ACTA CHEMICA SCANDINAVICA 21 (1967) 625–632

A Methane-Consuming Green Alga

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From enrichment cultures of photosynthetic sulphur bacteria, a *Chlorella* was obtained which combines capacities both for normal photosynthesis in a carbonate medium and for the utilization of methane as carbon source for its growth. This alga is adapted to anaerobic conditions, but the photosynthetic oxygen production enables the alga itself to affect the environment in this respect.

The first algal cultures in liquid medium contained quite a number of bacteria which could be removed by plating on the same medium solidified with agar and with 1 % glucose added. The remaining contamination of bacteria amounted to one bacterium per 10^4 algal cells. As the bacterial growth in batchwise experiments was insignificant, the methane consumption of the alga cannot be disputed. Thus, oxidation of methane to carbon dioxide by *Pseudomonas methanica* which might subsequently contribute to the growth of the alga did not occur, nor was it possible to indicate methane oxidizing bacteria on media specially suited for this purpose.

This alga may be of industrial importance for the production of cell mass on methane-carbonate basis, even without a supply of free oxygen. It could also be used in a closed system in order to remove CO_2 and CH_4 from the atmosphere and simultaneously bring about an oxygen enrichment.

The existence of methane-consuming algae may indicate that complete photosynthesis has occurred on Earth earlier than supposed and also that such photosynthesis is conceivable on planets with reducing atmosphere, assuming that the conditions in other respects would permit it.

When studying the growth of photosynthetic sulphur bacteria in atmospheres of different gases, a *Chlorella* was found in the enrichment cultures. The primary source was mud underlying stagnant water, deficient in oxygen. Enrichment cultures were repeatedly transferred, over a long period of time, on anaerobic medium (E_0' *ca.* -0.55 V at pH 8.0 in fresh medium) in closed and illuminated bottles. The algae ought to have become adapted to a milieu poor in oxygen and the numerous passages on the anaerobic medium must have maintained, and possibly accentuated, this adaptation.

Considering that this *Chlorella* had been cultivated for such a long time under the same conditions as the purple bacteria, it was felt to be of interest

to investigate the reaction of the alga towards different gases. In introductory trials with N_2, A, H_2, and CH_4, it was evident that CH_4 caused a slight increase in growth. The possibility that CH_4 acted as carbon source in the synthesis of algal substance prompted the following investigation.

EXPERIMENTAL

Organisms

Enrichment cultures of *photosynthetic sulphur bacteria* were kept for about one year in Medium 1 [1] in completely filled bottles containing 300 ml, illuminated from above by fluorescent lamps of about 4500 lux intensity. 10 % inoculation material was transferred every second week.

Medium 1.

Malic acid	2.0 g	NaCl	5.0 g
Na_2CO_3	0.1 g	$Na_2S_2O_3$	1.6 g
$(NH_4)_2SO_4$	1.0 g	Sodium thioglycollate	0.085 g
		Na_2S	0.18 g
K_2HPO_4	0.5 g	Tap water to 1 l	
$MgSO_4$, 7 H_2O	0.4 g	pH to 7.0 with NaOH	

In the first experiments dealing with the behaviour of these enrichment cultures towards different gases (half-filled bottles on a shaking table), it was shown that the purple bacteria could not grow on Medium 1 in methane atmosphere if malic acid and thiosulphate were omitted. In this case, the opaque cultures, which varied in shades between red and brownish yellow, changed into a thin, green suspension of micro-algae which, judging from the microscopical appearance, appeared to belong to the genus *Chlorella*. A slight, but obvious, difference was sometimes found between algae originating from different enrichment cultures when cultivated on agar medium (*cf.* p. 627).

Consequently these algae, together with the purple bacteria, had survived many passages on reducing medium. The number of algal cells was probably very small. They could not be observed in the routine microscopical control and the amount of oxygen which they produced during photosynthesis was negligible and consumed by other organisms in the culture.

Table 1. Results from cultivation of *Chlorella* G 5:3 with and without supply of methane.

	Experiment No.			
	1	2	3	4
Time of cultivation, days	10	13	18	17
Inoculation material, mg/l	70	126	60	37
$NaHCO_3$ added, mg/l	350	500	500	600
Growth, ash-free dry substance, mg/l				
without methane (a)	86	126	107	120
without methane (b)	77	134	105	124
with methane (c)	119	174	144	179
with methane (d)	117	176	146	179
Growth in per cent of the 'theoretical' value, calculated on the supply of carbon as $NaHCO_3$ and presuming 50 % carbon in ash-free dry algal substance				
without methane (a)	86	88	75	70
without methane (b)	77	94	73	72
with methane (c)	119	122	101	104
with methane (d)	117	123	102	104

Table 2. Survey of the number of cells in Experiment 4 (Table 1).

	Number of cells in millions per ml					
	I	II	III	IV	V	VI
	Algal cells		Bacterial cells In the beginning of the cultivation		Bacterial cells At the termination of the cultivation	
	In the beginning of the cultivation	At the termination of the cultivation	Incubation		Incubation	
			In air	In CH_4 and air mixture	In air	In CH_4 and air mixture
a) without CH_4	4.2	16	0.00019	0.00001	0.19	0.23
c) with CH_4	4.2	25	0.00019	0.00001	0.011	0.013

	The ratio between the number of different cells				
	II:I	I:III	I:IV	II:V	II:VI
a) without CH_4	3.8:1	2.2×10^4:1	4.2×10^5:1	84:1	70:1
c) with CH_4	6.0:1	2.2×10^4:1	4.2×10^5:1	2.3×10^3:1	1.9×10^3:1

After repeated transfers on Medium 1 (without malic acid and thiosulphate) and in CH_4 atmosphere, stable enrichment cultures of a number of alga strains with analogous properties were obtained. Among these strains, No. G 5:3 was chosen for all the following experiments.

Efforts to suppress the accompanying flora in the enrichment cultures by adding antibiotics gave no results of practical value. As it was desirable to eliminate possible methane oxidizing bacteria which might complicate the understanding of the CH_4 consumption of the algae themselves, terramycin, considered to be an active agent against pseudomonods, was particularly tested. The effect was studied by plating on agar prepared for methane oxidizing organisms according to Leadbetter and Foster.[2] The plates were incubated partly in a mixture of 50 % CH_4 and 50 % air, and partly in air alone. Even if the quantity of terramycin — up to 30 ppm which was the tolerable level for the algae — decreased the accompanying flora up to 75 %, this effect was practically meaningless, particularly as it was impossible to determine the extent to which presumable methane oxidizing bacteria were influenced.

A mixture of exceedingly small colonies of algae and contaminants was obtained in experiments aimed at purifying the algal cultures by plating on solidified algal medium (washed agar) in light. The algal growth on this medium was so poor that repeated plating was meaningless. On the other hand, the addition of 1 % glucose led to complete suppression of the accompanying flora whereas the algal growth was good. After five successive platings on this medium, the associated flora was almost entirely eliminated (*cf.* Table 2).

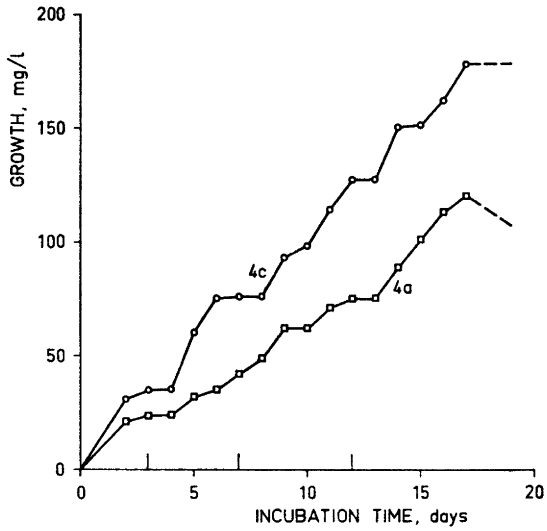

Fig. 1. Algal growth in Experiment 4. The formation of algal mass (total quantity of cells reduced by the inoculation material) as ash-free dry substance. Every day 50 mg/l of $NaHCO_3$ was added with the exception of three occasions indicated by short dashes on the abscissa.

Analyses

The algal concentration. Algal concentration was determined during the course of the cultivation by measuring the optical density at 620 mμ in a Linson 3 photometer using a diagram on the connection between light absorption and algal mass (as ash-free dry weight). At the conclusion of the cultivation, algal concentration was furthermore determined gravimetrically as described in the following.

Ash-free algal substance as dry weight. The high pH of the nutrient solution often caused an inorganic turbidity. This was dissolved in acetic acid and the sample (2 × 100 ml) centrifuged. The algal sediment was washed with water in the centrifuge tube, a troublesome detail, however, as the washed algae settled poorly in the centrifuge. The washed sediment was dried in a crucible at 105°C, weighed and afterwards incinerated in the furnace at 600°C. The difference was quoted as ash-free matter. Very good agreement between the samples was obtained (difference ± 1 %).

Cultivation conditions

The following Medium No. 2 was used throughout. The concentration of the nutrient salts chosen was based on a consideration of the conditions of the purple bacteria cultures from which the algae were obtained.

Medium 2.

$(NH_4)_2SO_4$	1.0 g	$MnCl_2,6H_2O$	0.4 mg
K_2HPO_4	0.5 g	$ZnSO_4,7H_2O$	0.007 mg
$MgSO_4,7H_2O$	0.4 g	$CuSO_4,5H_2O$	0.0005 mg
$FeCl_3$, H_2O	0.4 mg	$(NH_4)_6Mo_7O_{24},4H_2O$	2.2 mg
$CaCl_2$, anhydr.	6.0 mg	$Co(NO_3)_2,6H_2O$	0.0015 mg
H_3BO_3	3.4 mg	Distilled water to 1 l	

$NaHCO_3$ and CH_4 were added as carbon source as indicated below.

pH was adjusted to about 7.5 with NaOH and, after sterilization in the autoclave, further adjusted to 8.0 with sterile NaOH.

The cultures were grown in glass bottles (Pyrex) holding 1 l which were placed in a rectangular glass water bath kept at constant temperature (29°C ± 0.1°C). The bottles contained 900 ml of nutrient solution which was magnetically stirred. The illumination was provided by two pairs of fluorescent lamps along the vessel (General Electric, white, 20 W each), giving a light intensity of about 5000 lux at the wall of the bottle.

The bottles were sealed by rubber injection flask stoppers provided with an outlet valve and a sintered glass tube for the distribution of gas in the liquid. Samples were taken and additions made by injection-syringe.

The methane gas was of high quality (maximum limits of N_2 1.2 %, O_2 0.1 %, H_2 0.2 %, CO_2 0.3 %, and olefines 0.2 %). Before entering the culture vessel the gas was passed through gas washing bottles containing conc. H_2SO_4, alkaline $KMnO_4$, and KOH (35 %).

At the beginning of the cultivation, an addition of 50 mg/l $NaHCO_3$ was made, corresponding to a theoretical growth of about 14 mg ash-free dry cell substance. Calculated on the growth, portions of 45 mg $NaHCO_3$ were then added successively during the course of the cultivation. Thus, the maximum concentration of $NaHCO_3$ in the medium never exceeded 65—70 mg/l. Before terminating the cultivation all carbonate was consumed.

The pH rose regularly when the carbonate was consumed and was kept in the range 8.0—8.3 by the addition of H_3PO_4.

RESULTS AND DISCUSSION

In all experiments with the above arrangements, the results were analogous to those shown in Table 1. The addition of CH_4 caused an increase in algal growth of 35—45 % when the cultivation was run long enough for the growth to cease. The formation of new cells reached or exceeded the 'theoretical' value which is calculated on the basis that, from 1.0 g $NaHCO_3$, a maximum quantity of 0.29 g ash-free algal substance containing 50 % carbon can be formed. The latter value was obtained from analysis of *Chlorella* [3] grown in air containing 5 % CO_2. As algal cells have the same appearance whether they have grown in anaerobic or aerobic atmosphere, there is hardly any reason to suppose that their chemical composition should be considerably different in these two cases.

The principal fact is that the algae utilized CH_4 for the synthesis of their cell substance. This implies a new observation regarding the behaviour of green algae. If the process were run with the co-operation of the accompanying flora (which in that case should oxidize CH_4 to CO_2 with the aid of the oxygen produced by the alga in its normal photosynthesis), it would only imply a symbiotic phenomenon offering no fundamental contribution with respect to the anabolic capacity of algae.

Blue green algae growing in petroleum-mingled water have been noticed but the question is open if these algae really could utilize hydrocarbons.

It was evident that normal photosynthesis with the production of oxygen is involved under the present circumstances from the fact that the gas (100 % CH_4 in the beginning) above cultures in half-filled closed bottles, contained 6 % O_2 when the growth ceased. It is therefore probable that free oxygen, which may have acted in the oxidation of methane *via* presumed CH_4 oxidants in the accompanying flora, was present in the experiments.

Methane oxidants such as *Pseudomonas methanica* were suspected organisms in this connection. It is possible that methane oxidizing organisms were present in the original material of the enrichment cultures of purple bacteria. Theoretically they could have survived in anaerobic milieu for a long time in the laboratory and were later able to develop in the enrichment cultures of algae which were run with CH_4 as gas phase.

The probability that methane oxidants of known type (*cf.* Leadbetter *et al.*[2]) had been capable of surviving cultivation for years in a medium containing malate and completely lacking free oxygen is exceedingly small, however. Moreover, during their growth the algae excrete organic substances (preferably glycolic acid, *cf.* Moses and Calvin;[4] mono-, di-, and polysaccharides, *cf.* Maximova and Pimenova [5]) which inhibit the growth of methane oxidizing organisms.

By plating according to Leadbetter *et al.*[2] we have repeatedly tried to demonstrate the presence of methane oxidants in the enrichment algal cultures, but have never been successful. About the same number of colonies (generally very small) was obtained when incubating in a mixture of CH_4 and air, as in air alone. Obviously the chemicals and the agar of the medium contain sufficient carbon nutrition for these bacteria despite the fact that the former were of the highest purity available commercially (analytical reagent) and that agar washing was carried out. As no decisive evidence could be obtained in this way, attempts were made to obtain pure cultures of the algae by plating. Though absolutely pure cultures have not yet been obtained in this manner, the present degree of purity is so high that the insignificant role of the accompanying flora in the CH_4 assimilation can be estimated.

The problem was simplified by the fact that the immediate intention was not to obtain the alga in absolutely pure culture; it was necessary only to eliminate the bacteria to such a degree that it could be assumed that the negligible number of contaminants would not affect the CH_4 conversion in the culture. However, the question of whether all contaminating cells on the plates grew remains open.

Irrespective of faultiness in the process of determining the cell number in the accompanying flora, the repeated plating seems to have had an apparent effect of purification that was evident from the microscopical picture. The plates were incubated in air and light and it is highly improbable that presumed methane oxidants should under these circumstances have been able to survive five successive platings.

As mentioned earlier, we have been able to demonstrate the assimilation of CH_4 by the alga in all experiments arranged for this purpose. In no case does the accompanying flora seem to have influenced the results when cultivating with or without CH_4, although the number of contaminating cells at the end of the trial was, in some cases, as large as the number of algal cells and, in other cases, several thousandfold smaller. (If the calculation is made on the quantity of contaminating cell substance *versus* algal cell substance, the algal mass in the first case was at least 10 times larger than the contaminating cell mass, and in the second case at least 10^4 times larger).

It is further possible that the accompanying flora exerts an influence on the algal growth by oxidizing algal excretion products with oxygen from the

algal photosynthesis, thus producing CO_2 in larger or smaller quantities. This should give rise to increased algal growth irrespective of the presence or absence of CH_4. It is possible that such an effect can be deduced from Table 1. The cultures 1 and 2 were run in the presence of an abundant accompanying flora whereas 3 and 4 were performed with highly purified algal cultures. The growth in percent of the expected is actually somewhat lower in the later case.

It may at first seem remarkable that a *Chlorella* is able to utilize methane for its synthesis of cell mass under conditions that were initially anaerobic. However, in the present case the alga had developed in a milieu, that was both poor in oxygen and probably contained CH_4, whereafter it had been further adapted to similar conditions during a long period in the laboratory. As this alga is able to accomplish normal photosynthesis on carbonate basis parallel with its assimilation of CH_4, it creates its own milieu as regards the oxygen. Part of this free (or activated) oxygen can evidently be used by the alga for CH_4 oxidation.

As far as the author could deduce from literature studies information is lacking on the ability of green algae in general to utilize methane. If we consider which milieu is most likely to be associated with the occurrence of organisms such as *Chlorella*, methane as carbon source is hardly a first choice, and this possibility has therefore not incited any general interest.

The question of whether this newly discovered methane assimilation can be of technical value, cannot be answered until the conditions for maximum growth rate have been optimized and selection has been made between suitable algal strains. The phenomenon may be of value technically for the direct production of algal mass on methane-carbonate basis, even without a supply of free oxygen. It is also conceivable that methane-consuming algae could be used for gas exchange purposes in closed systems where their task would be to remove CO_2 and CH_4 from the air and enrich it with O_2.

The behaviour of this alga under anaerobic conditions and its capability to utilize CH_4, may also offer some aspects on the occurrence of life under extreme conditions. A 'reduced' atmosphere without oxygen, similar to that once comprising the gas envelope of the Earth and which is still present on the Jovian planets, obviously does not exclude *per se* the occurrence of photosynthetic organisms adapted to anaerobic environment but which, contrary to purple bacteria, are supplied with an enzyme system for complete photosynthesis. Even if the milieu is initially entirely anaerobic, the alga itself creates its own micro-milieu by oxygen production. At first, this atmosphere does not need to extend further than to the immediate proximity of the cell surface but which, at more intense growth, could, under suitable conditions, bias the macro-milieu in the aerobic direction.

The occurrence of organisms such as the alga under investigation indicates that a complete biological photosynthesis might have taken place during an earlier geological period than has hitherto been considered probable. Its anabolic record also supports the possibility that organisms with complete photosynthetic systems might even occur outside the Earth, in which case other factors such as temperature, the intensity and composition of radiation, and so forth are the critical points.

Acknowledgements. The author is very much obliged to Miss Maj Gullmetz for valuable experimental assistance. The investigation was supported by the *Research Institute of National Defence* which is gratefully acknowledged.

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Received October 31, 1966.