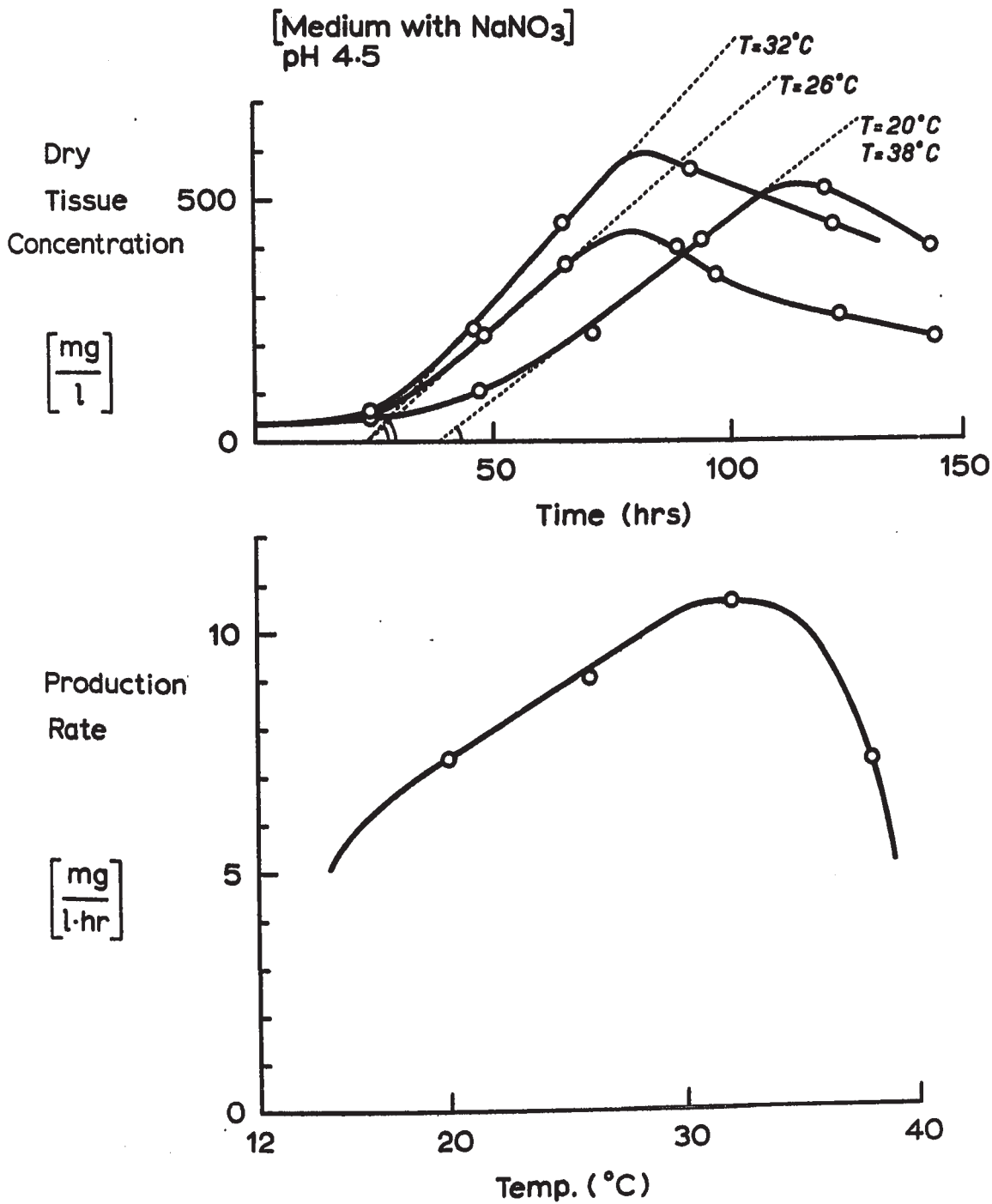
the superiority of ammonium sulphate as a source of inorganic nitrogen resulting in efficient growth of Graphium sp. culture.

### TEMPERATURE EFFECT

It has been shown that a considerable amount of heat is evolved during the oxidative fermentation of hydrocarbon substrate (Bennett et al., 1969). The quantity of heat theoretically evolved is approximately 2.5 times higher than that evolved during the fermentation of conventional carbohydrate substrate. 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  $\text{NaNO}_3$ . Figure 30 summarizes results of temperature experiments. Growth curves for each temperature are plotted against time. Temperature definitely effects the biomass production rate. Rates during experimental growth were:

$20^\circ$  and  $38^\circ\text{C}$ :  $7.5 \frac{\text{mg}}{\text{L}\cdot\text{hr}}$ ;  $25^\circ\text{C}$ :  $9 \frac{\text{mg}}{\text{L}\cdot\text{hr}}$ ; and  $32^\circ\text{C}$ :  $10.6 \frac{\text{mg}}{\text{L}\cdot\text{hr}}$ . Doubling times and specific growth rates at each temperature are summarized in Table 21. The shortest doubling time and best growth rates of 16.2 hr and  $0.0428 \text{ hr}^{-1}$  were observed at  $32^\circ\text{C}$ . 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 (Figure 30). Additional studies on Graphium show an optimum temperature for growth on natural gas between  $30\text{-}33^\circ\text{C}$ . This may vary slightly under certain cultural



*Effect of temperature on growth of Graphium sp. on natural gas.*

TABLE 21

EFFECT OF TEMPERATURE ON GROWTH OF GRAPHIUM SP.

Temperature [ $^{\circ}$ C]	20	26	32	38
* Doubling Time [hr]	22.5	18.6	16.2	22.8
* Specific Growth Rate [ $\text{hr}^{-1}$ ]	0.0308	0.0372	0.0428	0.0304

\*as estimated in exponential growth phase

conditions, but it is the recommended temperature range for growth on gaseous hydrocarbons.

#### BATCH CULTURE-STIRRED TANK

A few batch culture runs were completed in the same type of fermentor under the optimized conditions reported herein. If no antifoam was applied clumping and adherence of tissue was observed in the late exponential phase of growth occurring after 4 days of cultivation. The yield achieved under this condition averaged ~2 g/l. Prolonged fermentation resulted in heavy deposits of biomass on stationary surfaces of the fermentor and the broth cleared up gradually.

On addition of 0.3% yeast extract prior to inoculation, a tendency to foam was observed. Very heavy foam occurred later in the exponential phase of growth where it filled the whole space above the liquid and eventually contaminated the exhaust filter. A mechanical foam separator was used to prevent this from happening and the biomass concentration increased to ~ (4-5)g/l. Yeast extract did not seem to be entirely utilized when the fermentation was terminated after 4 days. However, this was not confirmed analytically. The optimal pH for batch culture with yeast extract added was observed to be slightly higher, approximately pH 5.5. However, further studies relating yeast extract addition and optimal culture pH must be completed.

Addition of antifoam HODAG F-28 resulted in improvement of fermentation conditions. The aeration pattern was greatly improved by dispersing

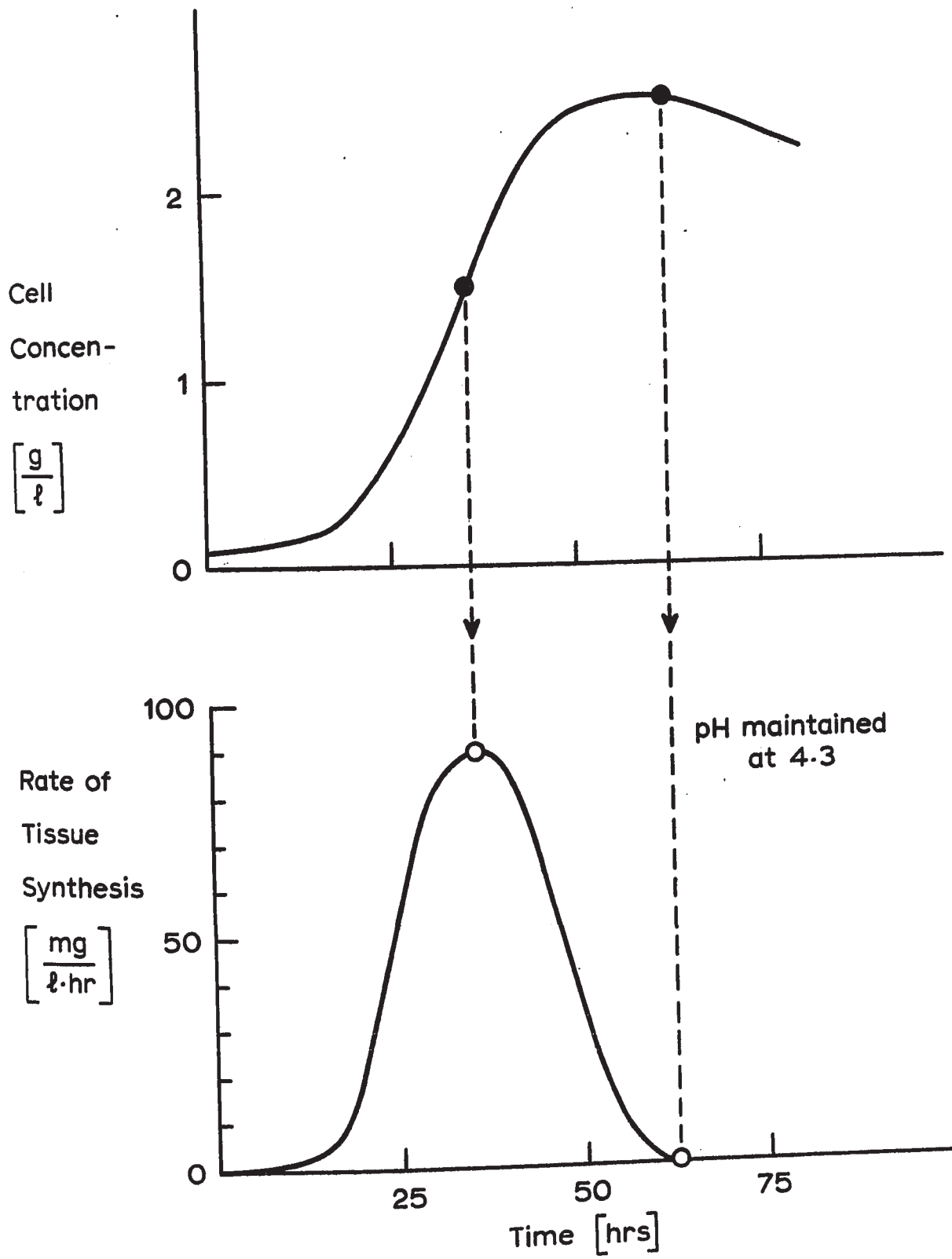
of decreased size bubbles of gaseous phase throughout the liquid broth. No foaming was observed until second day when a few additional drops of antifoam suppressed foam formation very effectively. The initial concentration of antifoam in the sterilized medium was ~ 100 ppm. No mass transfer inhibition was noted in the system and finely dispersed bubbles contributed to increased interfacial area for mass transfer.

These factors resulted in a higher cell concentration averaging from (2.5 - 2.9) g/l on synthetic medium. A typical growth curve is plotted on Figure 31 together with the rate of growth against the time of fermentation.

Increased biomass concentrations were observed on addition of 0.3% yeast extract and 100 ppm of F-28 antifoam. Between 5.5-7.8 g/l was the average biomass concentration for this type of fermentation. For all these batch tests the pH was continuously controlled by adding NaOH to the broth since C-medium with  $(\text{NH}_4)_2\text{SO}_4$  was used.

In addition to testing axenic growth of Graphium sp. in a 10 liter fermentor a mixed culture system was evaluated. The pH was maintained at 4.5 throughout the entire fermentation by adding  $\text{NH}_4\text{OH}$  as needed. Ammonium hydroxide was chosen because it also serves as additional source of inorganic nitrogen to support microbial growth. With no antifoam applied only very light foaming occurred and the biomass concentration reached 4.2 g/l on third day when the experiment was terminated.

In comparison to the axenic system higher biomass concentrations



*Batch growth characteristics of Graphium sp. on natural gas.*

were obtained in the mixed culture systems. A synthetic medium was used purposely for these studies and most studies described herein in order to make the basal medium as simple as possible.

#### PILOT PLANT OPERATION

With the main purpose of producing Graphium sp. biomass for animal feeding experiments, a 400 l stainless steel fermentor was operated. 300 l of non-sterilized C-medium [with  $(\text{NH}_4)_2\text{SO}_4$ ] was inoculated with 12 l of pure culture of Graphium sp. The resulting mixed culture system was operated under non-aseptic conditions.

The culture broth was mixed with 3 inch marine-type impeller, rotated at 1300 rpm on a slant shaft. There were no baffles in the culture vessel. A mixture of natural gas and air (60:40  $\text{V}/\text{V}$ ) was passed into the system by a bottom-located ring sparger at a rate of 35 lpm. pH was controlled throughout the entire fermentation at 4.3 by addition of 6N NaOH as required. Antifoam HODAG-F28 was applied to suppress foam formation and to improve an aeration pattern.

The biomass was recovered by filtration and freeze dried before further processing for toxicity and feeding experiments. Since the mixed culture system was not operated at optimal conditions, the terminal biomass concentration after 4 days averaged only from 2 - 3.5 g/l. No detailed studies were carried out on this type of operation.



## CHAPTER IX

### 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  $\text{CO}_2$  evolved as a product of respiration was measured. In one experiment it was necessary to remove  $\text{CO}_2$  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.

1. Gas Recycle With Oxygen Becoming Limiting. The fermentation vessel was sterilized together with inlet and outlet gas-phase filters for 40 minutes in an autoclave at 120°C (15 psi). A 5% by volume of pure culture of Graphium sp. was used to inoculate 6 l. of sterile broth. The composition of gaseous phase in the gastight system was adjusted initially to 17.3% by volume ethane, and 19.2% oxygen. Nitrogen was added to bring the gas volume to 100%. Approximately 0.2% CO<sub>2</sub> was present in the gaseous mixture. Nitrogen was used to replace gases utilized during the fermentation in order to maintain a steady pressure. The CO<sub>2</sub> produced by the growing culture was not removed. Parameters examined were cell concentration, concentrations of oxygen, ethane and CO<sub>2</sub> in the recycle gaseous phase. Plotting these against the time of fermentation, a curve for each variable was obtained (Figure 32). The upper part of the figure shows concentrations of the biomass and gases. Following a 19-hour lag period, exponential growth was observed. Tissue formation was accompanied by a corresponding increase in CO<sub>2</sub> 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 the Figure 32 where specific rates of oxygen and ethane utilization  $\left[\frac{\text{ml}}{\text{l.hr}}\right]$  are plotted against time. Also plotted are rates of CO<sub>2</sub> production and biomass production rate  $\left[\frac{\text{mg}}{\text{l.hr}}\right]$ . The lower part of Figure 32 is derived completely from the primary values measured and plotted in the upper diagram. All values

## F I G U R E 3 2

BATCH GROWTH OF GRAPHIUM ON ETHANE IN A CLOSED  
SYSTEM WITH RECYCLE OF GASES WITHOUT REPLENISHMENT

During the cultivation the following measurements  
were taken:

Concentrations of  $C_2H_6$ ,  $O_2$ ,  $CO_2$

Biomass concentrations

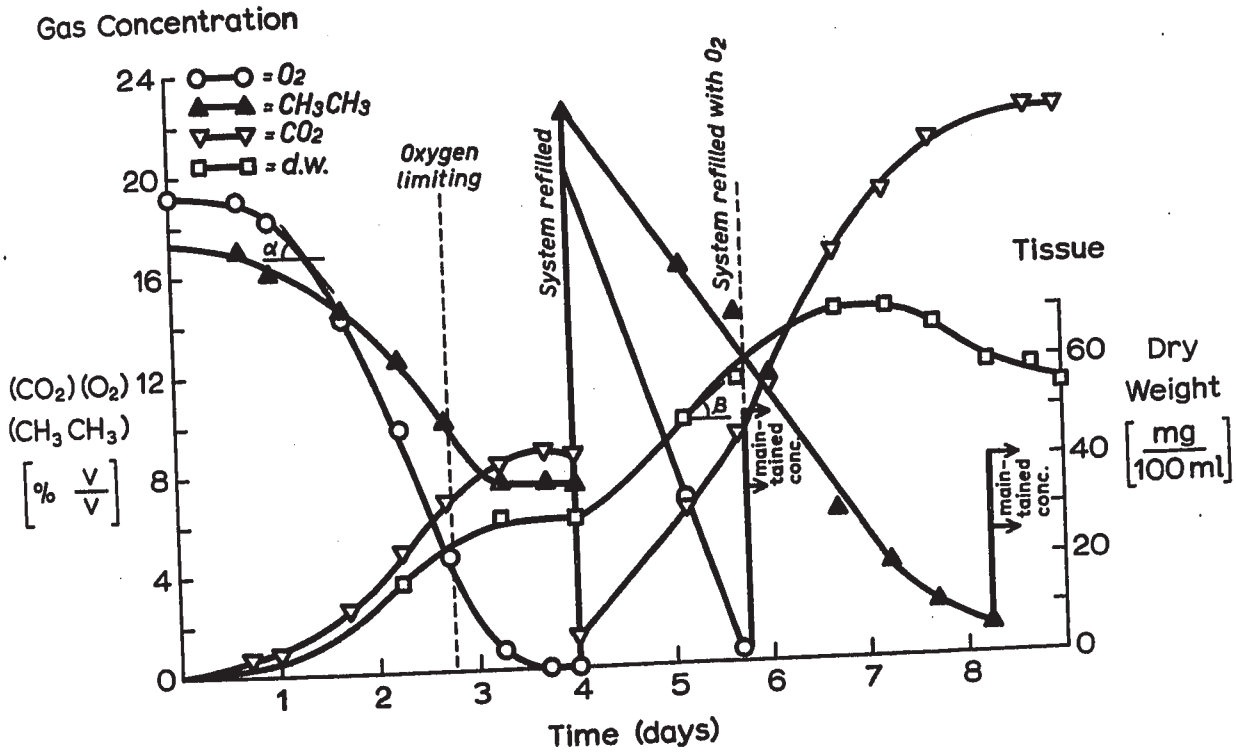
The calculated values were:

The rate of  $C_2H_6$  and  $O_2$  consumption

The rate of  $CO_2$  production

The biomass production rate

### BATCH FERMENTATION WITH CLOSED RECYCLE OF GASES (Graphium growing on ethane)



### RATES OF $O_2$ AND $C_2H_6$ CONSUMPTION AND $CO_2$ PRODUCTION RATE OF GROWTH OF GRAPHIUM IN BATCH CULTURE.

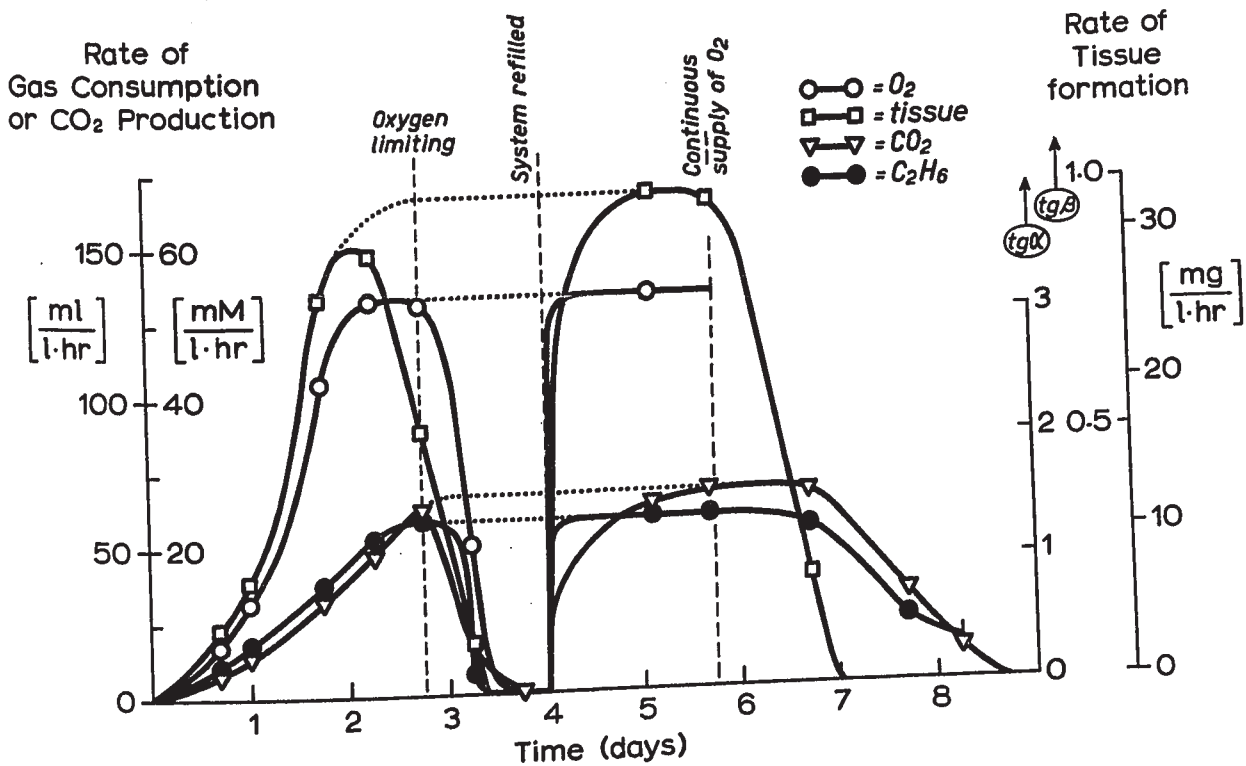


FIGURE 32.

are first derivatives (slopes) of the primary functions taken at selected points. The 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  $\text{CO}_2$  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  $5\frac{1}{2}$  days of fermentation, the oxygen level was adjusted again and maintained within a range of 8-10%  $\text{O}_2$ . 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 Figure 32 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.

2. Gas Recycle With Replenishment of Oxygen and Ethane. Another experiment was completed with Graphium sp. in 6 l 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, 0.2% CO<sub>2</sub> and the balance of the gas phase 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 CO<sub>2</sub> produced during the fermentation run was periodically removed by absorption on Baralyme. The CO<sub>2</sub> concentration was maintained between 3 to 5%. Cell concentrations and the amount of gases in the system were measured. The plot of these parameters against time of fermentation is shown in Figure 33. A third plot shows the rate of consumption in  $\frac{\text{ml}}{\text{hr}}$  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  $300 \frac{\text{ml}}{\text{hr}}$  as observed in the exponential growth phase. Calculated on a unit volume of broth basis, this value becomes  $50 \frac{\text{ml}}{\text{l.hr}}$  (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

## FIGURE 33

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:

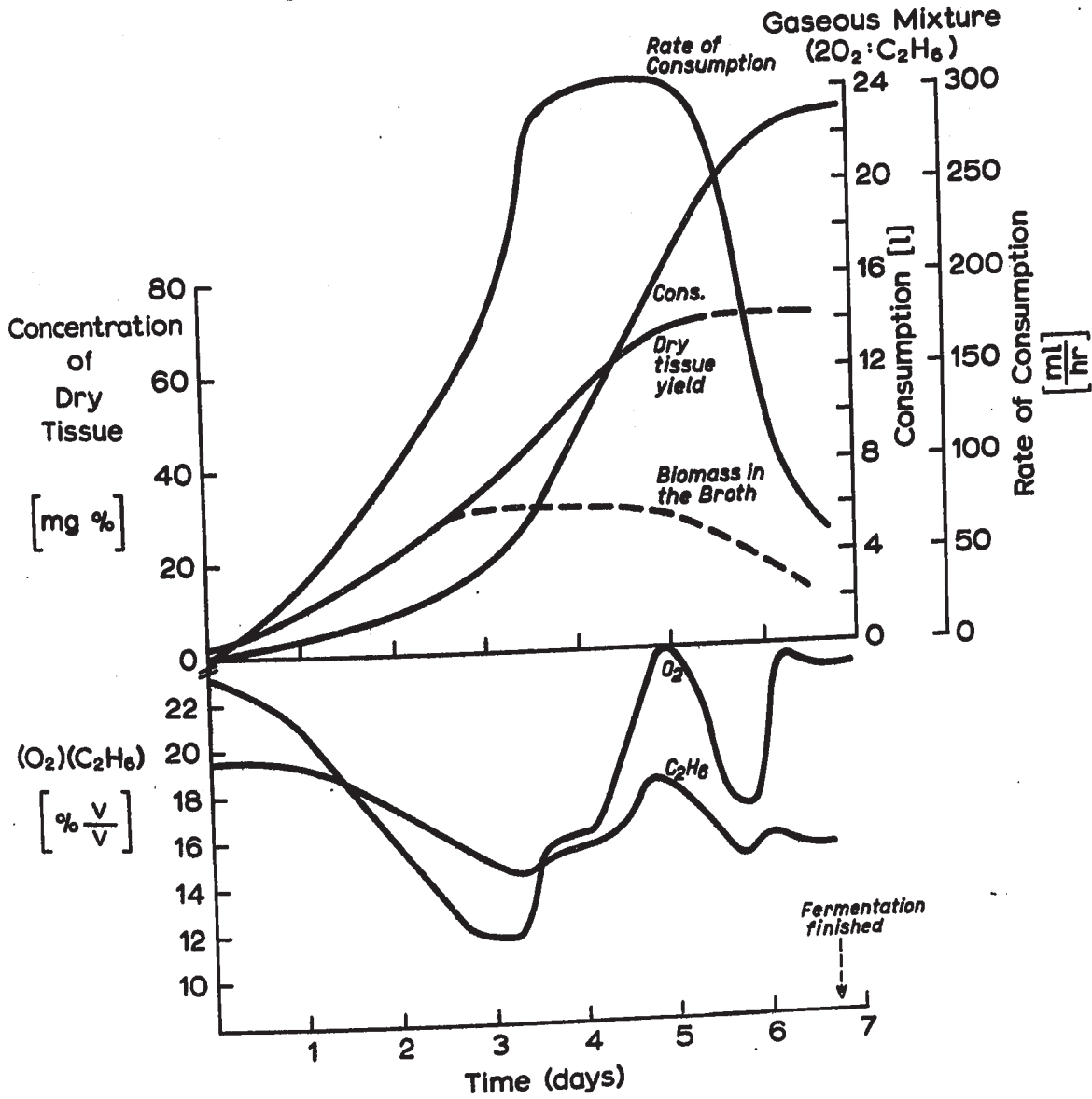
Biomass concentrations

Amount of gaseous mixture consumed

Concentrations of ethane and oxygen

The rate of gaseous mixture consumption was calculated.

**GRAPHIUM BATCH FERMENTATION  
WITH CLOSED RECYCLE OF GASES**  
(Gaseous mixture  $O_2 + C_2H_6 = 2:1$  fed in continuously)



CONCENTRATION OF OXYGEN AND ETHANE

FIGURE 33



to the stationary parts of the fermentation vessel later in the exponential growth phase. Total yield of the biomass was ~4.5 g dry tissue/6 l of broth.

The lower part of Figure 39 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 12-23% and ethane between 15-18%. Since there was a difference between the initial and final concentration of ethane, a correction was required. Knowing the consumption of the oxygen-ethane mixture and the total tissue produced the amount of ethane substrate utilized per unit weight was ~1.9 to 2.1 g per 1 g of dry tissue. The volumetric ratio of oxygen to ethane consumption averaged between 2.1 - 2.3 depending upon the phase of growth and physiological state of culture. It was closer to the higher value during the later phases of the fermentation.

## CHAPTER X

### CONTINUOUS CULTURE

Three continuous culture runs were completed using Graphium sp. culture. In continuous culture experiments I and II, natural gas was the sole source of carbon and energy while in continuous culture experiment III, conducted with a recycle of gaseous phase, pure ethane was used. Late in the latter experiment methane was added to study the response of the culture to this potential source of energy. In all three experiments the harvested biomass was recovered and weighed when dry. Changes in the biomass concentration in the system were plotted against time. The biomass production rate in mg/l hr was calculated from cell concentrations and the known dilution rate and was also plotted against the time of fermentation.

#### Continuous Culture I - 14l stirred-tank type fermentor

A 5% by volume of Graphium sp. culture was inoculated into 10l of sterile broth in a 14l fermentor. The basal C-medium was containing

$\text{NaNO}_3$ . Natural gas was the only source of carbon and energy. It was continuously passed through the mixed broth in mixture with air and exhausted without recycle. After initial period of 4 days of batch cultivation, continuous feed of fresh medium and harvest were commenced. The dilution rate was adjusted to  $0.02 - 0.017 \text{ hr}^{-1}$  which corresponds to a retention time of 50-60 hours in the fermentor.

When continuous culture was initiated the cell concentration approached 400 mg/l. The system responded to fresh medium input by a decrease in biomass concentration to ~ 280 mg/l. This is seen on Figure 34. After 7 days of cultivation a steady increase in cell concentration was observed reaching its peak at 10 days. A slight temporary increase in retention time to 80 hrs. at 10 days caused a corresponding increase in cell concentration, however the production rate averaged 10 mg/l.hr over the whole period between 8 and 14 days. Steady state was established between 10-14 days with cell concentration levelling at ~ 650 mg/l.

During this period there was a foam layer about 1-2 inches thick on the surface of the fermentation liquor. To prevent foaming, an antifoam agent HODAG K-20 was applied dropwise as needed. It was very effective in very small amounts in suppressing the foam. However, the culture responded by a sharp decrease in growth activity as measured by cell concentration and production rate. The antifoam was previously tested in shake flask culture and did not show any toxic effects to the culture (Chapter VII). The inhibition of growth in the fermentor may be contributed to decreased mass transfer coefficient due to the antifoam addition. The culture of

## FIGURE 34

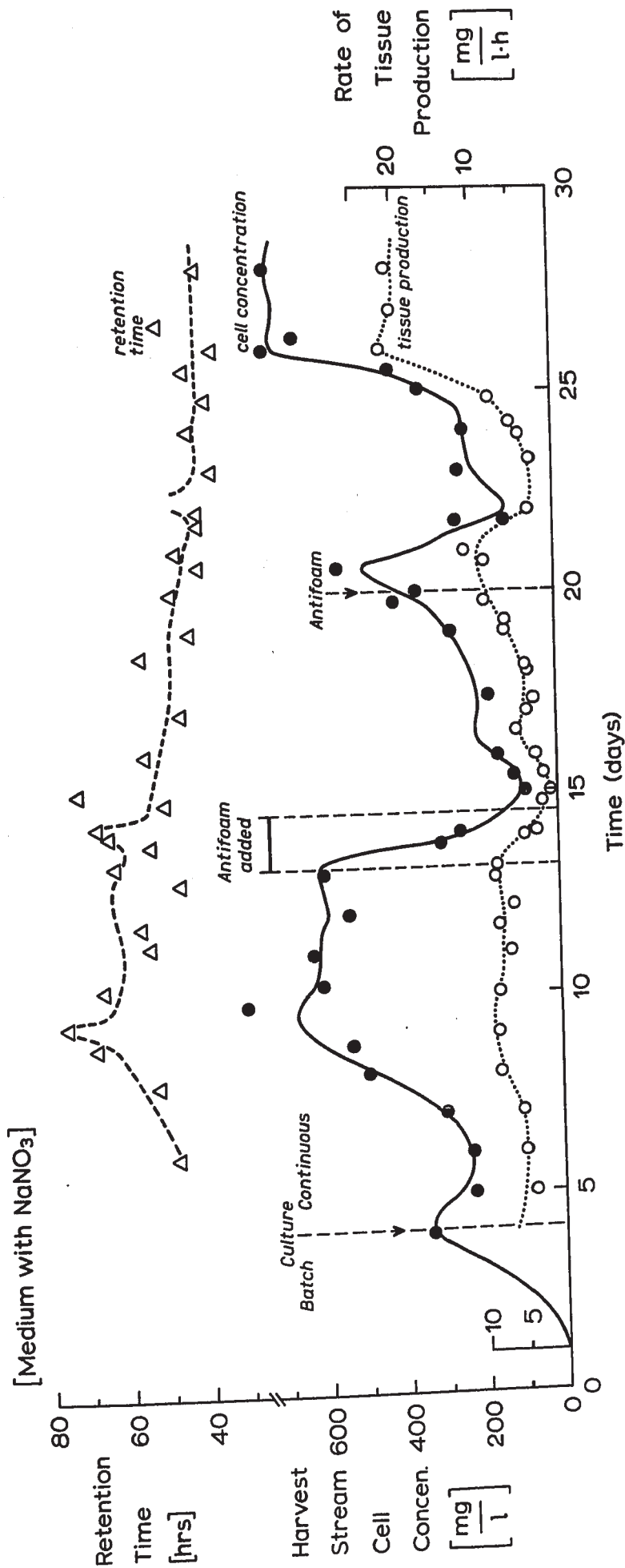
## CONTINUOUS CULTURE I

The culture of Graphium sp. was cultivated in a continuous culture system in 14 l stirred tank-type fermentor. Supporting aqueous C-medium contained  $\text{NaNO}_3$  .

pH was maintained throughout the entire fermentation at 4.0 . The experiment was conducted for 28 days. The antifoam added was HODAG - K20.

The biomass concentration was measured on dry weight basis.

The production rate was calculated.



Continuous culture of *Graphium* sp. on natural gas

Graphium may be especially sensitive to this since the growth substrate as well as necessary oxygen are supplied in the gaseous phase. Upon discontinuance of the antifoam addition the culture in fermentor recovered as the antifoam was washed out. After the 20th day when the previous activity was almost achieved a few drops of antifoam K-20 was applied. The culture responded the same way showing dramatic decrease in cell concentration with minimum of 150 mg/l on the 22nd day. Antifoam addition was stopped and the system permitted to recover. At this time another perturbation of the system was introduced by cutting off the continuous feed and harvest for a period of 6 hours. This batch culture period delayed the recovery progress and the culture plateaued for half a day at the steady values of cell concentration of 280 mg/l. When continuous-flow culture conditions were resumed the recovery process and residual antifoam washout proceeded resulting in high cell concentration of 750 mg/l after 2 days. A new steady state was established on the 25th day at a dilution rate of  $0.025 \text{ hr}^{-1}$  (retention time 40 hrs) and corresponding tissue production rate of 21 mg/l hr. Continuous culture was terminated after 29 days of operation due to excessive growth and tissue build-up on all stationary parts of the fermentor vessel (Figure 35) which made proper control of the system impossible.

#### Continuous Culture II - 28 l stirred-tank type fermentor

A 28 l stirred-tank type fermentor was used with 18 l operated volume. C-medium with ammonium sulphate as a nitrogen source was the liquid supporting medium while natural gas was the only source of carbon

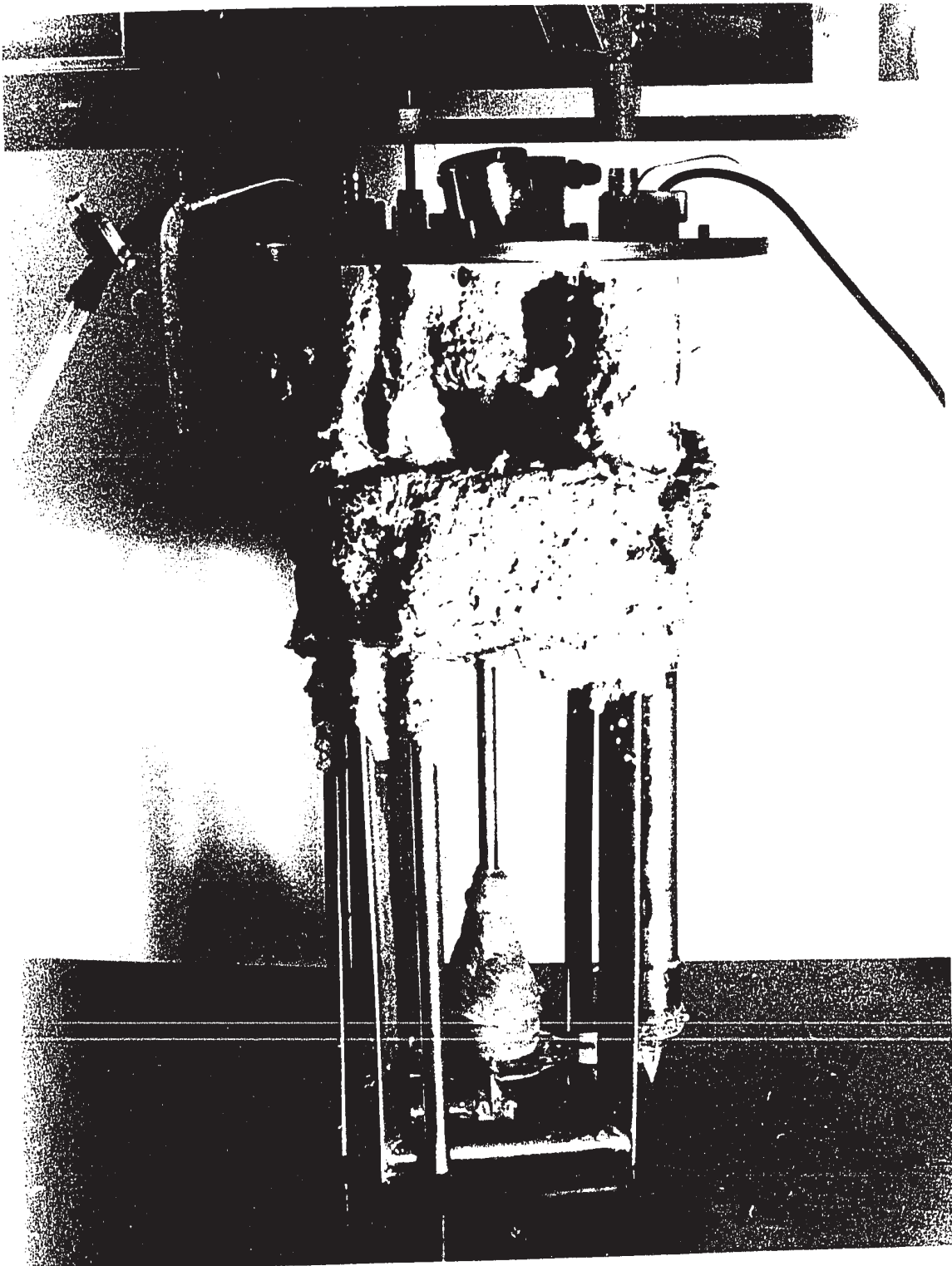


FIGURE 35

Antibiotic culture experiment 1 - all mass solidified in the fermentation vessel after 30 days of operation.

and energy. 5% by volume of a culture of Graphium sp. was applied as inoculum.

Cell concentration was measured throughout the run and, combined with dilution rate, was used to calculate the production rate. These are plotted against time on Figure 36. After 2.5 days of initial batchwise cultivation the culture was in advanced exponential phase of growth giving 800 mg/l. At this time continuous feeding and harvesting was commenced. The dilution rate was adjusted to  $0.0312 \text{ hr}^{-1}$  ( $0.77 \text{ day}^{-1}$ ) which corresponds to a retention of 32 hrs.

Initially slow response of the fermentation system as measured by the biomass concentration change was observed. After 2 days very sharp decrease in the biomass concentration was the result of introducing continuous-flow culture technique.

A steady state was established on the 6th day when cell concentration reached 330 mg/l and cell production rate 10 mg/l.hr. These values were maintained for another 3 days.

On the eighth day a continuous addition of yeast extract concentrated solution was commenced to bring the yeast extract concentration in the fermentation broth to 0.1%. The overall dilution rate was slightly increased by this additional input to  $0.0358 \text{ hr}^{-1}$ , which represents the retention time of 28 hrs. The system responded, with corresponding delay, by decreased cell concentration reaching its minimum on the tenth day. On subsequent culture recovery, the cell concentration reached a previous



## FIGURE 36

## CONTINUOUS CULTURE II

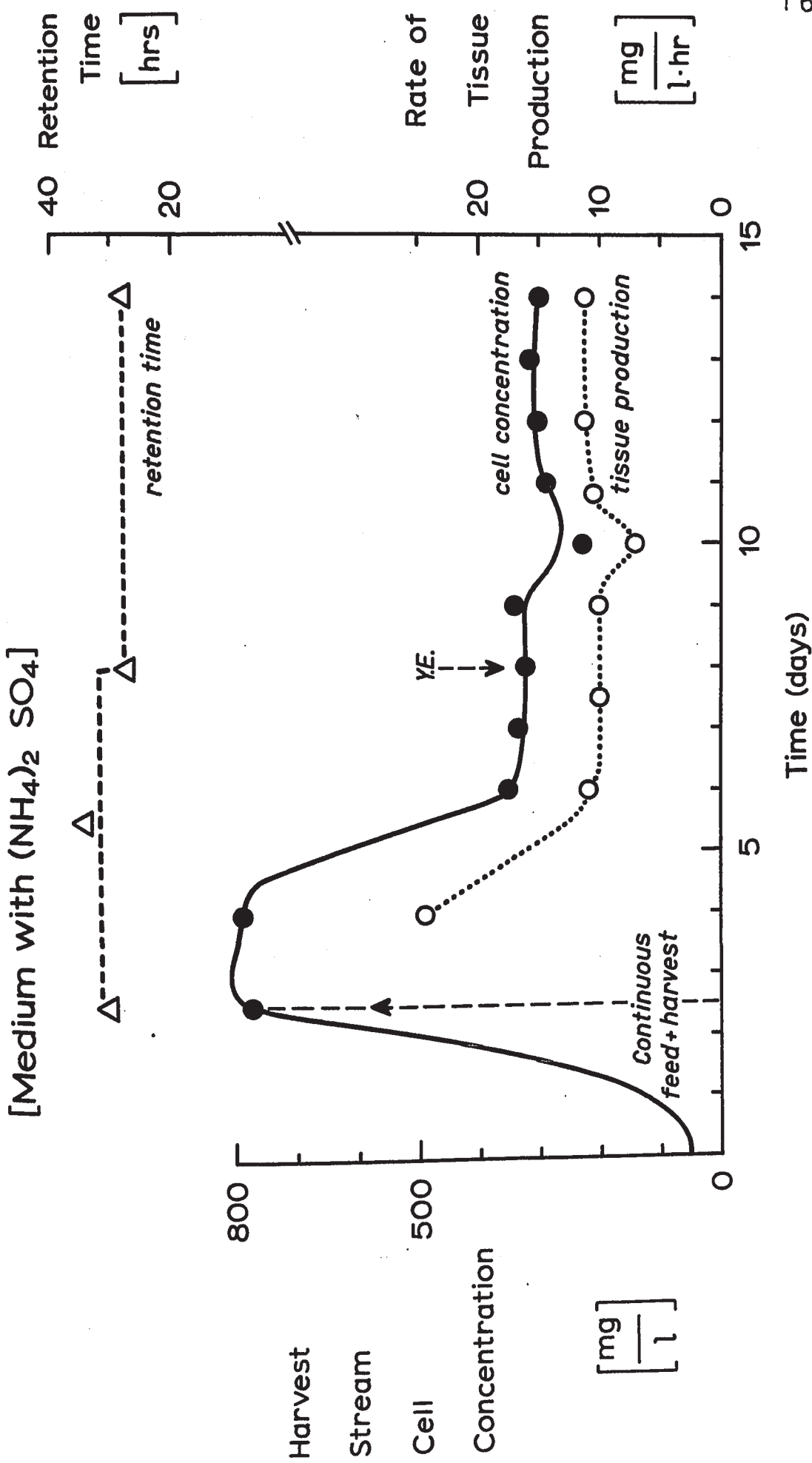
The culture of Graphium sp. was cultivated in a 28 liter stirred tank-type fermentor. Continuous-flow culture technique was used. Aqueous supporting C-medium contained  $(\text{NH}_4)_2\text{SO}_4$ . Natural gas was the only source of carbon and energy.

pH was maintained throughout the entire fermentation at 4.5 . The experiment was conducted for 14 days.

The concentration of biomass was measured on dry weight basis.

The biomass production rate was calculated.

Y.E. - yeast extract addition commenced,  
its concentration in the broth was  
brought to 0.1 % .



Continuous culture of *Graphium* sp. on natural gas.  
 (medium with  $(\text{NH}_4)_2 \text{SO}_4$ ).

value of 300 - 330 mg/l on the 12th day where a new steady state was established. This represented an increased value of cell production rate averaging ~ 12 mg/l.hr. The increased cell production rate was attributed to the yeast extract addition effect (as shown in Chapter VI). However, this result was much lower than anticipated. This was due in part to the excessive tissue formation above liquid surface inside the fermentor which brought the fermentation process to a termination after 14 days of operation.

#### Continuous Culture III - with recycled gaseous phase

A 12 liter magnetic bottom-driven fermentor was employed in this experiment. Because of the closed head arrangement of this fermentor one can achieve a gas-tight system. This makes it possible to quantitatively evaluate the gas consumption. In order to prevent the biomass from adhering to the stationary parts of the fermentation vessel protruding through the liquid level were restricted to pH electrodes, harvest tube and aeration tube. No baffles were inserted. A silicone base antifoam HODAG-F28 found beneficial in earlier studies was added to the feed medium. It effectively controlled foam formation and improved an aeration pattern. Small size bubbles were well dispersed throughout the fermentation broth.

The fermentor was operated at 8 l volume after initial inoculation with 5% (v/v) of Graphium sp. culture. After 2 days of batch culture, continuous feed and harvest were commenced. The dilution rate was gradually increased to  $0.0278 \text{ hr}^{-1}$  after 70 hours of continuous operation. The cell concentration was increased during this period, however a slight

decrease in biomass concentration before steady state was established.

Cell concentration in the fermentor was measured throughout the continuous run. It is plotted together with dilution rate and the biomass production rate (calculated) against time in Figure 37. A tendency towards oscillation is apparent from the diagram as a response of the system to perturbations. There is a definite response of the system to a temporary increase in retention time after ~ 160 hours of culture. Good steady state was observed between 200 and 350 hours of continuous operation. The cell concentration averaged 700 mg/hr during this time while the production rate was ~ 20 mg/l.hr at a retention time of 36 hours. 18% oxygen and 18% ethane concentrations were maintained in the system. The CO<sub>2</sub> evolved during the oxidation process was continuously removed and its concentration did not exceed 2%. The ratio of oxygen to ethane consumption averaged 1.75 over this period of culture.

After 360 hours of continuous culture the composition of recycle gaseous phase was altered. Concentrations of both ethane and oxygen were adjusted to 40%. Increased concentrations of the two gases resulted in immediate increase in biomass synthesis. A cell concentration of 1060 mg/l was observed and the production rate calculated at a retention time of 38 hours was 30 mg/l hr.

There was an initial sharp increase in oxygen to ethane consumption ratio for this period which was followed by a steady value of 1.55. This ratio was stable as long as the steady state was maintained.

## F I G U R E 3.7

CONTINUOUS CULTURE-EXPERIMENT III  
 WITH GASEOUS PHASE RECYCLE AND CONTROL

VIRTIS 12 l fermentor with 8 l volume of culture broth was operated under aseptic conditions. Experimental arrangement is shown in FIGURE 13 and schematic flow diagram in FIGURE 11.

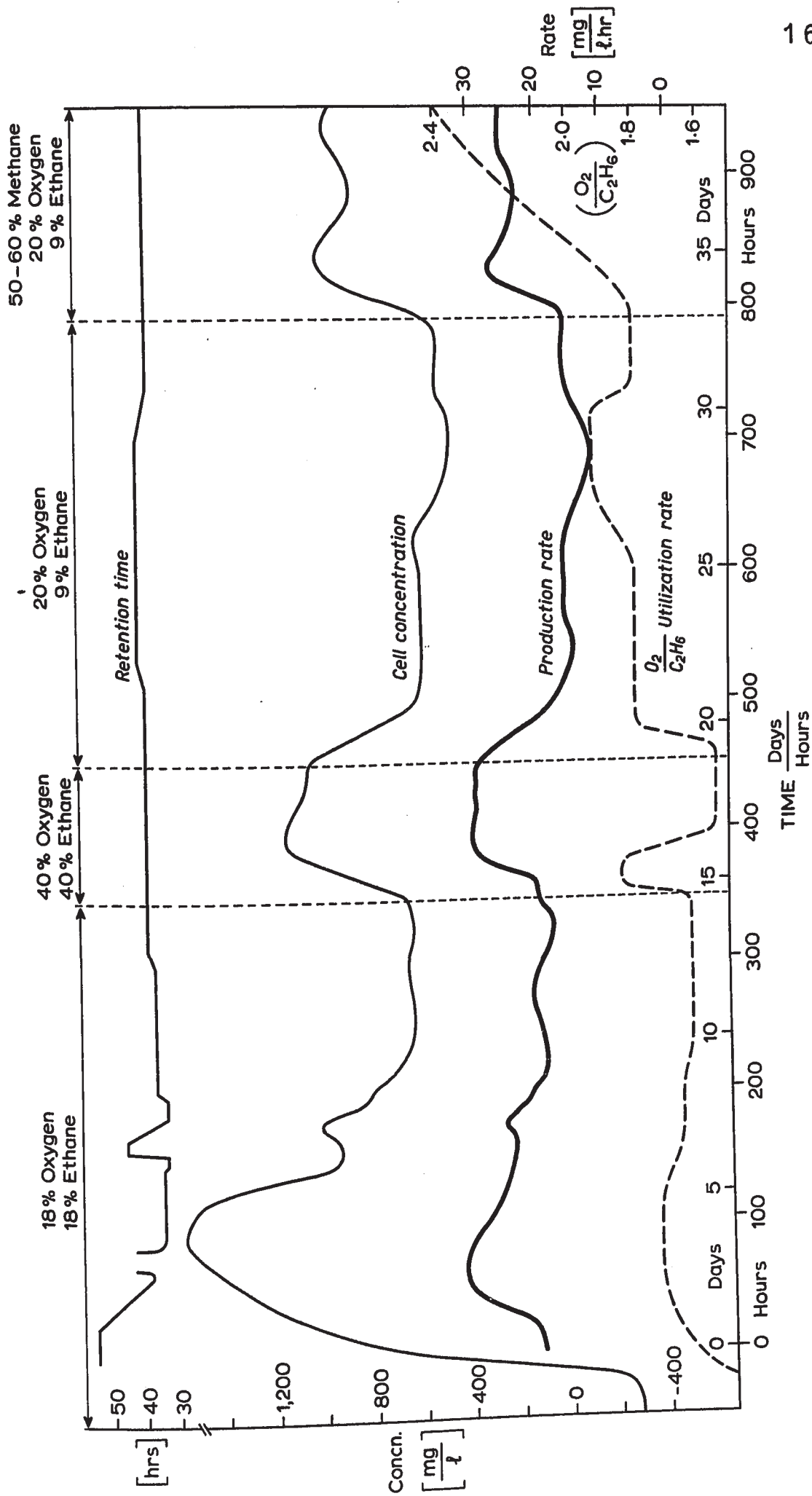
Experiment was conducted for 39 days with steady pH 4.5. Retention time (dilution rate) was adjusted as shown on the diagram.

Depending on the concentration of gases maintained in the recycle gaseous phase, the experiment was divided into 4 time periods:

<i>1st period</i>	-	<i>18% oxygen, 18% ethane</i>
<i>2nd period</i>	-	<i>40% oxygen, 40% ethane</i>
<i>3rd period</i>	-	<i>20% oxygen, 9% ethane</i>
<i>4th period</i>	-	<i>20% oxygen, 9% ethane, 50-60% methane</i>

Nitrogen balanced the gaseous mixture to 100%. Oxygen-ethane mixture was metered into the system as required to maintain the concentration of gases.

- The biomass concentration was measured (dry weight)
- The gaseous mixture utilization rate was measured
- The production rate was calculated



Continuous culture with gaseous phase recycle and control

FIGURE 37

The composition of the gases was altered again after 455 hours of continuous culture. Adjusting oxygen concentration to 20% while ethane was adjusted to 9% by volume a perturbation to the system was introduced. This resulted in oscillations in biomass concentration until a new steady state was established. The biomass concentration decreased in this period to an average of 550 mg/l which gave a production rate of 13 mg/l.hr. There was a slight increase in retention time over this period which varied from 37 to 40 hours (dilution rate  $0.027 - 0.025 \text{ hr}^{-1}$ ). The change in gas phase composition was also followed by an increased oxygen to ethane consumption ratio. The value was 1.8. A further increase in this ratio to 1.9 corresponds to a minimum in cell concentration as seen in the Figure 37.

After 790 hours of continuous culture, pure methane was introduced to the system replacing part of nitrogen and making up 50-60% by volume of the recycle gaseous phase. The oxygen and ethane were 20% and 9% by volume respectively. The presence of methane resulted in a significant increase in biomass synthesis. After an initial oscillatory response the cell concentration reached 930 mg/l and the production rate, based on a 37 hours retention time, averaged 25 mg/l hr. The oxygen to ethane consumption ratio increased to ~ 2.4 during this period. However, accurate quantitative evaluation was not possible during this study because of difficulties encountered in controlling the multicomponent gaseous mixture.

According to the recycle gaseous mixture composition in the system the entire continuous culture experiment can be divided into four

periods.

During the first period when the concentrations of both oxygen and ethane were 18% the biomass yield was ~ 0.68 g per 1 g of ethane utilized. The same yield was observed also during the third period with oxygen and ethane concentrations being 20% and 9% respectively. When concentrations of oxygen and ethane were maintained at 40% each (second period) the biomass yield observed was 0.46 g of dry mycelial tissue per 1 g of ethane.



## CHAPTER XI

### ANALYSES OF GRAPHIUM SP. BIOMASS

The Graphium sp. was grown under specific cultural conditions on different substrates. The tissue recovered from each fermentation run was recovered by filtration and freeze-dried. Samples were taken and numbered as described in the chapter on Materials and Methods paragraph on Biomass analyses - culture conditions. Each of eight samples of biomass grown under specified conditions was analysed for organic nitrogen by micro-Kjeldahl method and another portion was, after acid hydrolysis, assayed for the complete spectrum of amino acids present in its protein fraction.

In addition the lipid portion of the biomass recovered from Experiment 1 (Chapter IV, Table 11) was analysed in some detail. In this experiment the culture of Graphium sp. was grown under typical growth conditions with natural gas being the only source of carbon and energy.

The amount of inorganic substances present in the biomass

was determined by ashing several samples. Ash ranged from 9 to 12% of dry weight of the cellular material. The amount of carbohydrate and fibrous matter was calculated by difference.

#### NITROGEN DETERMINATION AND AMINO ACID SPECTRUM OF GRAPHIUM PROTEIN

The samples were analyzed in triplicate for determination of organic nitrogen in the tissue of Graphium sp. culture. Calculation of the amount of crude protein was made by multiplying the figure for nitrogen by 6.25. Averaged results are shown in Table 22.

The highest amount of protein (~55%) has been detected in the mixed culture which usually runs about 5% higher than the pure culture protein content. A typical value for protein content of Graphium biomass recovered from pure culture fermentation averaged from 46 to 50% of dry tissue weight. The biomass grown on ethyl alcohol as carbon substrate was shown to contain surprisingly low amount of protein of ~36.6%. It should be kept in mind, however, that this was the only fermentation completed with ethanol substrate. Under other type growth condition the ethanol substrate might possibly give improved yields.

Amino acid spectrum was taken for all eight samples each of them cultivated under different conditions. Analysis was done with TECHNICON AUTOMATIC AMINO ACID ANALYZER, except for tryptophan which is destroyed on acid hydrolysis. Tryptophan was assayed by the method of Miller (1967). Table 23 summarizes the results on the amino acid analyses.

TABLE 22

NITROGEN CONTENT OF GRAPHIUM SP.

Culture Experiment No.	Culture Conditions	Amt. of Nitrogen [%] dry weight	Calculated Amt. of Protein [%] dry weight
1	Pure culture on natural gas	7.45	46.5
2	Mixed culture on natural gas	7.22	45.1
3	Pure culture on natural gas + 0.4% Y.E.	7.74	48.3
4	Pure culture on ethanol	5.86	36.6
5	Mixed culture on n.g. in pilot plant	7.55	47.2
6	Pure culture on ethane	7.80	48.7
7	Continuous culture I on natural gas	7.75	48.4
8	Continuous Culture II on natural gas	8.02	50.1
	Mixed culture from continuous-flow enrichment system	8.78	54.9

TABLE 23

AMINO ACID COMPOSITION OF GRAPHIUM PROTEIN

Sample No. Amino Acid	% of crude protein (nitrogen x 6.25)								F.A.O.
	1	2	3	4	5	6	7	8	
Aspartic Acid	8.26	8.37	7.81	7.17	7.70	7.11	11.61	11.7	
Threonine	5.09	5.3	4.84	5.09	4.91	3.81	1.81	1.73	2.9
Serine	4.12	4.76	4.41	4.64	4.62	3.48	0.37	0.29	
Asparagine									
Glutamic Acid	11.42	12.3	12.10	13.12	9.84	11.02	13.96	13.92	
Glutamine									
Proline	4.07	3.95	3.31	4.38	3.81	3.93	4.9	4.89	
Glycine	4.47	4.25	4.26	4.34	4.01	5.71	6.82	6.98	
Alanine	5.47	5.53	6.02	5.32	5.86	5.87	6.92	6.81	
Cystine	0.3	0.41	t	1.47	0.49	-	0.41	0.16	
Valine	5.14	4.93	4.81	4.28	4.86	5.51	6.66	6.61	4.3
Methionine	0.8	1.05	0.85	1.01	1.21	1.01	1.98	1.45	2.3
Isoleucine	4.61	4.53	4.0	3.47	4.21	3.92	5.22	5.12	4.3
Leucine	9.04	10.1	8.45	8.76	9.91	5.88	8.23	8.39	4.9
Unknown	4.31	5.98	4.68	6.57	6.87	-	-	-	
Tyrosine	4.30	3.75	5.05	3.95	3.99	3.46	2.64	2.19	
γ Amino But. A.	0.15	t	0.95	0.49	1.06	-	0.55	0.57	
Phenylalanine	3.68	3.55	3.14	2.66	3.30	3.21	4.63	4.65	2.9
Histidine	4.84	3.98	3.84	4.07	3.74	3.42	2.66	2.57	
Tryptophan	1.21	1.23	1.12	0.98	1.33	0.96	1.23	1.19	1.4
Ornithine	0.18	0.18	0.27	0.28	0.24	-	0.58	0.83	
Lysine	6.38	6.47	6.13	4.79	6.14	7.47	8.12	8.91	4.3
Ammonia	4.34	3.35	4.31	4.80	3.85	7.37	5.65	6.75	
Arginine	5.46	5.62	4.92	4.94	4.98	5.12	5.71	5.36	
Unknown	1.46	0.65	2.47	1.57	1.01	-	-	-	

It is seen that the composition of amino acids in the Graphium biomass differs slightly with changes in culture conditions. In comparison with the "ideal protein" as recommended by F.A.O. one can see that essential amino acids are even higher than required with lysine being particularly attractive in the range from 6.1 to 8.9% of crude protein of Graphium. Methionine and tryptophan are slightly lower which is commonly encountered in microbial proteins.

In general this chemical analysis establishes that the single cell protein from Graphium sp. culture is nutritionally sound. However, its true nutritional value remains to be investigated in vivo since feeding efficiency and toxicity are not known.

#### LIPID FRACTION OF BIOMASS EXTRACTION AND ANALYSIS

##### 1) Extraction of lipids

A sample of biomass recovered from experiment 1 (Table 11) was extracted with a chloroform-methanol mixture (2:1). Three different extraction procedures were applied:

- 1) The cellular material was sonicated for 10 minutes in the solvent mixture and then extracted twice for three hours with fresh solvent in an Erlenmeyer flask placed on a shaker.
- 2) The cellular material was vigorously shaken for 48 hours in a round bottom flask containing the solvent, glass powder and glass beads.

3) The biomass was extracted in a Soxhlet reflux apparatus for 48 hours. During this period the extracted material was heated at approx. 70°C.

After finished extraction the solvent was evaporated under vacuum at 35°C, and each sample redissolved in chloroform. The proportions of chloroform-soluble and insoluble materials extracted by each method are presented in Table 24. The chloroform-soluble extract amounted to approximately 9 - 9.8% of the total dry weight of the biomass. This fraction of lipids was further analysed.

Solvent extraction was also carried out with the sample of biomass from Experiment 6 (Table 11). The mycelial tissue of Graphium sp. cultivated on pure ethane (Exp. 6) gave considerably lower amounts of lipid extract (~4% by weight) as compared to the results of extraction of the biomass recovered from Experiment 1 where natural gas was the source of carbon and energy. No further lipid analysis was completed on the biomass from Experiment 6.

## 2) Column chromatography

The chloroform-soluble lipid fraction obtained by extraction of the biomass from Experiment 1 was chromatographed on an acid-treated Florisil (ATF) column. Three fractions were eluted from the column with three different solvents:  $\text{CHCl}_3$  (fraction A),  $\text{CHCl}_3$ -MeOH (1:1) - fraction B, and  $\text{CHCl}_3$ -MeOH (3:1) - fraction C, respectively. The amounts of lipid material in each of the A, B, and C fractions are shown in Table 25 for

TABLE 24

SOLVENT EXTRACTION OF GRAPHIUM BIOMASS

Fraction	SAMPLE - EXP. 1		
	Sonified	Glass Powder	Soxhlet
CHCl <sub>3</sub> -MeOH biomass extract [% w/w]	13.35	12.2	13.61
CHCl <sub>3</sub> -soluble biomass extract [% w/w]	9.85	8.92	9.25
% of chloroform - soluble fraction in the CHCl <sub>3</sub> -MeOH extract	74	73	68

TABLE 25

NEUTRAL AND PHOSPHOLIPID FRACTION PROPORTIONS  
Acid-treated Florisil chromatography of CHCl<sub>3</sub>-soluble extract of the lipids from Graphium sp.

Fraction	SAMPLE - EXP. 1	
	Soxhlet	Sonified
Amount of material applied [mg]	185	197
CHCl <sub>3</sub> eluted fraction "A" [%]	18.7	25.3
CHCl <sub>3</sub> + MeOH (1:1) eluted fraction "B" [%]	63.4	56.7
CHCl <sub>3</sub> + MeOH (3:1) eluted fraction "C" [%]	17.9	18.0
Phospholipids (B+C) [%]	81.3	74.7
Recovery [%]	90	86

two extraction methods (Soxhlet and sonification).

The extraction of the sonicated biomass gave greater amounts of lipids eluted in fraction A (25.3%) as compared to the size of fraction A (18.7%) obtained from the Soxhlet extracted biomass. The phospholipids in the Soxhlet extract constituted 81.3% of the total chloroform-soluble fraction, while the phospholipids of the sonicated biomass comprised only 74.7%.

### 3) Thin layer chromatography (TLC)

All lipid fractions obtained by column chromatography as well as the non-fractionated chloroform-soluble extract were analysed by TLC.

The fraction A (eluted from the column with chloroform) and the fraction B (eluted with 1:1  $\text{CHCl}_3$ -MeOH mixture) were chromatographed in a petroleum ether-ether-acetic acid system. Standards applied were cholesterol,  $\text{C}_{15}$  fatty acid, diglyceride and triglyceride (tripalmitin). The results of this TLC are shown in Figure 38, plate 1

Three major components were found in the fraction A with  $R_f$  values corresponding to those of triglycerides, free fatty acids and cholesterol respectively. This, however, cannot be considered as the confirmatory evidence of the standard compounds being identical with the three components in the fraction A. Particularly the bottom spot of the fraction A could be considered as being 1,2- diglyceride although its  $R_f$  value in



this system is identical with that of cholesterol. Plate 2 in Figure 38 developed in a  $\text{CHCl}_3$ -MeOH-Ammonia system, offers more evidence that one of the components in the fraction is diglyceride. Cholesterol is not very likely to be found in microbial lipids and further specific tests would be required to establish its presence in the lipid extract of the biomass of Graphium sp. Fraction A was consisting mainly of neutral lipids but detail analysis was not completed.

The phospholipids eluted in the fraction B were composed of many components as seen in Figure 39. A whole spectrum of components was shown on the TLC plate developed in a  $\text{CHCl}_3$ -MeOH- $\text{H}_2\text{O}$  system (plate 1). The major component had the same  $R_f$  value as phosphatidyl ethanolamine. The identity of the two compounds was further confirmed in a MeOH- $\text{CHCl}_3$ -Acetone-Acetic Acid-Water system (plate 2).

The fraction C (eluted with methanol) contained only a single component. Its  $R_f$  value was identical with that of phosphatidyl choline (PC) in a  $\text{CHCl}_3$ -MeOH-Water system (Figure 39, plate 1) as well as in a  $\text{CHCl}_3$ -MeOH-Ammonia system (Figure 40, plate 1). Further confirmation of the fraction C consisting of PC only was done by two-dimensional TLC. The two compounds were not separable as shown in Figure 40, plate 2.

#### 4) Gas-liquid chromatography of fatty acids (GLC)

The chloroform-soluble lipid extract of Graphium biomass

## F I G U R E 3 8

THIN LAYER CHROMATOGRAM OF FRACTION "A" FROM  
CHCl<sub>3</sub> - SOLUBLE PORTION OF GRAPHIUM SP. BIOMASS

Fraction "A" eluted from ATF column with CHCl<sub>3</sub>  
is compared with authentic standards on the two  
TLC chromatograms.

Plate 1 was developed in  
pet. ether - ether - acetic acid (65:40:1)

Plate 2 was developed in  
CHCl<sub>3</sub> - MeOH - 28% Ammonia (65:35:5)

The spots were visualized by H<sub>2</sub>SO<sub>4</sub> spray.

B - fraction "B"  
Chol - cholesterol standard  
FA - fatty acid standard (C<sub>15</sub>)  
A - fraction "A"  
DG - 1,2 - diglyceride standard  
TG - triglyceride standard (tripalmitin)

THIN LAYER CHROMATOGRAPHY  
*Graphium* sp. chloroform-soluble  
 lipid fractions.

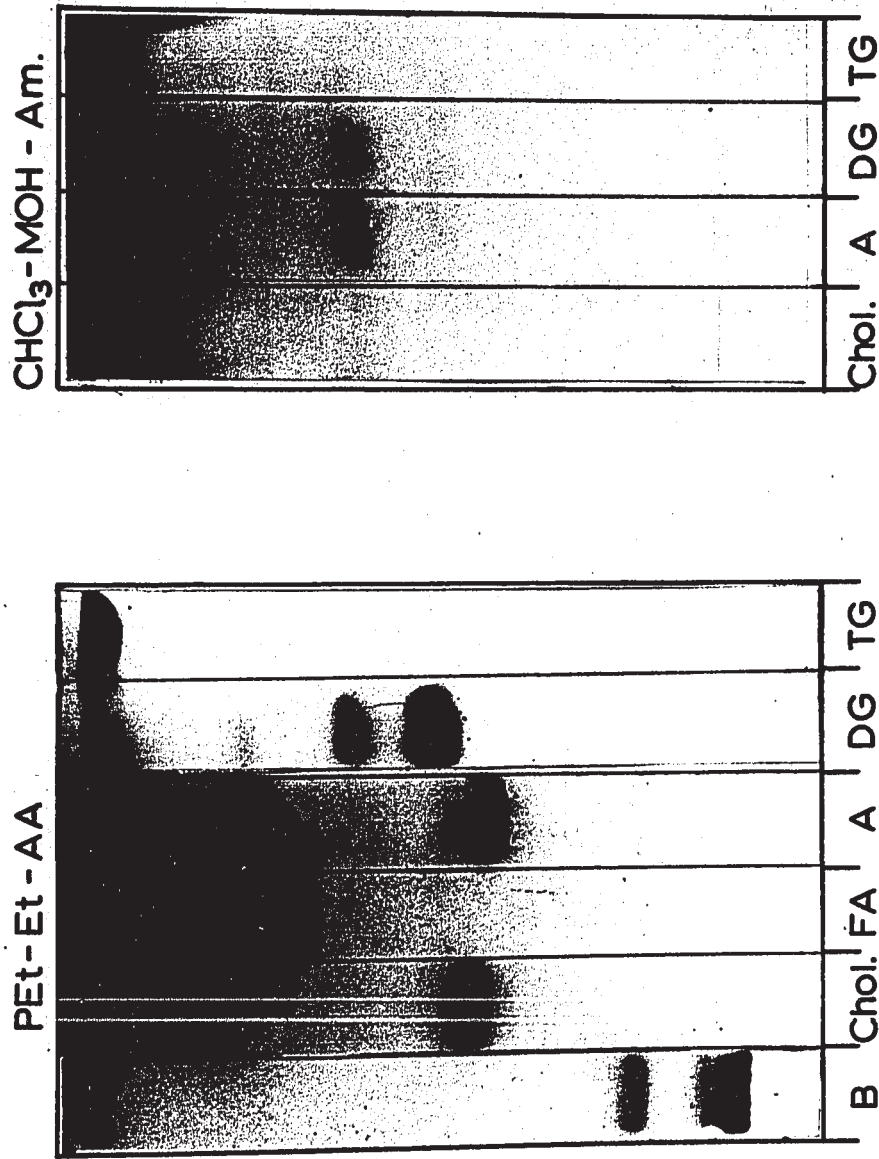


Plate 1

Plate 2

FIGURE 38

## F I G U R E 3.9

THIN LAYER CHROMATOGRAM OF FRACTION "B" AND "C"  
FROM CHLOROFORM-SOLUBLE PORTION OF GRAPHIUM SP.

## BIOMASS

The fractions separated by ATF column chromatography as compared with authentic standards are presented on the two TLC chromatograms.

Plate 1 was developed in

$\text{CHCl}_3$  - MeOH -  $\text{H}_2\text{O}$  (65:20:3)

Plate 2 was developed in

$\text{CHCl}_3$  - Acetone - MeOH - Acetic Acid -  $\text{H}_2\text{O}$   
(5:2:1:1:0.5)

The plates were visualized by  $\text{H}_2\text{SO}_4$  spray.

Chol - cholesterol standard

A - fraction "A"

B - fraction "B"

C - fraction "C"

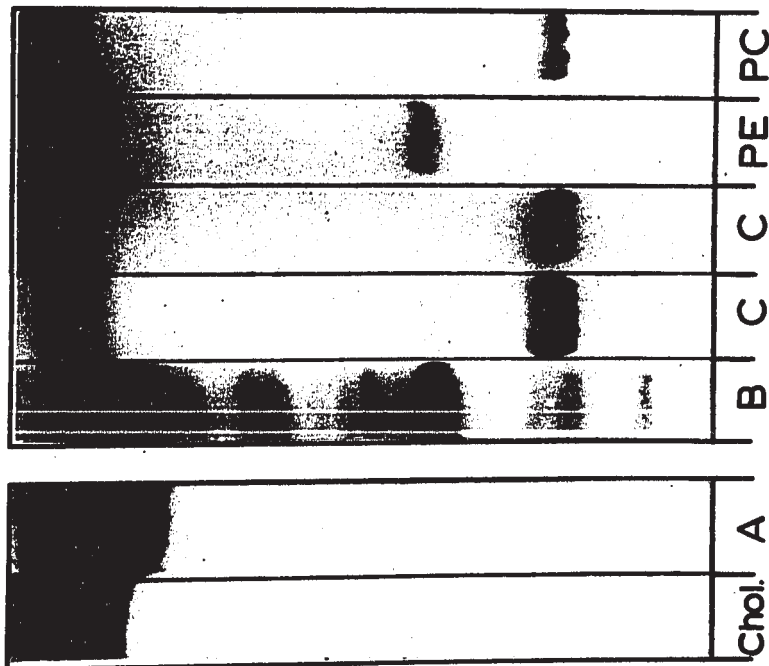
PE - phosphatidyl ethanolamine standard

PC - phosphatidyl choline standard

THIN LAYER CHROMATOGRAPHY

Graphium sp. chloroform-soluble lipid fractions

CHCl<sub>3</sub> - MOH - H<sub>2</sub>O



CHCl<sub>3</sub> - MOH - Act - AA - H<sub>2</sub>O

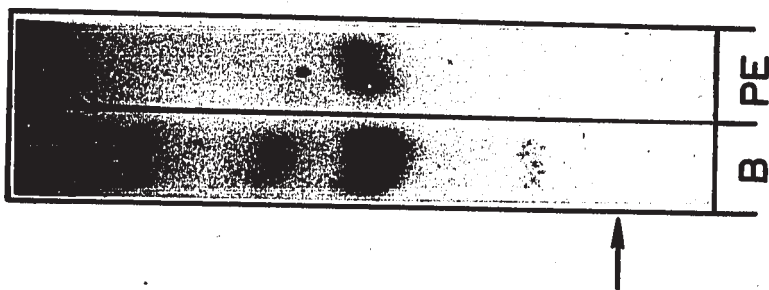


Plate 1

Plate 2

## F I G U R E 4 0 -

THIN LAYER CHROMATOGRAM OF FRACTION "C" FROM  
CHCl<sub>3</sub> - SOLUBLE PORTION OF GRAPHIUM SP. BIOMASS

Plate 1 was developed in CHCl<sub>3</sub> - MeOH - 28% Ammonia  
(65:35:5)

Plate 2 was developed in two dimensions :

First dimension: CHCl<sub>3</sub> - MeOH - 28% Ammonia  
(65:35:5)

Second dimension:

CHCl<sub>3</sub> - Acetone - MeOH - Acetic Acid - H<sub>2</sub>O  
(5:2:1:1:0.5)

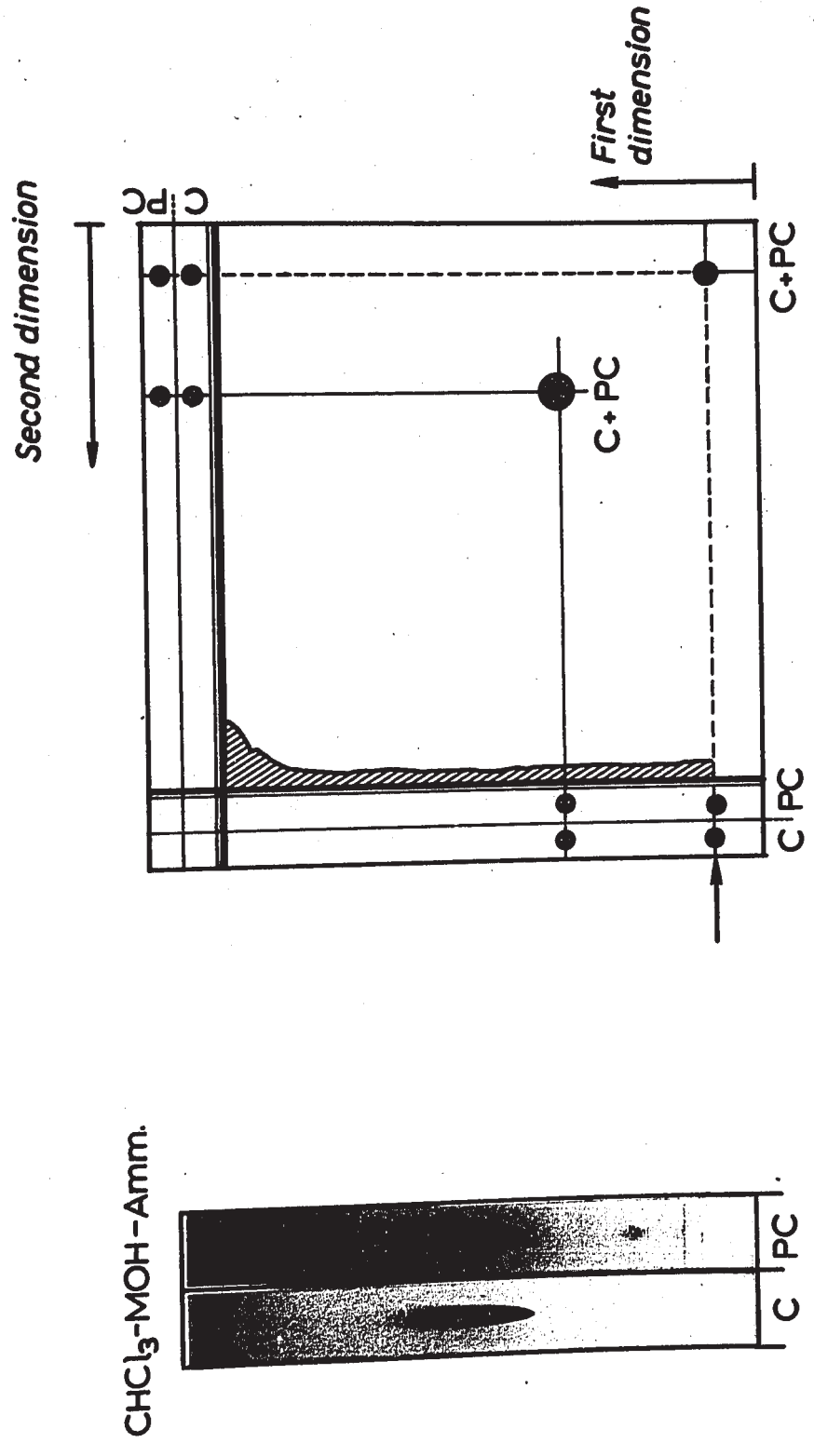
The plates were visualized by H<sub>2</sub>SO<sub>4</sub> spray.

C - fraction "C"

PC - phosphatidyl choline standard

THIN LAYER CHROMATOGRAPHY

Graphium sp. chloroform-soluble lipid fractions



TWO-DIMENSIONAL TLC

FIGURE 40



was analysed for the content of fatty acids. The fraction A composed of neutral lipids and the fraction C containing phospholipids (phosphatidyl choline) were also examined. Following transmethylation of the lipid extract the resulting mixture of methyl esters of fatty acids was injected into a Beckman gas-liquid chromatograph model GC-45. Separation of the C<sub>6</sub> to C<sub>18</sub> methyl esters was obtained on 5% EGSS-X with Chromosorb W column at 184°C.

Quantitative evaluation of the amount of major fatty acids in the Graphium lipid extract is shown in Figure 41.

Oleic acid (18:1) is the major fatty acid averaging 47% of the total fatty acid content while stearic (18:0) and palmitic (16:0) are 19.9% and 18.7% respectively. One of the essential fatty acids, linoleic acid (18:2) makes ~3.5%. Other fatty acids have been detected in small or trace quantities. The sum of the unsaturated fatty acids makes up ~56%, while saturated fatty acids account for ~44% of the total fatty acid content.

The amount of fatty acids in the lipid extract as compared to the fraction A and fraction C fatty acids is shown in Table 26.

Considerably lower amounts of palmitic acid was found in the fraction C (8.85%) in comparison to that in the fraction A whereas oleic acid in the fraction C was higher (56.5%) than in the other two samples analysed. The essential linoleic acid was highest in the fraction A (4.28%).



**FATTY ACID COMPOSITION OF  
GRAPHIUM TISSUE LIPIDS**

Total lipids in the tissue :  
4-10% of dry weight.

(Amount of fatty acids in % of total)

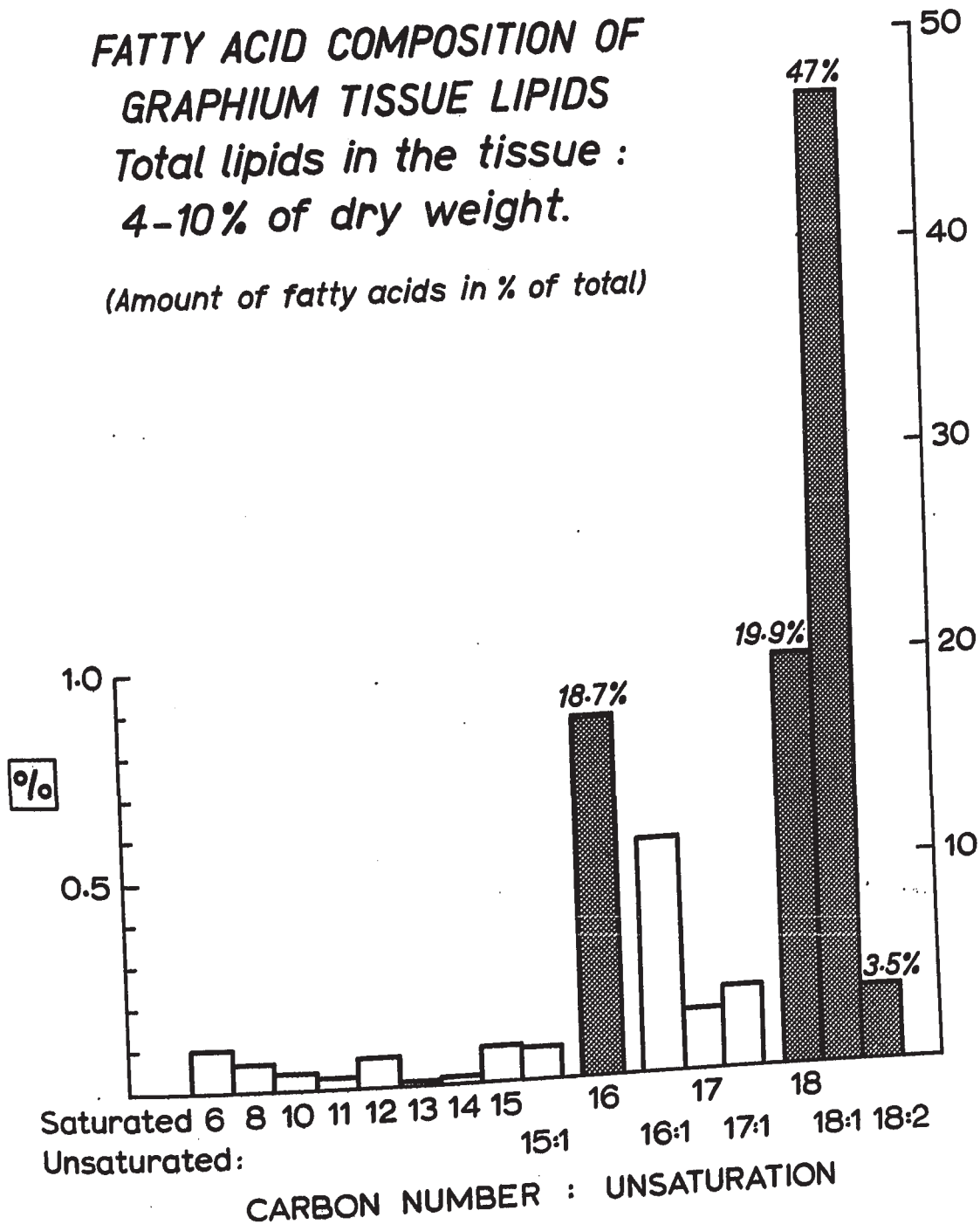


FIGURE 41

TABLE 26  
 FATTY ACIDS OF THE LIPIDS OF GRAPHIUM SP.  
 [% of total amount]

Fatty Acid	Lipid Extract	Fraction A	Fraction C
C <sub>6</sub>	0.10	-	-
C <sub>8</sub>	-	-	0.10
C <sub>10</sub>	-	0.10	-
C <sub>12</sub>	-	0.47	-
C <sub>14</sub>	0.16	0.31	-
C <sub>15</sub>	-	0.14	-
C <sub>16</sub>	18.70	18.31	8.85
C <sub>16</sub> 1	0.57	0.82	0.61
C <sub>17</sub>	0.15	0.21	0.11
C <sub>17</sub> 1	0.20	0.20	0.31
C <sub>18</sub>	19.90	22.73	21.5
C <sub>18</sub> 1	47.00	44.55	56.5
C <sub>18</sub> 2	3.49	4.28	3.9

Graphium sp. grown on natural gas (EXP. 1 - Table 11)

## CHAPTER XII

### SAFETY EVALUATION OF GRAPHIUM SP. BIOMASS PRELIMINARY FEEDING STUDY IN RATS

Short term screening tests in rats for both obvious toxic or ill-effects and for the nutritive value are described.

#### Experimental

The biomass examined in this study was that obtained from mixed culture cultivation of Graphium sp. growing on natural gas in aqueous C-medium containing  $(\text{NH}_4)_2\text{SO}_4$ . A 400 l pilot plant fermentor was used for both continuous-flow as well as batch cultivations. The biomass was recovered by filtration, freeze-dried, powdered and mixed to attain uniform composition.

The biomass was incorporated into the experimental synthetic diets at specified levels (Table 27). Fresh batches of diet were prepared every second week and kept at room temperature.

Two experiments were conducted one being short term (7 days), the other was extended over a period of 5 months. At the end of

TABLE 27  
DIET COMPOSITION FOR SHORT TERM  
TOXICITY STUDIES

Group	PROTEIN		CARBOHYDRATE	FILLING
	Source	Amount [%]	Dextrose [%]	CellufLOUR [%]
A (control)	Casein	20	60	20
B	<u>Graphium</u> Biomass	40	60	-
C	<u>Graphium</u> Biomass	100	-	-
D	<u>Gr.</u> Biomass Casein	10 15	55	20
E	<u>Gr.</u> Biomass Casein	20 10	50	20
F	<u>Gr.</u> Biomass Casein	30 5	45	20
G	<u>Gr.</u> Biomass Casein	40 0	40	20

Salt mixture and water soluble vitamins were added to each diet in even amounts.

The average amount of crude protein in raw dried Graphium Biomass was ~47%.

5 months those rats not sacrificed for study were transferred back to a standard ration with no Graphium biomass added. They will be under observation for longer periods of time (~1 year) should any tumors develop.

A) Short term toxicity study

One month old male rats (Sprague-Dawley) were used for this experiment. The diet was prepared for each group of animals as shown in Table 27. There were two rats in each group. The experiment was conducted for 7 days. The animals were weighed and the food intake was measured every day over the period of one week. Table 28 shows the total weight gain and the amount of food eaten by the rats in a 7 day period.

In the course of these experiments no abnormalities were observed in animal behaviour or appearance. Not even rats living on a diet of 100% Graphium biomass showed any ill signs. They, however, lost weight but this was attributed to very small amounts of the diet actually eaten.

After 1 week both animals from each group were killed by chloroform and examined at an autopsy for gross pathological changes. No abnormalities were observed. Based on these experiments it was concluded that there were no acute toxic effects of a diet of a biomass of Graphium which would appear during the short term exposure.

TABLE 28  
 SHORT TERM TOXICITY STUDIES WITH RATS  
 WEIGHT GAIN AND FOOD INTAKE [g]  
 OVER 7 DAY PERIOD

GROUP RAT	A		B		C		D		E		F		G	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Initial Weight	65	70	63	72	70	69	112	102	98	110	105	86	84	86
Terminal Weight	106	116	73	78	62	51	153	136	120	132	140	110	104	106
Weight Gain	41	46	10	6	-8	-18	41	34	22	22	35	24	20	20
Average Wt. Gain	43		8		-12		38		22		30		20	
Av. Food Intake (per 2 rats)	166		126		52		207		174		162		133	

B) Preliminary feeding value assessment

Male rats (Wistar) 4 weeks old were used for this experiment. The diet was prepared for each group of six animals as shown in Table 29.

TABLE 29

DIET COMPOSITION FOR PRELIMINARY GRAPHIUM BIOMASS NUTRITIONAL VALUE ASSESSMENT

Group	PROTEIN		CARBOHYDRATE	FILLING
	Source	Amount [%]	Dextrose [%]	CellufLOUR [%]
1	Casein	20	60	20
2	<u>Graphium</u> Casein	20 10	50	20
3	<u>Graphium</u> Biomass	40	40	20

Salt mixture and water soluble vitamins were added in even amounts.

The experiment was conducted over a period of 5 months. Three animals of each group were killed after 100 days and the rest maintained on their respective rations. The animals were weighed every week and the resulting average weight for each group plotted (Figure 42). Although not showing any ill signs the group on 40% Graphium (No.3) gained

# RATS ON Graphium BIOMASS

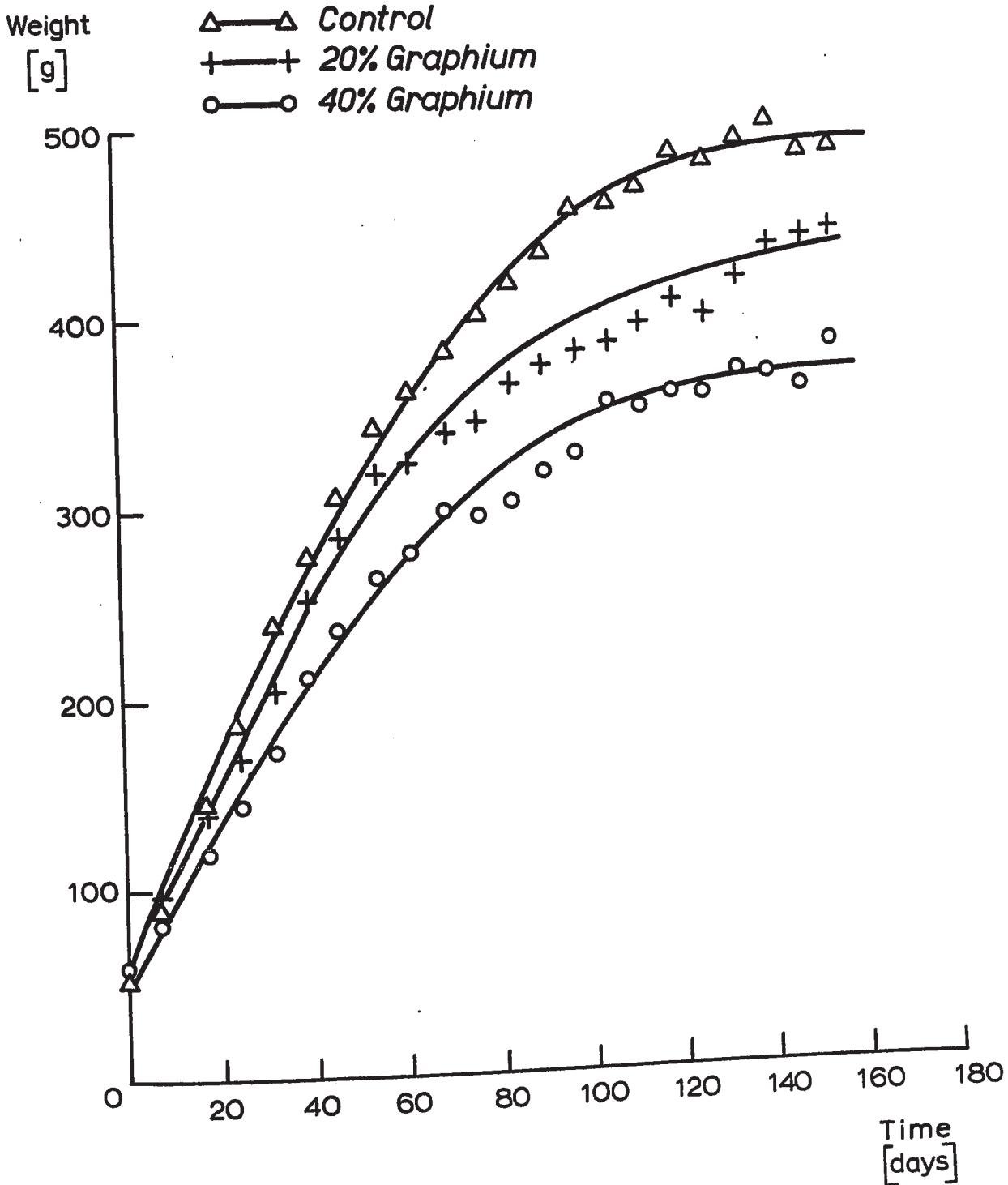


FIGURE 4.2.



weight at a lower rate as compared to group No. 2 and the control group, No. 1.

By increasing the amount of Graphium biomass in the ration and decreasing the casein in the diet, the weight gain decreased.

The animals sacrificed after the 100 day period were examined by an autopsy and no apparent pathological changes of any of the organs were observed. The weights of selected organs were recorded and average is shown in Table 30. Differences in absolute and relative organ weights were randomly distributed between the various groups and none of the minor changes appeared to be related to diets containing Graphium sp. biomass.

Sections of the tissues and organs embedded in paraffin from all the animal groups were stained with haematoxylin and eosin for microscopic observation. No gross histological changes were observed of the examined tissues which were comparable to those of the control animals.

The remaining rats were kept on their respective diets to complete a 5 month feeding study. Appearance of the test animals as compared to the control ones' after this period was quite normal as seen in Figure 43. The animals in group 3 which had the lowest average weight were slightly smaller. The difference, however, can only hardly be seen in Figure 43d where the control rat can be compared to the one from group 3 fed by 40% Graphium.

TABLE 30

WEIGHT OF RAT ORGANS

ORGAN GROUP	TOTAL BODY WEIGHT	LIVER	HEART	KIDNEYS	PANCREAS	SPLEEN	TESTES	ADRENALS	PITUITARY	THYMUS
CONTROL (1) Average wt. [mg]	454000	16529	1264	2967	1400	866	3162	55	8	517
20% BIOMASS (2) Average wt. [mg] % of control	381000 84	13392 81	968 77	2882 97	1326 95	620 72	3100 98	56 102	8.5 106	451 87
40% BIOMASS (3) Average wt. [mg] % of control	328000 73	13961 84	938 74	2759 93	1301 93	516 60	3193 101	58 105	8 100	418 81

The results of the preliminary feeding studies completed so far did not reveal any deleterious effects of feeding rats on diets containing up to 40% of fungal biomass grown on natural gas.

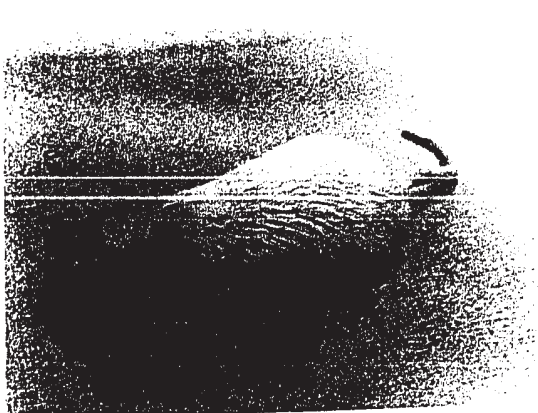


(a) Control on casein

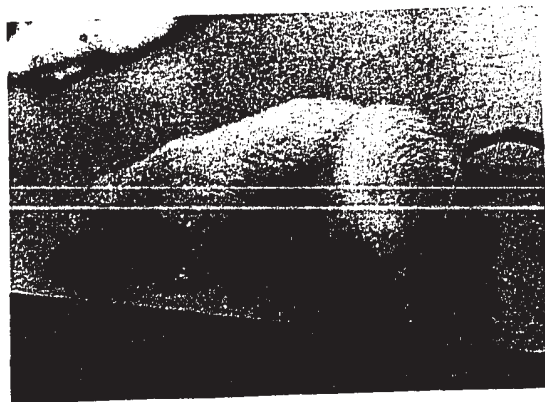


(c) 40% Graphium biomass

RATS AFTER 5 MONTHS ON THE DIET



(b) 20% Graphium biomass



(d) Control and 40% G.biomass

FIGURE 43

## CHAPTER XIII

### DISCUSSION

A culture identified as a species of Graphium has been isolated which is capable of oxidizing and growth upon natural gas, ethane, propane and butane as a sole source of carbon and energy. The fungus was selected by a continuous-flow enrichment technique. The system was originally inoculated with raw sewage and was placed in operation primarily to isolate some natural gas-oxidizing bacteria. Bacteria capable of utilizing methane or natural gas have been known since the beginning of this century (Sohngen, 1906). They were subsequently re-classified by Orla-Jensen (1909) and new strains and varieties described mainly by Foster and Leadbetter and Dworkin (1956, 1957, 1958, 1959). The unique continuous-flow enrichment mixed culture system lead to isolation of the first fungal culture growing on natural gas and also provided a mixed culture ecological system with special properties. A methane-oxidizing bacteria actually predominated in the mixed system during the initial phase of study. A decrease in the pH of the ferment-

tation broth and an increase in aeration introduced by more vigorous mixing resulted in selection of a fungus. Broader studies on the mixed culture system were regarded as beyond the scope of this thesis. The main interest has focussed on the new fungal culture which proved to be the predominating culture in the natural gas continuous-flow enrichment system. This culture was capable of utilizing gaseous hydrocarbon substrates as a sole source of carbon and energy.

It was tentatively identified as a species of Graphium (Zajic et al., 1969; Appendix). No attempt could be made at this stage to identify to the species level the new isolate.

It was isolated from raw sewage several times in repeated attempts using natural gas continuous-flow enrichment technique. There have been no previous reports on fungi able to grow on natural gas and capable of using gaseous hydrocarbons as a sole carbon source.

Graphium sp. grows on a mineral salts medium with a 40% natural gas and 60% air mixture sparged into the fermentation broth. The dispersed type of growth is suitable for large scale production. The biomass concentration and the production rate of biomass can be further increased by optimization of various growth parameters. This has not been completed at this stage of work and further efforts should be directed towards improving biomass production process if it is to be considered feasible for further scale-up.

Because of its high protein content (43-55%) Graphium sp. has been considered to be a new potential source of single-cell protein.

BATCH CULTURE TECHNIQUE

Studies on batch growth of Graphium sp. were carried out in two subsequent stages - in shake flasks and in laboratory stirred tank-type fermentors.

A supporting aqueous mineral salt medium (Johnson and Temple, 1962; Coty 1967) was selected to keep the supporting medium simple, no trace metals were added and tap water was used as make up water. Further optimization of medium components has not been made since objectives were directed toward engineering problems of the growth of Graphium sp. rather than medium optimization.

The optimal initial pH in batch system is 4.0 - 4.5 with good growth observed over a pH range of 3.5 - 7.5. This optimal pH if maintained throughout the entire fermentation gives the highest concentration of biomass in broth and also the highest rate of growth. The low pH is attractive because not many microorganisms tolerate this low pH and secondary contamination is reduced.

Ammonium sulphate is superior to any other source of nitrogen. However, in a shake flask system higher terminal concentrations of tissue were observed on C-medium containing nitrate. This was obviously due to the pH change which followed the pattern as depicted in Figure 26 which gave a terminal value of ~7.6. The use of ammonium sulphate

in C-medium resulted in sharp decrease of pH value in shake flasks, where no pH control was applied. The low pH values ~2.8 - 3.2 affected inversely the terminal tissue concentration.

The type of growth was also dependent on the culture pH and source of nitrogen used. Clumping was more pronounced in nitrate containing media.

The experiments carried out in the stirred tank-type fermentor where pH control was applied throughout the whole fermentation period were directed at determining optimal pH and other parameters for growth. This information was needed for continuous-flow technique application where knowledge of growth rate, specific growth rate and other parameters is required to established optimal conditions for maintaining effective steady state by adjusting the proper dilution rate. Most of the basic data for continuous culture system are derived from preliminary batch culture studies (Malek and Fenc1, 1966).

Under favorable conditions an exponential phase in batch culture is completed in less than 4 days in shake flasks. A shorter time of 2 to 2.5 days is needed in stirred tank-type fermentor. This is assoc-



iated with better mixing resulting in improved mass transfer between a mixture of oxygen and substrate in gaseous phase and the growing micro-organism. Shear forces exerted by the impeller also help keep the culture growth in a dispersed form rather than growing in pellet form as it does in shake flasks. Dispersed growth pattern is also aided by the addition of suitable surface active agent which also contributes to better bubble dispersion accompanied by increased interphase area resulting in improved gas-liquid mass transfer. Selection of the surfactant (antifoam) is very important for it should not have any secondary effects which would inhibit the growth of Graphium. The culture is particularly sensitive towards gas-liquid transfer rates since it is fully dependent on a gaseous substrate and oxygen.

Various fermentation systems like cyclone-type fermentor (Mueller, 1969), non-agitated Waldhof type fermentor, non-symmetrically shaped aerated fermentor (Humphrey, 1968) or perforated plate multistage tower fermentor (Kitai et al., 1969) using unique principles of gas-liquid contacting would be worth testing for this particular culture, although the mycelial type of growth could cause some problems which may not occur in the case of bacterial culture.

Air is a good source of oxygen. Carbon dioxide is a normal by-product of the fermentation reactions carried out by the culture of Graphium sp. It is known for example that the oxidation of methane by Pseudomonas methanica is directly influenced by the presence of carbon dioxide (Leadbetter and Foster, 1958; Johnson and Temple, 1962). The natural gas used as the sole source of carbon and energy for the culture

of Graphium sp. contains a small percentage (0.25%) of carbon dioxide as well as air that normally is used in a mixture with the gaseous substrate, so that the initial fermentation reaction described in this thesis inevitably was exposed to this constituent. This amount of CO<sub>2</sub> was found adequate to initiate good growth. However, slightly elevated concentrations of CO<sub>2</sub> (~2%) were shown to shorten lag phase. When pure gases (O<sub>2</sub> and C<sub>2</sub>H<sub>6</sub>) were used with no CO<sub>2</sub> present in the mixture, a slightly prolonged lag phase of Graphium sp. culture was noted, and variable growth responses were observed. The effect of CO<sub>2</sub> on the fermentation pattern does not appear to be related to the effect which its impact has on the pH of the supporting medium.

Pure methane if present as the only source of carbon and energy is not oxidized by the Graphium sp. Ethane mixed with air is oxidized readily. Propane and butane are also utilized. Methane added in conjunction with ethane is oxidized along with the ethane. Similar studies were not made with combinations of methane and propane and with methane-butane. This is probably another example of co-oxidation which was first described by Foster (1956). Even co-utilization of methane is possible in the system. Increased biomass concentration was observed if both ethane and methane are present in the gaseous mixture. The conclusive proof on methane presence effect could be carried out only by using C<sup>14</sup> labelled methane and by revealing the metabolic fate of the labelled carbon. This type of experiment has not been carried out in this stage of work.

Conventional carbohydrates as glucose and sucrose are readily metabolized by Graphium sp. The culture does not lose the ability to utilize gaseous hydrocarbons for growth when cultivated on carbohydrates as some of hydrocarbon-oxidizers do (Davis, 1967). Strict methane oxidizing bacteria only grow on methane and methanol (Dworkin and Foster, 1956; Foster and Davis, 1966; Ribbons et al., 1970).

Corresponding primary alcohols derived from low molecular weight gaseous hydrocarbons are utilized by Graphium. However, almost negligible growth was observed on odd number carbon alcohols while ethanol and butanol gave good yields of biomass of Graphium. This would imply that metabolic mechanism of the culture of Graphium sp. is well set for oxidizing and incorporation of two-carbon fractions.

The optimal amount of oxygen required for oxidation of ethane by microbial reaction has been theoretically calculated (Darlington, 1964; Klass et al., 1969; Bennett et al., 1969). The topic is discussed in following paragraph on tissue biosynthesis efficiency. The optimal amount of oxygen required for the oxidative reaction carried out by Graphium sp. has been determined in experiments with the recycle of gaseous phase, where it has been possible to control the composition of gaseous phase. However, in the preliminary shake flask studies various concentrations of  $O_2$  and  $C_2H_6$  were tested on a batch basis. It should be noted that the growth system in shake flask filled with certain gaseous mixture is conducted under unsteady conditions. Oxygen and the gaseous

substrate (ethane) are utilized while  $\text{CO}_2$  is liberated as the respiratory product. Concentrations of gases as well as the pressure in the system is continuously changing making conclusive evaluation of the system difficult. Also the solubility of each component of the gaseous mixture in the liquid phase should be taken into consideration. Solubilities of all the components are known (International Critical Tables, 1928; Perry, 1950). It has been suggested by Klass et al. (1969) that the composition of the gaseous phase be adjusted so that the gases dissolved would be present in stoichiometric proportions. The exact stoichiometry of the fermentation reaction has not, however, been fully revealed. The highest concentrations of Graphium sp. tissue have been observed when high concentrations of both oxygen and ethane were used (40%), although the mixture of 20%  $\text{O}_2$  and 20%  $\text{C}_2\text{H}_6$  gave satisfactory results and better economy. The conclusions drawn from the results obtained from shake flask experiments are comparable to those based on continuous culture with controlled gas phase recycle.

Increased mycelial synthesis and utilization of natural gas has been obtained by adding some growth factors such as those present in yeast extract. The culture of Graphium sp. was able to utilize yeast extract as a source of carbon and energy when grown in liquid medium on air only. If natural gas was added in conjunction with air, the yield of biomass was even higher. The effect of yeast extract addition is best described by calculating the "relative increase" in biomass which is defined as "I". The maximum relative increase in biomass concentration

was observed on addition of ~0.4% yeast extract expressed by value  $I = 6$  fold. At this particular concentration of yeast extract, the absolute increase in biomass concentration over the control was ~10 fold. Yeast extract represents a very complex mixture of variety of growth factors, organic compounds like amino acids, vitamins, etc. Components in yeast extract causing this improvement have not been studied. It was only ascertained that mineral components of yeast extract obtained by ashing did not show any effect upon the culture of Graphium sp. when added.

Excessive foaming became a serious problem in stirred tank-type fermentor where yeast extract was added into the synthetic C-medium. The conditions were greatly improved when suitable antifoam agent was found (HODAG F-28) which did not adversely affect gas-liquid mass transfer within the system. Hopefully the yields of biomass should increase even more than 10 fold as nutritional and growth factor requirements of the culture are investigated and bioengineering progress is made in handling the system.

#### CONTINUOUS CULTURE TECHNIQUE

Growth rates, rates of ethane and oxygen utilization and many other parameters had to be thoroughly studied in batch culture process to establish engineering requirements for continuous-flow production. The rate of change of some growth characteristics have been expressed in terms of first derivatives of their basic functions. Graphical methods

were used entirely to obtain the derivatives (Chapter IX). Although some error is encountered with this approach it was found satisfactory. Known mathematical expressions (logistic function) which describe a sigmoid type growth curve are known (Edwards, 1967; Edwards and Wilke, 1968) and could be used. In conjunction with computerized curve fitting program employing non-linear least squares technique experimental data can be expressed in the form of mathematical formula. Derivative functions for "rates" could be then obtained on purely mathematical basis.

Clumping and excessive biomass growth covering all stationary parts inside the fermentor has been already shown to be a common problem encountered in most fungal fermentations. This makes introduction of continuous culture techniques to this type of fermentation especially difficult from a technical and engineering viewpoint. The surface growth of mycelium eventually plugs addition ports, control devices and electrodes. Termination of the whole continuous process is often unavoidable for the above reasons. Baffled stirred-tank type fermentors do not seem to be a satisfactory arrangement for fungal fermentation. Smooth walls are often recommended for a fermentation vessel where this problem is encountered. Also, all the stationary parts extended through the liquid surface should be restricted to minimum and necessary electrodes and other sensing probes placed under the liquid level. Vigorous mixing provides necessary washing to prevent tissue from growing on submerged surfaces. Almost no mycelial deposits were observed on submerged parts of the fermentor during long term continuous cultivation. In small volume

fermentations of the type conducted herein a small bar of magnetic stainless steel can be placed into the vessel prior sterilization. By sliding this bar along the walls of the vessel using powerful magnet from outside growing tissue adhering to the sides can be scraped back into the solution.

Another way of improving the growth pattern and avoiding heavy biomass deposits is the application of suitable chemical agent serving a multiple purpose: as an antifoam, improving mass transfer, and giving an improved dispersed growth pattern. It was shown in the 14 l continuous culture (I) that the choice of proper antifoam agent is very important, especially for the culture of Graphium sp.

The defoamer used was HODAG K-20. In shake flask cultures, it showed no toxic effects at all (Chapter VIII). On the application in stirred-tank type fermentor, it depressed foams very effectively but had a negative effect upon mass transfer within the system resulting in dramatic decrease in growth activity. This effect was even more pronounced in the culture of Graphium sp. which is dependent even upon the transfer of substrate (ethane, natural gas) supplied in gaseous form. On the other hand HODAG-K20 could be used as growth inhibitor to study dynamic responses of the continuous-flow culture system.

During batch stirred-tank cultivations (Chapter IX) HODAG-F28 silicone based antifoaming agent was shown to have most of the desired features. It even enhances the growth of Graphium sp. slightly by possi-

bly affecting the surface tension properties of the broth. This resulted in smaller size of aeration bubbles and thus increased the surface area available for gas-liquid mass transfer which appeared not to be inhibited.

This type of antifoam was applied to the continuous culture with gas recycle (III) with very satisfactory results. The experiment was conducted over a five week period without any deposits of biomass. It was terminated after this time but could have been easily extended. It was impossible to obtain exacting kinetic data because in the closed system operating on a continuous-flow principle some gas is carried away by harvest stream. However, this could be calculated from solubility data if known. Since the harvested broth consists of microbial cells suspended in aqueous phase the solubility data would have to be determined first for this suspension. Otherwise considerable error could be introduced especially in calculating the biomass yield value when gaseous substrate concentrations are high.

When concentrations of oxygen and ethane were both 40%, it was calculated that 2.3 g of ethane was utilized for 1 g of biomass synthesized. Due to the errors of the type described for the quantitative evaluation of the yield, ethane utilization may even be lower than 2 g per gram of biomass.

These studies did show that the elevated concentrations of oxygen and ethane tested did not have any inhibitory effects upon Graphium



sp. in continuous culture.

More detailed kinetic studies were also difficult to accomplish when methane was added to the system. Resulting increases in biomass concentration and production rate as well as increased oxygen uptake would imply possible methane co-utilization. To handle and control on quantitative basis, the multicomponent gaseous mixture would require a more sophisticated experimental arrangement. However, this stage of the continuous culture with recycle gases confirmed some previous observations from shake-flask studies (Chapter VII) since it helped to confirm that methane had a stimulating effect and emphasized again the possibility of co-utilization of this substrate.

#### EFFICIENCY OF FUNGAL TISSUE SYNTHESIS

The yield of cellular material produced per unit weight of substrate utilized is one of the most important characteristics of a fermentation process. An ideal organism could be defined as one which could convert 100% of the substrate carbon to cellular carbon. In actual practice, a large fraction of the natural gas is converted to CO<sub>2</sub> by respiratory activity and some of the carbon is incorporated into extracellular by-products. This reduces the overall yield of tissue.

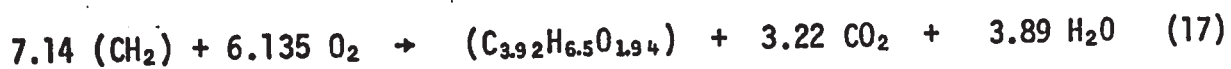
The metabolic oxidation of hydrocarbons proceeds to yield cellular energy for carrying out numerous bio-synthetic reactions e.g. protein, carbohydrates and lipid synthesis and production of CO<sub>2</sub> and H<sub>2</sub>O.

Other mineral inorganic nutrients present in the medium are taken up by the culture and on tissue analysis are usually expressed in terms of ash. The problem of determining the stoichiometry of a hydrocarbon fermentation process is made difficult because of the number of reactions which occur simultaneously and are dependent upon the microbial culture. Thus an empirical approach is required to develop overall stoichiometric relations.

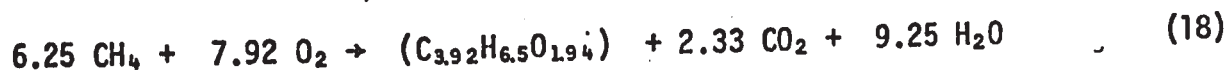
Hydrocarbon substrates furnish only C and H for cell growth and by definition contain no oxygen. Being completely reduced substrates, more oxygen is needed for this kind of fermentation. This increased oxygen requirement is resolved by improved aeration and agitation. On the other hand, hydrocarbons give generally higher yields of biomass per unit weight of substrate than conventional carbohydrates. Results reported by various authors vary considerably, ranging from 50 - 130% (wt. tissue/wt. hydrocarbon). Lower values of 50 - 90% by weight of substrate utilization were observed for some bacteria (Douros, 1968). The highest of 110 - 130% were reported for the bacterial genera of Micrococcus, Nocardia, (Perkins and Furlong, 1967; Wagner et al., 1969), whereas yeast grown on petroleum give a yield of 80 - 100% (Dostalek et al., 1968; Miller and Johnson, 1966; Takeda et al., 1965). Cell yields differ according to the microorganism 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 (Darlington, 1964; Humphrey, 1967; Norris,

1968).

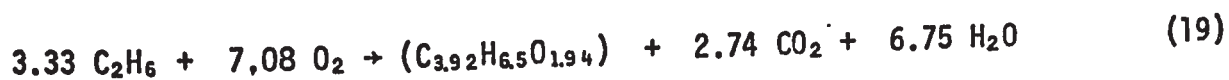
The overall stoichiometric equation as suggested by Darlington (1964) for hydrocarbon fermentation is:



Based on his assumptions Klass et al (1969) modified the above formula for the methane fermentation:



Assuming the same formula for biomass as shown above, the equation for ethane fermentation would be:

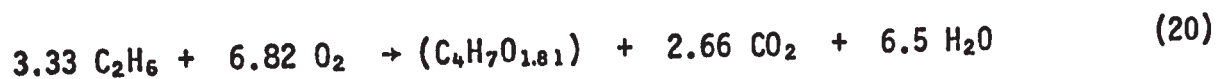


The cell tissue composition synthesized on hydrocarbons does not differ significantly from cells grown on carbohydrates. A typical composition of microbial tissue grown on hydrocarbon substrate may be considered (Wagner et al., 1969):

C	-	48%,
H	-	7%,
N	-	8%,
Ash	-	8%, and
O	-	29% (by difference)

This agrees with the composition of Graphium sp. This particular composition gives a slightly modified general formula for cellular material

( $C_4H_7O_{1.81}N_{0.57}Ash$ ) which, when substituted in simplified form in the above equation, gives the relation:



This assumes the weight of tissue synthesized is equal to the weight of hydrocarbon substrate utilized. Such an expression has theoretical significance, but would probably not be valid for quantitative data measured and obtained at different growth phases in an actual fermentation.

It is obvious that the above formula is valid only for certain physiological states of a culture as the proportions of the tissue synthesized, the  $CO_2$  produced and the eventual excretion of by-products changes as the fermentation proceeds. Any exact stoichiometric calculation is very difficult to make for a complex fermentation process and far more data are needed before all reaction mechanisms are known. From the experimental results obtained with Granthium 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. This corresponds to a yield between 48 - 53% of tissue formed per substrate utilized, expressed on a weight per weight basis.

The actual overall efficiency of the tissue biosynthesis can be calculated using a stoichiometric relation in which an ideal conversion is taken as the theoretical base. Here ideally 100% of the sub-

strate carbon is converted to cellular material and no carbon dioxide is formed. For ethane this relation would be:



Expressing this on a weight basis, 60 g of  $\text{C}_2\text{H}_6$  is required for 100 g of tissue formed, which would include the weight of nitrogen and ash making up 16% of dry tissue weight. Nitrogen and ash data are not included in the general formula above.

For theoretical purposes, if one again assumes conversion of ethane-carbon to microbial tissue, a conversion factor  $f_E$  for expressing the biosynthetic process efficiency can be calculated.

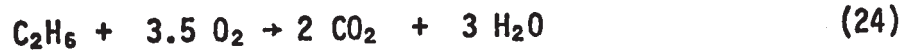
$$f_E = \frac{60 \text{ g C}_2\text{H}_6}{100 \text{ g Tissue}} \times 100\% = 60\% \quad (22)$$

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

$$\eta_T = \frac{1}{\text{Amt. of substrate utilized in grams per gram tissue}} \times f_E \quad (23)$$

Graphium sp. grown on ethane utilizes 1.9 - 2.1 g  $\text{C}_2\text{H}_6$  per g tissue synthesized. Using the above formula this corresponds to an efficiency of  $\eta_T = 28.6 - 31.6\%$ . The efficiency can also be calculated from heats of combustion. A comparison of the heats of combustion of ethane and tissue of Graphium gives:

## 1. Ethane (Substrate) Combustion:



Heat of Combustion = 368.4 kcal/g mole.

2. Graphium Tissue (Product)

Composition: 52% protein, 4% fat, 9% ash,  
35% carbohydrate (by difference)

	Heat of Combustion	Total/100 g tissue
Protein	4.1 kcal/g	213.2
Fat	9.3 kcal/g	37.2
Carbohydrate	4.1 kcal/g	<u>143.5</u>
	Total heat of combustion:	393.9 kcal/100 g tissue

Referring to the hypothetical equation (21) which was suggested to define 100% efficiency of the fermentation process, one can see that another factor ( $f'_E$ ) is required for a calculation of the efficiency of tissue synthesis based on heats of combustion.

According to the equation (21) 60 g of ethane which gives 736.8 kcal on combustion, is needed to produce 100 g of tissue yielding 393.9 kcal on combustion. This is defined as 100% "tissue synthesis efficiency".

Then:

$$f'_E = \frac{\text{combustion heat of 60 g of ethane}}{\text{combustion heat of 100 g of tissue}} \quad (25)$$

$$f'_E = \frac{736.8}{393.6} = 1.87$$

This factor should be used to multiply the actual tissue efficiency as calculated from the data based on the actual process. From the results reported in this thesis ~200 g of ethane yielding 2455.75 kcal on combustion is needed to produce 100 g of tissue.

The actual efficiency of the tissue synthesis in Graphium is expressed as ratio of heats of combustion of tissue and substrate multiplied by factor  $f_E$  :

$$\eta_T = \frac{\text{Tissue } H_T}{\text{Substrate } H_{C_2H_6}} \times f_E \quad (26)$$

$$= \frac{393.9}{2455.75} \times 1.87 = 0.299$$

$$\eta_T = 29.9\%$$

This calculation of 29.9% for efficiency of tissue synthesis based on heats of combustion corresponds very well to that based on stoichiometric weight to weight conversion basis.

#### ANALYSES OF GRAPHIUM SP. BIOMASS

Freeze dried biomass of Graphium sp. possessed a general composition of 7.4 - 8.1% nitrogen, corresponding to 46 - 51% of crude protein (N x 6.25), 9 - 11% lipids, 10 - 12% ash and the balance of dry weight of carbohydrates and fibrous material. Certain error may be involved in the calculation of the amount of protein from nitrogen.

Total nitrogen as determined by micro-Kjeldahl analysis was multiplied by a factor 6.25. Using this factor assumes that the typical structure of proteins contains 16% of nitrogen. This is very dependent on the type of amino acid residues which constitute the protein structure and on the nitrogen contribution of other sources of nitrogen such as nucleic acids, purines, pyrimidines and amino sugars. The latter occur very commonly in some microbes in the cell wall structure. The cell walls of imperfect fungi may contain significant amounts of chitin (Litchfield, 1968). However, nucleic acids and amino sugars are very often lower in fungal cells than in bacteria (Miller, 1968; Litchfield, 1967).

A more accurate evaluation was obtained by direct analysis for the constituent amino acids of the biomass of Graphium sp. The sum of all amino acids was found to be from 3 - 7% lower than the amount of crude protein calculated on total nitrogen basis. This difference is contributed to the non-proteinaceous compounds present in the biomass. On the whole the pattern of amino acids in the biomass is reasonably good when compared to conventional protein sources and also other sources of single cell protein. It is, however, somewhat lower in sulphur amino acids, which represents perhaps the major amino acid deficiency of SCP (Bressani, 1968).

Lipid fraction of the Graphium biomass was lower (4% by weight) in the culture grown on pure ethane as compared to the lipid extracts obtained from the biomass grown on natural gas (8 - 10% by



weight). Fat contents of various SCP's vary greatly as reported by many authors. In fungi higher protein contents in the mycelia are usually associated with lower fat contents (Litchfield, 1968). It is also important to determine the carbon-to-nitrogen ratio in the medium that gives the highest protein and lowest fat contents in the biomass. Available data on analysis of fungal lipids are very scarce.

Informative analyses were completed on the lipid fraction of the Graphium sp. cellular material.

Several investigators have observed that lipids, and especially phospholipids, solubilize into organic solvents many non-lipid materials such as inorganic salts, proteins, amino acids, sugars, water soluble phosphate esters, etc. (Ansell and Hawthorne, 1964; Baer et al., 1956; Marinetti, 1962). This effect is partly due to the solubilization of these substances in lipid solvents but it is mostly due to complex formation and such complexes may not be easily dissociated. Between 20 - 30% of the material originally extracted was found not to be soluble in pure  $\text{CHCl}_3$  on resolubilization. Only the chloroform soluble fraction was further analyzed. The lipids of Graphium sp. are composed mainly of phospholipids, although triglycerides, diglycerides and free fatty acids are present as well. Two major phospholipid components were identified as phosphatidyl choline and phosphatidyl ethanolamine.

Quantitative evaluation of the spectrum of fatty acids

showed the highest relative amount of palmitoleic acid. Appreciable amounts of palmitic and stearic acid were also detected. Of the essential fatty acids linoleic was 3.5 - 4.2% of the fatty acid total. No higher essential fatty acids could be detected due to the analytical procedure used.

#### PRELIMINARY SAFETY EVALUATION OF GRAPHIUM SP. BIOMASS IN RATS

Although yeast and other fungal products have been consumed by man for years, in view of the unusual nature of the fungal single-cell protein derived from natural gas, it was felt that the product should be subject to some preliminary toxicological tests. Thus feeding studies were needed to establish its nutritional value. Decreased digestibility of many SCP products has given rise to many of the problems associated with SCP feeding (Miller, 1968; Bressani, 1968).

Young albino rats have been regarded as the species of choice (Oser, 1968) for feeding experiments. It was, however, realized that rats do not, for example, develop kidney stones upon long term feeding of high-purine diets. This is probably one of the long-term problem areas of feeding SCP (Miller, 1968).

The studies conducted with feeding Graphium sp. biomass were directed rather towards determining acute toxic responses, if any, than towards investigation of chronic problems. Short term screening tests in rats for both obvious ill-effects and for the preliminary

assessment of the nutritive value of Graphium SCP were carried out in this study.

The animals were observed for effects on growth, food consumption, efficiency of food utilization, physical appearance and behaviour such as changes in posture or locomotion, appearance of excreta, etc. External manifestations frequently provide important evidence of toxic effects. These include the condition of fur, tails, anal region, nares and particularly the eyes of the test animals.

Two experiments were conducted, short-term six-day and another, where the rats were kept on the specific diet for 5 months. In the first experiment no toxic effects were observed even in animals fed solely on the Graphium biomass. They lost weight slightly over the six-day period but did not show any ill signs. No abnormalities were also observed on autopsy. The other experiment also did not reveal any deleterious or toxic effects upon the animals. Weight gain of the group living on 40% Graphium biomass in their diet as a sole protein source was, however, slightly lower as compared to the control group on casein diet. In all experiments as the amount of Graphium in the ration increased the weight gain decreased and the less of the ration was actually consumed. It was impossible to show whether the decrease was nutritionally based or just from the lack of eating. Further nutritional studies would be required for assessment of the protein quality, its biological value and feeding efficiency.

The rest of the animals not sacrificed after 5 months on the Graphium diet were transferred onto a standard rat diet and will be observed for possible long-term effects. Since carcinogenic properties of the fungal biomass, if any, often result in tumors developing in periods not shorter than one year it is proposed to maintain the remaining rats for at least one year. Only small groups of animals were used for each test due to the insufficient supply of biomass. The freeze dried biomass was not processed prior to ration preparation and this could contribute to its lower consumption by the animals due to certain flavour and odour components present.

It must be emphasized that these feeding experiments were preliminary and that greater emphasis must be made in researching this area if certain engineering breakthrough can be surmounted.

## APPENDIX

### MORPHOLOGY OF GRAPHIUM SP.

from "Growth of Graphium sp. on Natural Gas"  
(Zajic et al., 1969)

#### "Characteristics of Graphium sp."

When grown on solid media, hyphae are hyaline, regularly septate, 1.5 - 4.5  $\mu$  wide (average 3.0  $\mu$ ). Hyphal morphology of the Graphium sp. in aqueous broth culture on mineral salts C-medium in a gaseous atmosphere in 40% natural gas and 60% air was similar to that on solid media. The mycelium develops as many discrete small cottony colonies which do not clump or become dense (Figure 44a). With natural gas as the carbon source, the vacuolation of the hyphae is very marked (Figure 44, b and c). Conidia are produced abundantly in liquid and on solid media.

## F I G U R E 4 4 (A)

MORPHOLOGICAL CHARACTERISTICS OF GRAPHIUM SP.

- (a) Loose network of mycelium (x130).
- (b) Apical hyphae (x425).
- (c) Conidia (x950).
- (d) Conidial formation (x950).

The culture was grown in liquid C-medium with natural gas as a source of carbon and energy.

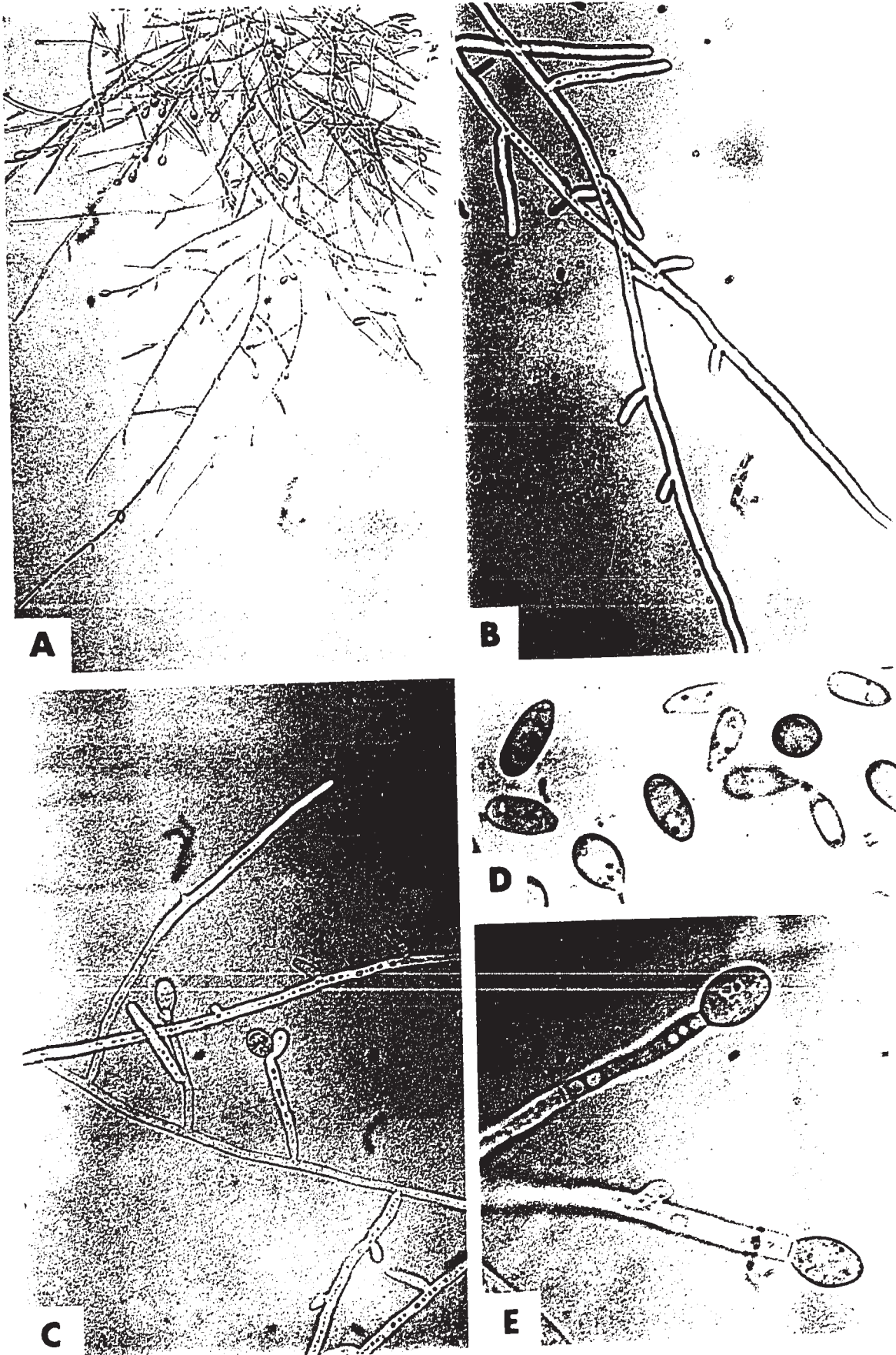


FIGURE 44 (A)

Mononematous conidia are one-celled, uninucleate, hyaline or faintly golden-colored on oatmeal agar; ovoid, subglobose to pyriform or clavate, rounded at the apical end, truncate at the basal end, sometimes with an annular frill ( $3.5 - 5.6 \times 4.9 - 12.6 \mu$ , average  $4.4 \times 8.9 \mu$ , Figure 44d).

The development of conidia occurs in the following manner. On solid media single apical conidia arise at the tips of leader hyphae or on short or long lateral branches (Figure 44, c and d). The conidia separate readily from the parent hyphae by the dissolution or fracture of the hyphal wall immediately proximal to the spore. Further proliferation of the sporogenous cell through the apex (Figure 45a) results in the formation of a second conidium. As this process is repeated, conidia gather as a cluster or spore ball. Occasionally in slide culture the conidia do not separate from the parent hyphae and each other as they are formed, thus chains of two, three, or more spores are formed (Figure 45b). The sporogenous cells are therefore typical annellophores. The node-like annellations on the sporogenous cell and the broad basal scar and annular frill on some conidia are quite distinct. Sometimes the conidiophore renewed growth by sympodial extension just behind the terminal annellospore (Figure 45c), rather than by proliferation through the cross wall or scar of the terminal annellospore. The conidium produced on the short sympodial swelling could be interpreted as a sympodulospore and as an annellospore. In liquid culture, this is the usual method of conidial production. As the terminal spore is delimited by a septum, a second spore usually arises subapically (Figure 44c). The conidia are released readily from the par-



## F I G U R E 4 5 (A)

## GRAPHIUM SP. - CONIDIAL FORMATION

- (a) First formed annellospore displaced and second spore developing by renewed growth of sporogeneous hypha through the apex. Note annellation on sporogeneous hypha (x950).
- (b) Annellospores in a chain (x950).
- (c) Second spore forming by sympoidal growth of hypha behind apical spore (x950).
- (d) Intercalary chlamydospores (x425).

The culture was grown in slide culture on cornmeal agar.

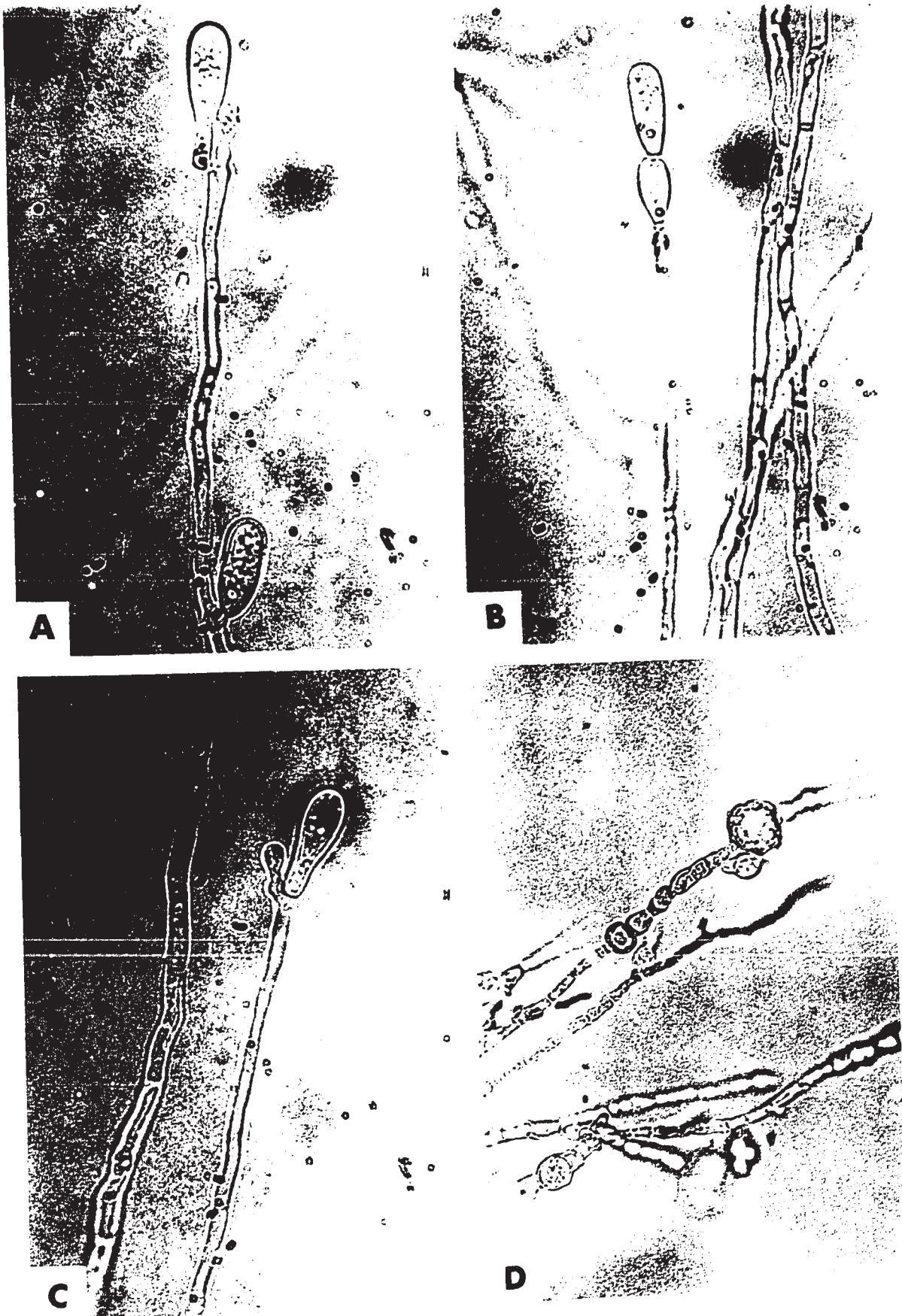


FIGURE 45 (A)

ent hyphae and dispersed into the aqueous medium where they germinate readily. Hyphae also fragment and give rise to rapidly growing colonies. Both these means are used in liquid culture for reproduction. In older mycelium both in liquid and on solid media intercalary chlamydo-spores of variable size ( $10.5 - 22.4 \times 11.2 - 25.2 \mu$ , average  $15.1 - 17.4 \mu$ ) and shape (Figure 45d) are common.

A synnematosus state was formed in 3-week old cultures on corn or oatmeal agar. From a cluster of hyphae with large barrel-shaped 'cells', aerial hyphae arose which grew parallel to one another into an erect column which became darkly pigmented and branched at the top to form a fan of sporogenous annellophores (Figure 46, a and b). The coremia may be up to  $580 \mu$  high and  $180 \mu$  broad, and are produced singly or in groups. Each annellophore produces a single apical conidium which readily separates from the parent hypha. Elongation of the sporogenous hypha and successive proliferation of further conidia through the apex occurred, resulting in the formation of node-like annellations on the sporogenous cell. Conidial development in the synnema was therefore similar to that described for the mononematous state, but no sympodulospore formation was observed in the synnematosus heads. The long, cylindric annellospores, rounded at the distal end and truncate at the basal end, ( $2.4 - 4.6 \times 6.5 - 12.9 \mu$ , average  $3.7 \times 9.8 \mu$ ) (Figure 46c) accumulate in a drop of exudate at the apex of the coremium as a ball or "slime head". The synnematosus state has not been observed in liquid culture. No perfect stage has been produced by this isolate under the cultural conditions used. Further studies are in progress.

## F I G U R E 4 6 (A)

## GRAPHIUM SP. - SYNNEMATOUS STATE

(a) Group of coremia (x135)

(b) Fanshaped apex of coremium, sporogeneous  
hyphae with annellations (x425)

(c) Conidia (x950)

(d) Base of coremium (x425)

The synnematosus state was produced on cornmeal  
agar.

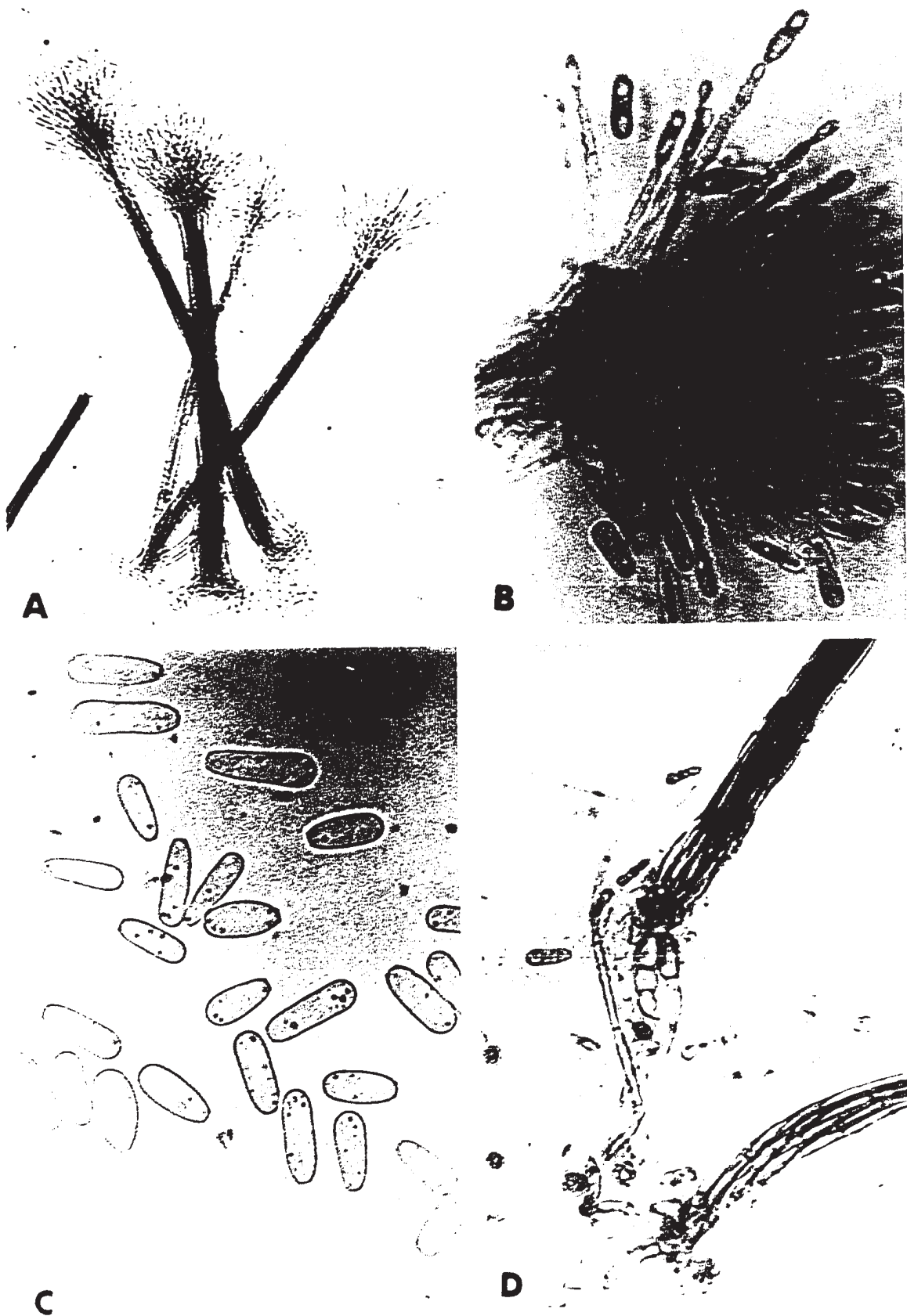


FIGURE 46 (A)

### Identification of the Graphium sp.

Since no perfect state has yet been produced by our isolate and because it possesses more than one kind of asexual spore, its generic placement in the form genera of the Fungi Imperfecti, based on the most distinctive or more conspicuous spore type (Hughes, 1953, 1958; Carmichael, 1962), would be determined from the synnematos state. However, the production of coremia may not be a constant characteristic since it varies with the environment, and in cultures with coremia there are many conidiophores which are not aggregated. Using Morris' key to the synnematos genera of the Fungi Imperfecti (which is based on the Saccardoan system), the isolate would be placed appropriately within the section Phaeostilbae-Amerosporae in the form genus Graphium (Morris, 1963, p. 10 and 62); this assumes the method of conidial formation is interpreted as not being catenulate. Superficially the conidial development does not appear to be catenulate because the conidia separate from the hyphae as they are formed. Only from a developmental study in slide culture is it apparent that the conidia, when undisturbed, form chains. Furthermore, the genus Graphium is a "heterogeneous assemblage of forms in which the sporogenous cells may be annellophores, sympodulae or phialides" (Barron, 1968) which is in need of taxonomic revision, so the assignment of our fungus to that genus is considered provisional. In addition it is clear that no attempt can be made at this time to identify to the species level the isolate described in this chapter. There is a close resemblance in size and morphology between the Graphium state of our isolate and G. cuneiferum (Berk. and Br.) Mason and Ellis (Mason and Ellis, 1953), IMI isolates 84732 and 87750(b).

The slender coremia are also similar to the Graphium states of species of Petriella (Barron, Cain, and Gilman, 1961).

The variability observed in the development of mononematous conidia of this isolate creates some difficulty in comparing the spore type with other mononematous form genera of the Fungi Imperfecti. The mononematous conidial form, in which apical annellospores were formed in succession from the sporogenous hyphae in a similar manner to that described for the synnematous state, resembles Scopulariopsis (Cole and Kendrick, 1969); however, usually the spores separate readily from the parent hypha and from one another thus forming spore balls. The chain of annellospores, which is a diagnostic feature of Scopulariopsis, has been observed in slide culture when the conidia do not separate from one another. The other type of conidial development described, in which the conidiophore renewed growth by sympodial extension just behind the terminal annellospore, fits the popular concept of Sporotrichum (Barnett, 1960), but the lectotype species of Sporotrichum (S. aureum) is unlike the popular concept of this genus (Hughes, 1958; Carmichael, 1962; Barron, 1968). The two types of mononematous conidial formation - apical or apical and sympodial annellospores - cut across the distinction made by Barron (1968, p. 285) between the two genera Sporotrichum (with aleuriospores) and Sporothrix (with sympodulospores). The criteria of color of mycelium and habit used by Carmichael (1962) to distinguish between Sporotrichum and Chrysosporium would suggest a relationship with Chrysosporium, but the mononematous state of the Graphium isolate does not fit exactly the description of any of the species of Chrysosporium described by Carmichael (1962). It most closely

resembles C. tropicum. Until the relationships between Chryso sporium and Sporotrichum and Sporothrix are more precisely defined, the mononematous state of the Graphium isolate could be assigned to any of these three genera. The alternate methods of conidial formation described for the Graphium-isolate have been observed previously in the conidial states of some species of Graphium (Hedgcock, 1906, pp. 81 and 95) and in the conidial states of an unidentified cleistothecial ascomycete (Barron, personal communication).

#### Occurrence and Economic Importance

Whether Graphium is a common component of the fungal flora of sewage treatment plants or is a chance contaminant has not yet been determined. Graphium species are commonly found on woody substrata or on plant debris (Morris, 1963) and have also been recorded for soil (Gilman, 1957; Goos and Timonin, 1962; Barron, 1968). Graphium is not listed in Cooke's Laboratory guide to fungi in polluted waters, sewage and sewage treatment systems (Cooke, 1963), but other synnemmatous genera of Fungi Imperfecti such as Stilbella bulbicola Hennings, Stysanus stemonitis (Persoon) Corda, and Isaria cretacea van Beyma, are listed. Mononematous genera such as Scopulariopsis brevicaulis Bainier, Scopulariopsis spp., Sporotrichum sp., Chryso sporium pannorum (Link) Hughes, and a 'Monosporium' state of Allescheria boydii Shear, which shows some resemblances to this isolate, have also been recorded from sewage. Trichoderma viride Link ex Fries was found in all sewage habitats examined."



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