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Methanogenesis Mediated by Methylotrophic Mixed Culture

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ABSTRACT

Enrichment of methanogenic cultures on methanol from the microbial population in the anaerobic digesters operated on agricultural wastes revealed a high rate of biomethanation efficiency. Routine maintenance of this enrichment in a minimal basal medium at room temperature resulted in maximal growth in 40–50 d, and indicated pigment production toward the end of the growth phase. The cultures grown in three different media, with different substrates under light and dark conditions, were analyzed for protein, pigment, and gaseous products, and morphological studies were carried out by light, phase-contrast, fluorescence, and electron microscopy. In different media with methanol as substrate, growth and pigment production were maximal for the light-grown cells, decreasing in the order: phototrophic (PS(m)) > mineral > basal medium. Methanation and phototrophic growth were inversely correlated under light-grown conditions. In contrast, growth in the dark was predominently methanogenic in the decreasing order: mineral > basal > PS (m). Among other growth conditions tested, utilization of phototrophic substrates under light and dark conditions indicated the following:

1. Basal and mineral media were supportive of methanogenic growth under both light and dark conditions, although methane yields under light-grown conditions were low;

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2. Among the different substrates tested, methanol-grown cells gave the highest methane yield in the dark and;
3. Phototrophic growth in PS medium with succinate, malate, and pyruvate was better than that with methanol.

Absorption spectra of light-grown cells indicated the presence of bacteriochlorophyll a (Bchl a), as a doublet in the 800–850 nm region, which was absent in the dark-grown cells. Spectra of extracted pigments confirmed the presence of Bchl a with a 770-nm peak and carotenoid absorption bands in the 400–500 nm region indicative of the presence of a pigment of the spirilloxanthin type. Collective evidence for the predominant growth of a phototrophic organism under light-grown conditions and microscopic examination under all conditions indicated the possible presence in the mixed culture of purple nonsulfur bacteria of the *Rhodopseudomonas* type. In addition, the enrichment culture was found to contain other morphological forms, such as short and long rods, both individually and in clusters and coccoid cells.

The presence of such different forms of microbial population in a methylotrophic enrichment along with phototrophic bacteria is interesting and is of ecological significance. Considering the uphill task of methanol oxidation under anaerobic conditions, the studies on the present enrichment signify metabolic partnerships in the methylotrophic biochemical mechanisms operative toward energy recovery.

Index Entries: Mixed culture; methanogenesis; metabolic partnership; metabolic control; methanogenic; phototrophic.

INTRODUCTION

Natural habitats contain a wide variety of characteristic niches consisting of mixed microbial populations. These microbes have evolved to coexist and metabolically interact for their mutual benefit, directing the flow of carbon, energy, and other intermediates (*1*). These mixed cultures in nature are very versatile, are capable of efficient biodegradation of many complex forms of organic matter, and the bacterial populations in such habitats are highly resilient toward stress conditions (*2,3*). Establishment of mixed culture may also arise to meet a particular metabolic challenge, such as the presence of xenobiotic compounds, whose removal is a metabolic necessity (*4–7*).

Anaerobic digestion is an extreme example of a microbial interrelationship in nature, with immense potential in the context of both energy and environment (*8–11*). The process is biocatalytically brought about by the involvement of four different groups of bacteria, viz., hydrolytic, fermentative, acidogenic, acetogenic, and methanogenic types, maintaining anaerobic conditions toward methane formation. In this complex process, the action of the fermentative organisms is to direct in the flow of

reducing equivalents arising from the oxidation of various substrates toward methane formation by the methanogens, negotiated through mechanisms of interspecies hydrogen transfer (*12*). Recently, we have shown that during active biomethanation, an interactive metabolic control of the intermediates is mediated by the groups of cells, and it is only at the optimal load rate of the substrate that a high-rate methanation ensues (*13,14*).

Studies on mixed cultures broadly fall into two catagories: (1) use of culture-enrichment techniques to adapt suitable organisms from the natural inoculum toward utilization and degradation of specific compounds, and (2) using defined pure microorganisms in cocultures to effect desired biotransformation reactions. Studies with enrichment cultures using polymeric substrates toward methanogenic growth were reported (*15–18*), whereas specific defined cocultures were used in understanding methanogenic conversion of simpler substrates, such as glucose (*19*).

In our laboratory, mixed methanogenic cultures have been developed and used to effect degradation of specific biopolymers (*20*). Methanogenic enrichments on one-carbon compounds yield simpler microbial systems to study and understand the mode of metabolic regulation of electron flow toward methane formation. The present study is on one such enrichment culture maintained on methanol as carbon source, which gave a high methane yield of 16–20 mmol/L, in about 40–50 d, besides augmenting methanation during the anaerobic degradation of various biomass substrates (unpublished data). In this article, results are presented with regard to growth-related changes in this culture toward the understanding of methylotrophic metabolic partnership influenced specifically by culture conditions.

MATERIALS AND METHODS

All chemicals were AR-grade from E. Merck (Bombay, India). Yeast extract, peptone, vitamins, and caseamino acids were from Himedia (Bombay, India). Glutathione, sodium salts of succinate, malate, pyruvate, and phosphotungstic acid were purchased from Sigma Chemical Company (St. Louis, MO).

Enrichment of the Methanogenic Culture

The source of inoculum was the digested slurry from laboratory reactors of 2-L capacity operated semicontinuously on leaves of *Leucaena leucocephala* as input feed stock at 2.0 g input VS yielding output gas at 0.8–1.1 L/L/d with 85% methane content.

Methanogenic enrichment was carried out in a minimal medium containing (g/L): NH_4Cl, 0.9; K_2HPO_4, 1.45; KH_2PO_4, 0.75; $MgCl_2 \cdot 6H_2O$, 0.2; and methanol, 5 mL, and will be referred to as the basal medium. Digester slurry as the source of inoculum was subjected to 10-fold serial dilutions in the basal medium. The highest positive dilution of the original inoculum, as indicated by growth and gas analysis, was inoculated into roll tubes containing a formulated mineral medium and 2% (w/v) agar, and incubated at room temperature. Colonies picked from roll tubes were grown in 5 mL basal medium, before further enumeration in the same medium at room temperature. Based on the assessment of growth and methane yield, one of the colonies enumerated was chosen for the present study.

Growth Conditions

All experiments were carried out in 35-mL serum vials containing 20 mL of sterile medium and maintained anaerobically using butyl rubber stoppers (Nathany Industries, Calcutta, India). All media were prepared under an atmosphere of N_2, using the anaerobic techniques described by Hungate (*21*) and Bryant (*22*). Traces of O_2 were removed by passing the gas through a heated copper column. The pH of the media was adjusted to 7.4, before autoclaving at 121°C for 20 min. The final pH of the autoclaved media was also checked and, if necessary, adjusted to 7.4. Before inoculation, sterile anaerobic solutions of $MgCl_2 \cdot 6H_2O$ and methanol were injected into the vials.

Composition of the Media

Mineral medium: Mineral medium was formulated having the composition: KH_2PO_4-K_2HPO_4, 10 m*M*; $MgCl_2$, 1.0 m*M*; NH_4Cl, 20 m*M*; methanol, 100 m*M*; NaCl, 50 m*M*; glutathione, 2 m*M*; 1 mL of stock mineral solution; and 1 mL of stock vitamin mixture.

The stock mineral solution contained (g/L) $CoCl_2 \cdot 6H_2O$, 0.36; $MnCl_2 \cdot 4H_2O$, 0.29; $NaMoO_4 \cdot H_2O$, 0.48; $FeCl_3 \cdot 6H_2O$, 0.1; $NiCl_2 \cdot 6H_2O$, 1.19; $ZnCl_2$, 0.055; Na_2SeO_3, 0.06; $CaCl_2 \cdot 2H_2O$, 36. Vitamin stock solution contained (g/100 mL): riboflavin, 0.5; biotin, 0.2; thiamine, 0.5; folic acid, 0.2; and vitamin B_{12}, 0.05.

Photosynthetic (PS) Medium

Two different photosynthetic media, viz. PS (m) and PS, were used for the growth studies. The PS (m) medium contained the following (per liter): Casein acid hydrolysate, 3 g; stock vitamin solution, 1 mL, methanol, 5 mL. The PS medium was composed of (g/L): yeast extract, 4; peptone, 3. Various carbon compounds used as substrates were added from concentrated sterile anaerobic stock solutions.

Experimental Culture Conditions

Dark growth in different media was monitored at 37°C in an incubator (Gambaks Instrument Co., Madras, India), whereas for the light growth, the cultures were exposed to a 100-W tungstun lamp placed at a distance of 20–25 cm at 25°C. In separate experiments, carbon dioxide was included in the gas phase of PS medium to a final concentration of 5% v/v, other conditions remaining the same. The concentration of methanol used was 100 m*M*, whereas the other carbon sources, succinate, malate, and pyruvate were added to final concentration of 5–20 m*M*. Growth under light condition was monitored for 12 d, whereas cultures in the dark were followed up to 30 d. A 10% v/v of mixed culture was used as inoculum for the experiments.

Analytical Procedures

Growth studies: Cell growth was monitored by measuring the optical density at 540 nm using a Beckman (Geneva, Switzerland) model DU-54 spectrophotometer. Protein was estimated using the procedure of Lowry et al. (23). F_{420} levels during methanogenic growth were estimated both in the culture supernatants and in the solubilized cell pellets (24).

Pigment Analysis

Absorption spectra of whole cells of the light-grown cultures were recorded under anaerobic conditions using appropriate blanks (25). For extraction of pigments, 100 mL of the light-grown mixed culture in the basal, mineral, and photosynthetic media were harvested at 8000*g* using Remi centrifuge model C24 (Madras, India). Cell pellets were washed twice with phosphate buffer, pH 7.4, followed by 90% acetone and stirred at 4°C for 2 h. The colored supernatants after acetone extraction were used for recording the spectra on Shimadzu model UV-3100 double-beam spectrophotometer (Kyoto, Japan).

Gaseous Product Analysis

Gas analyses were done using a dual-injector, dual-detector Tracor model 540 gas chromatograph (Austin, TX). Methane was separated on a fused silica capillary column and detected by flame ionization (FID), whereas H_2 and CO_2 were separated on a stainless-steel column packed with Poropak Q (50 × 80 mesh) connected to thermal conductivity detector (TCD). The flow rate of the carrier gas (N_2) was 40 mL/min. The oven, port, and detector temperatures were 100, 100, and 140°C, respectively. The peak areas at different retention times were standardized and quantified using an on-line Nelson interface.

Microscopic Observations

Light microscopy of a wet drop of well-grown culture was done on Leitz (Wetzlar, Germany) model Ortholux microscope, equipped with an Orthomat camera attachment for photography. Epifluorescence of the cultures was checked using a Nikon (Tokyo, Japan) episcopic fluorescence microscope with excitation filters for 300–380 nm and 400–440 nm and the pairing barrier filters BA 420 and 470, respectively. For scanning electron microscopy (SEM), the samples were fixed with 2% glutaraldehyde and dehydrated as described by Albrecht et al. (*26*). The dehydrated samples were sputter-coated with gold for 1 min and viewed under a JEOL scanning electron microscope operated at 10 kV. Glutaraldehyde fixed samples were washed three to four times with phosphate buffer (pH 7.4), placed on a collodion-coated copper grid, stained using 1% phosphotungstic acid (pH 3.5), and viewed under a JEOL transmission electron microscope, operated under standard conditions at 80 kV.

RESULTS

Enrichment cultures from anaerobic digesters degrading agricultural wastes were mainatined on different methanogenic substrates, which included formate, acetate, methylamine, and methanol, under room temperature conditions in a minimal basal medium. Among various enrichment cultures thus obtained, the methanol-grown cultures routinely yielded methane 16–20 mmol/L culture in 40–50 d. These cultures could also be used as a source of methanogenic inoculum for augmentation of methane yield during the degradation of complex biomass substrates (unpublished data). Apart from the methanation efficiency, this enrichment culture exhibited a pale reddish pigmentation toward the end of the growth phase. Further investigations on the nature of this culture and growth under different conditions were carried out using a formulated mineral medium and a nutrient medium containing yeast extract and peptone, in order to understand: (1) whether the pigment associated with the enrichment culture in basal medium was light dependent and (2) the effect of medium composition, if any, on the extent of pigment synthesis and methanation efficiency thereof. The effect of light on the growth as measured by turbidity and the increase in the levels of protein content thereof with time (Fig. 1) in the three media were similar. Exponential growth, was preceded by an initial lag period, which was 4, 6, and 10 d in PS(m), mineral, and basal media, respectively, followed by a rapid increase in turbidity within 48 h in the PS(m) medium whereas a steady increase in the mineral medium and a gradual increase up to 24 d in the basal medium were noted. Growth in all the three media was apparently phototrophic, as indicated by the associated pigmentation observed, al-

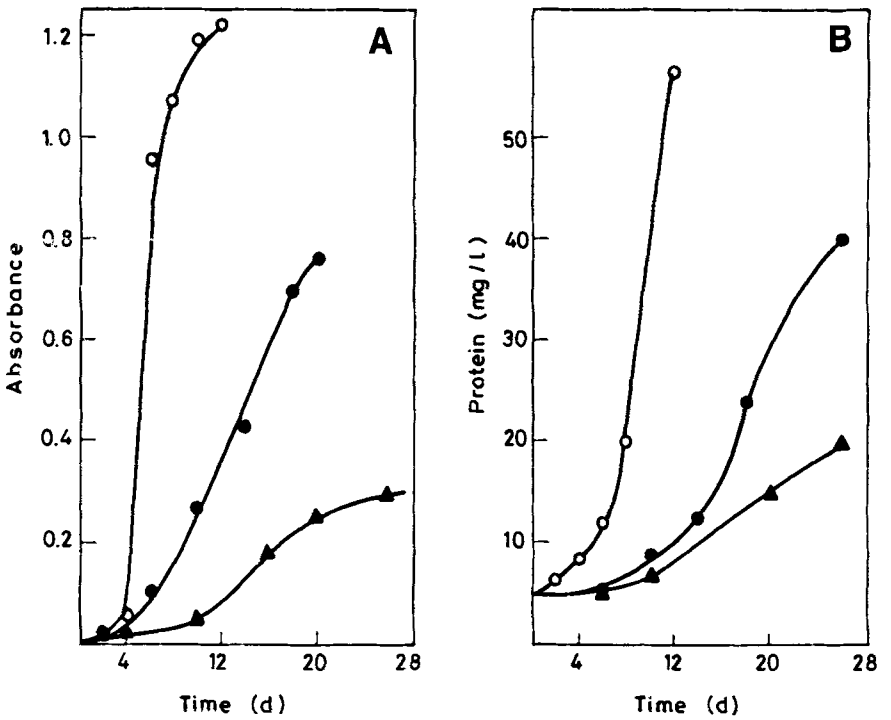

Fig. 1. Growth characteristics of the light-grown mixed methanogenic culture with methanol as substrate. (**A**) turbidity measurement at 540 nm and (**B**) protein level. ○—○, PS(m) medium; ●—●, mineral medium, and ▲—▲ basal medium.

though the extent of pigments formed in basal and mineral media was generally much lower. The observed pattern with respect to changes in the protein levels with time also indicated an initial lag period similar to that observed with the pattern of growth.

Corresponding to the increase in the growth measured on the 4th d in the PS(m) medium, the protein levels increased from 8.6 mg/L in 4 d to 55 mg/L in 10 d. In contrast, growth in mineral and basal media resulted in a protein level of 20 mg/L attained in a prolonged period of 16 d in the former and 26 d in the latter. The extent of growth and protein levels for the cells under dark conditions is shown in Fig. 2. In contrast to the light-grown cells, the dark-grown cells lacked pigmentation. There was no lag during the growth in the PS(m) medium, whereas a lag of 6 and 10 d was observed for the cells in mineral medium and basal medium, respectively. The rate of growth was better in the mineral medium than in the basal medium, attaining maximum measurable turbidity by 18 d in the former and 28 d in the latter. Turbidity measurement indicated that the pattern of OD values observed in both the mineral and basal media tended to decline after 12 d, whereas the protein levels continued to increase apparently owing to the aggregation of the cells observed during the growth

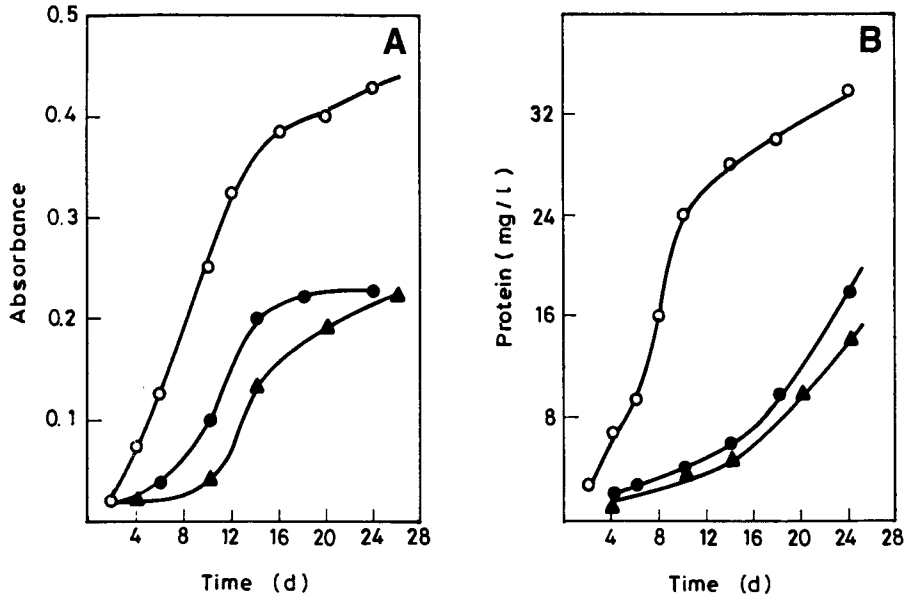

Fig. 2. Growth characteristics of the dark-grown mixed methanogenic culture using methanol as substrate. (**A**) turbidity measurement at 540 nm and (**B**) protein level, ○—○ PS(m) medium; ●—● mineral medium, and ▲—▲ basal medium.

after 10 d in both the media. Interestingly, after the exponential growth phase, a stationary phase was not immediately attained in the PS(m) medium, and growth was slightly decelerated even after 16 d. In general, the maximal growth attained in the dark-grown condition in all the three media was one-third that of the light-grown cells.

The nature of the phototrophic growth observed in the mixed culture with pigmentation under light-grown conditions was studied further, and the absorption spectra of the cells and that of the extracted pigments were recorded (Figs. 3 and 4). The absorption spectra of the light-grown cells showed maxima at 866, 806, and 590 nm, a pattern typical of bacteriochlorophyll a (27). The absorbance at 806 and 866 nm in the PS(m) medium was about 10 times higher than that in the mineral and basal media respectively. This is in accordance with the growth and protein levels in the three media. Absorption spectra of organic solvent extracts of the cells gave peaks at 770 nm besides having absorption maxima at 468, 496, and 526 nm, and the pattern indicated the presence of carotenoids of the spirilloxanthin series (*28*). The growth pattern and the pigment analysis together indicate the possible presence of purple nonsulfur phototrophic bacteria in the enrichment culture.

Toward understanding the growth in the three media, gaseous products were analyzed after maximal OD was attained in the respective media (Figs. 5 and 6). For the light-grown cells, the gaseous products

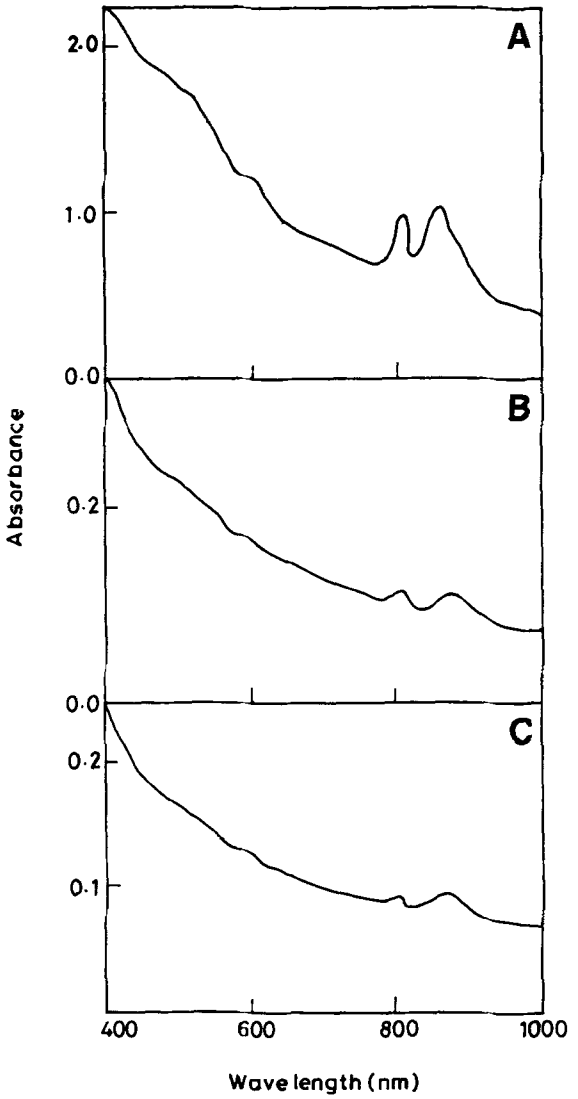

Fig. 3. Absorption spectra of intact cells of light-grown methanogenic mixed culture using methanol as substrate. (**A**) *PS(m)* medium, (**B**) *mineral* medium, and (**C**) basal medium.

detectable in the PS(m) medium were only H_2 and CO_2, whereas methane was undetectable. The production of H_2 accelerated slowly reaching 2.5 mmol/L between 8 and 10 d, correlating with maximum growth achieved during this period. In contrast, the H_2 produced was minimal for the growth under light in mineral medium and remained negligible, whereas the methane levels progressively increased to 1.4 mmol/L by 18 d. In the basal medium, however, there was no change in the percentage gas composition. In the dark-grown cells, the methane yields in both the mineral and the basal media up to 20 d were comparable, whereas by 24 d, there

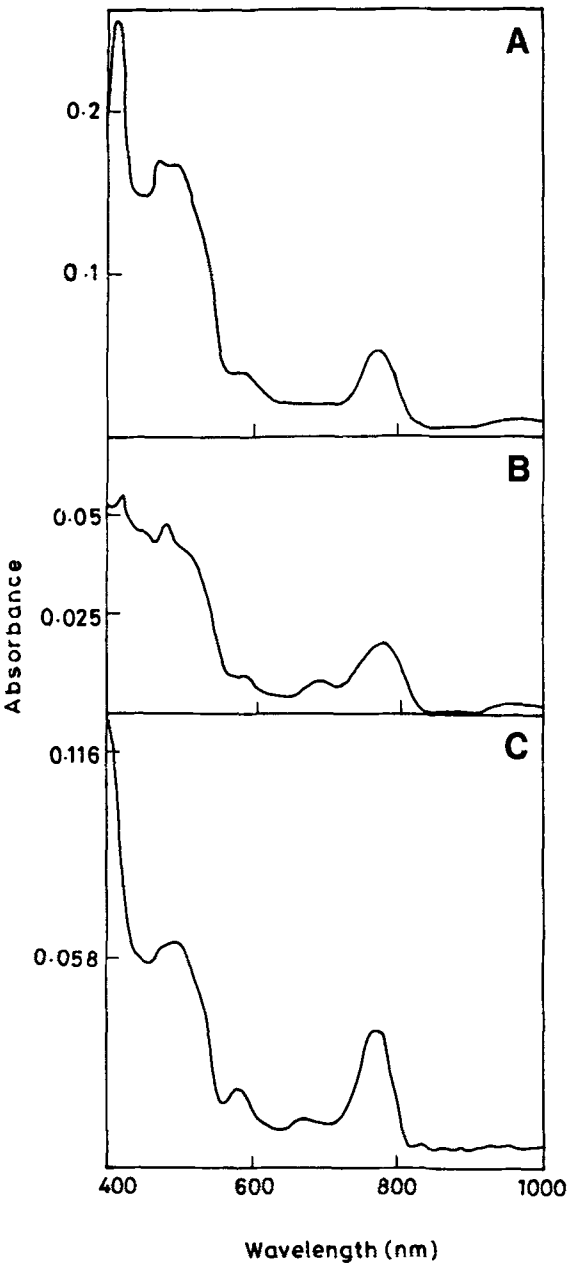

Fig. 4. Absorption spectra of extracted pigments from light-grown methylotrophic mixed culture. (**A**) PS(m) medium, (**B**) mineral medium, and (**C**) basal medium.

was a spurt in methane yield to 22 mmol/L, in mineral medium compared to basal, indicative of a possible role for minerals in enhancing methanogenesis. In the dark, H_2 levels in the basal and mineral media were insignificant, whereas in the PS(m) medium, they accelerated to 1 mmol/L by 10 d.

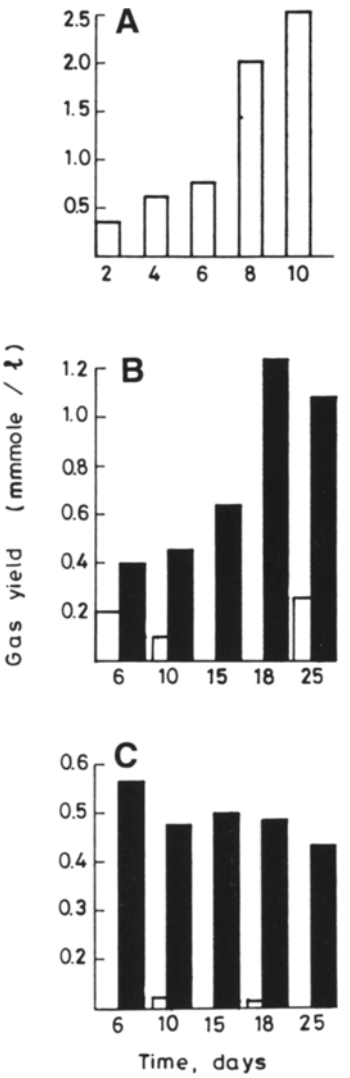

Fig. 5. Gas analysis of the light-grown methylotrophic mixed culture. The open and shaded rectangles represent the level of hydrogen and methane, respectively. Gas yields on the days shown on the *x*-axis alone have been indicated in the bar diagram. (**A**) PS(m) medium, (**B**) mineral medium, and (**C**) basal medium.

Effect of Phototrophic Substrates on the Growth and Methane Generation by the Mixed Culture

Owing to the mixed microbial population present in the methanol-grown enrichment culture with the possible presence of phototrophic bacteria (as indicated by spectral evidence for Bchl a under light-grown condition), it was of interest to study the growth of the mixed culture

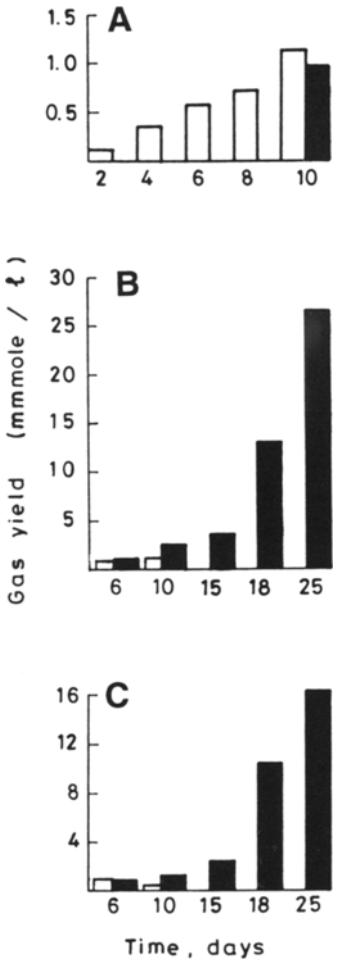

Fig. 6. Gas analysis of the dark-grown methylotrophic mixed culture. The open and shaded rectangles represent the level of hydrogen and methane, respectively. Gas yields on the days shown on the *x*-axis alone have been indicated in the bar diagram. (**A**) PS(m) medium, (**B**) mineral medium, and (**C**) basal medium.

with other carbon sources as substrates normally used for typical phototrophic growth, under light and dark conditions and the extent of methanation thereof. Succinate, malate, and pyruvate in the presence and absence of methanol were used as growth substrates. Apart from the three media described earlier, PS medium supplemented with CO_2 was also studied to determine if CO_2 in the head space gas could enable methanogenic growth under light. The cultures were grown in the light for 12 d and in the dark for 24 d, before analysis of the gaseous products formed. The gas composition of the culture grown in the PS medium, under light and dark conditions, is given in Table 1. Cells grown in the PS

Table 1
Gas Analysis of the Methanogenic Mixed Culture Grown in Photosynthetic Media with Different Substrates[a]

	Gaseous products mmol/L										
	PS					PS + 5% CO_2					
	Light		Dark			Light			Dark		
Substrate	H_2	CO_2	H_2	CO_2	CH_4	H_2	CO_2	CH_4	H_2	CO_2	CH_4
Succinate	—	11.54	1.61	—	—	—	17.7	2.26	—	20.36	—
Succinate + methanol	—	9.64	—	9.86	1.89	—	11.5	—	0.77	13.93	—
Malate	—	—	—	18.3	1.39	—	42.1	2.29	—	26.18	1.4
Malate + methanol	0.63	8.34	—	21.9	5.68	—	36.8	2.93	—	23.18	—
Pyruvate	—	19.21	—	19.86	—	—	26.9	—	—	31.8	1.99
Pyruvate + methanol	0.93	9.32	2.61	16.8	—	2.97	25.8	—	1.83	11.78	—
Methanol	2.5	17.7	0.32		—	0.72	11.2	—	—	18.15	—

[a]Values are average of duplicates from three sets of experiments. Mixed culture grown in 35-mL serum vials.

medium phototrophically were not associated with methanogenesis, yielding H_2 and CO_2 only, whereas the same medium with CO_2 in the head space gas contributed to low-rate methanation even under light-grown conditions. Analysis of the gaseous products for the culture grown in the basal and the mineral media, under light and dark conditions, is given in Table 2. Comparing CH_4 yelds in mineral medium, in the dark, the culture with methanol gave 22.3 mmol/L, whereas the corresponding light-grown culture gave only 1 mmol/L. In contrast, succinate, malate, and pyruvate with or without methanol gave higher yields in light than in the dark. In the basal medium, the utilization of succinate and malate was better under light-grown conditions, and contributed to low rate methanation, whereas in the dark-grown conditions, maximal methanation ensued with methanol as substrate, which was further enhanced by the supplementation of minerals. Under light-grown conditions, with methanol, it was observed that methanation was better in mineral medium compared to basal medium. In contrast, with either succinate, malate, or pyruvate, it was the basal medium that supported methanation compared to the mineral meidum. With respect to the growth of the mixed culture with different carbon sources, the following observations emerged from these studies:

1. Methane formation and phototrophic growth had an inverse correlation, and methane yields under light were in the order basal medium > mineral medium > PS medium;
2. Utilization of phototrophic substrates toward methanogenesis in basal and mineral media was better in the presence of methanol;
3. The growth in the PS medium, exposed to light, was predominently phototrophic, and pigment synthesis was better with succinate, malate, or pyruvate than with methanol;
4. The phototrophic growth was better with medium containing yeast and peptone followed by mineral medium and least in the basal medium; and
5. Addition of CO_2 to the head space of the PS medium contributed to low levels of methanation under phototrophic conditions.

Thus, the coexistence of the mixed methylotrophic population was best supported in the basal medium at room temperature. In the dark-grown conditions, the methanogenic population had an accelerated growth that, in fact, required mineral supplementation and subsequent transfers of the culture, resulted in the elimination of the phototroph (unpublished data). On the other hand, growth in the complex medium with nonmethanogenic substrates was predominently phototrophic.

To confirm the predominant growth of a particular group of bacteria as influenced by the culture conditions and to understand the morphology of the cells, the enrichment cultures maintained under different

Table 2
Methanogenesis by the Mixed Culture with Various Carbon Sources Under Different Conditions[a]

	Gaseous products mmol/L					
	Basal				Mineral	
	Light		Dark		Light	Dark
Substrate	H_2	CH_4	H_2	CH_4	CH_4	CH_4
Methanol	—	—	—	11.0	1.1	22.3
Succinate	—	—	—	—	6.38	3.17
Succinate + methanol	—	4.48	—	2.53	3.76	3.06
Malate	—	5.67	—	3.85	4.14	—
Malate + methanol	—	5.78	—	4.56	5.18	4.6
Pyruvate	—	3.84	0.24	1.89	2.64	1.44
Pyruvate + methanol	0.45	6.58	2.15	—	4.58	5.16

[a] Values are average of duplicates from three sets of experiments. Mixed culture grown in 35-mL serum vials.

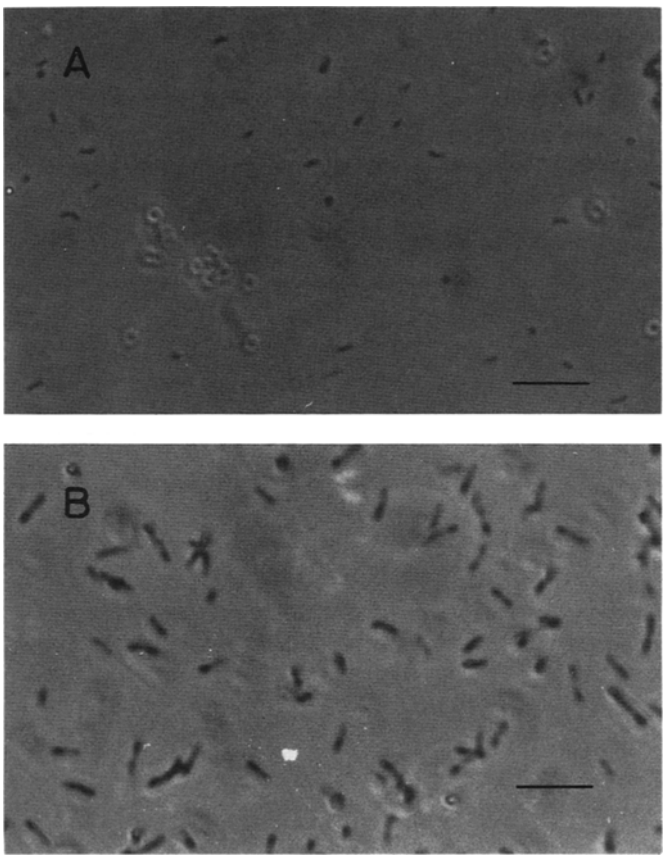

Fig. 7. Phase-contrast photomicrograph of the methanol utilizing methanogenic mixed culture. (**A**) At room temperature in basal medium; (**B**) under light in PS medium. Bar represents 6 μm.

conditions (viz., at room temperature and when grown in the different media in light and dark) were examined under the light and phase-contrast microscope, as well as by scanning and transmission electron microscopy. In addition, the cultures were also examined by fluorescence microscopy, for identifying the methanogenic population.

The photomicrograph of a well-grown enrichment culture (OD_{max} = 0.3–0.4) in basal medium, maintained at room temperature, consisted of a relatively high population of cells, of which the predominent morphological forms were short and long rods occurring individually, as well as in small and large clusters. There are also sarcina-like clusters present along with some individual coccoid cells (Fig. 7A). The rod-like cells in clusters as well as some individual rods had a bright blue-green fluorescence, as seen under fluorescence microscope. The photomicrograph of light-grown cells had a predominently motile rod population (Fig. 7B) that was 1–2 μm long and 0.2 μm in width. The scanning elec-

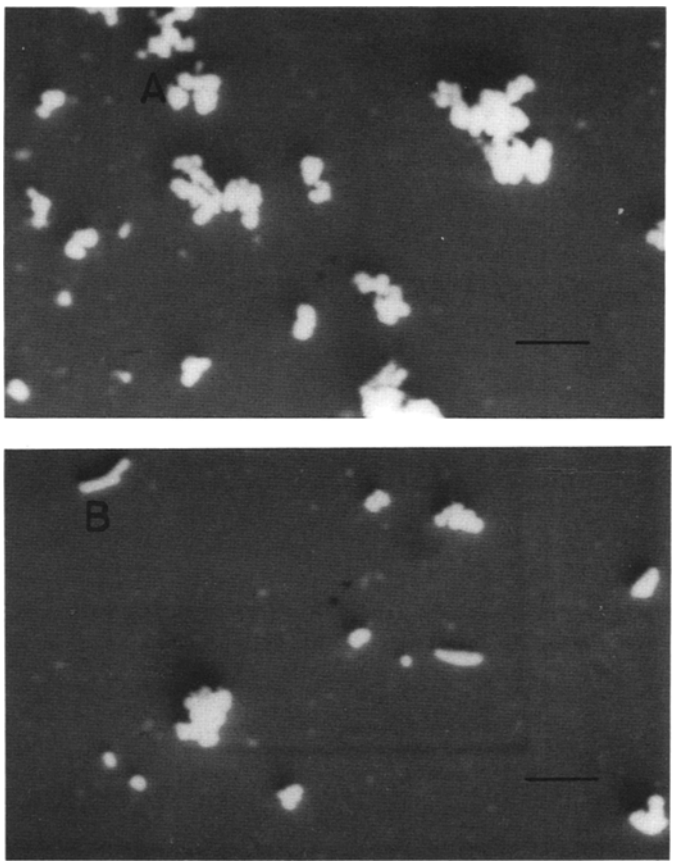

Fig. 8. Scanning electron micrograph of the dark-grown methanogenic mixed culture with methanol as substrate, in mineral medium. **A** (top) and **B** (bottom) denote two different fields of the same sample. Bar represents 10 μm.

tron micrograph of cells grown in mineral medium consisted of many clusters, as well as rods that were short and oval (Fig. 8). The physical association of rods and coccoid cells in the mixed population grown in mineral medium can be seen in the transmission electron micrograph (Fig. 9A and B). In contrast, the light-grown culture consisted predominently of rod-type cells (Fig. 9C and D), which appear to be morphologically similar to the purple bacteria described by Van Niel (*29*), based on the results presented.

DISCUSSION

Mixed cultures defined as cocultures capable of metabolizing complex substrates, including cellulose (*30,31*), and simpler substrates, such as glucose (*32*), have been advantageously used in model studies for

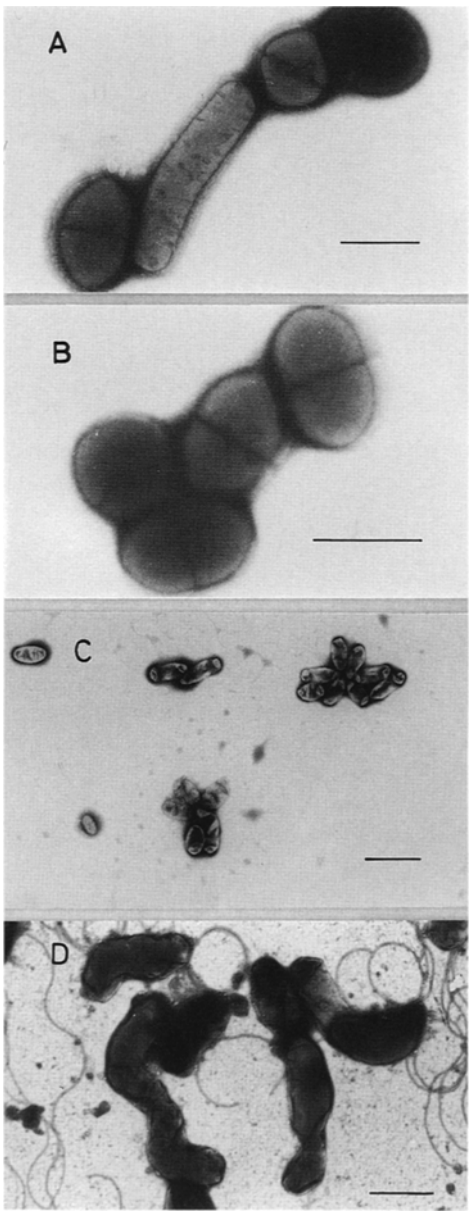

Fig. 9. Transmission electron micrograph of the methanol-utilizing mixed culture. Cells in mineral medium grown at room temperature consist of: coccoid cells in association with a rod-type cell in the center (**A**) and a group of coccoid cells (**B**). In the PS medium in light, the predominant population was rods (**C**), which appear flagellated (**D**). Bar represents 0.5 μm (A and B), 2.5 μm (C), and 1 μm. (D).

biomethanation, since the metabolic features of the individual organisms were known. The present study aims at understanding the microbial interactions in a methanol-grown mixed methanogenic culture, which was stably maintained at room temperature in a simple basal medium during repeated transfers over a period of 2 yr. Growth, pigment, and gaseous product analysis, and microscopic examination revealed the presence of a complex microbial consortia consisting of several morphological forms in the basal medium, including purple nonsulfur bacteria of the *Rhodopseudomonas* type. During routine maintenance, the enrichment culture gave a high methane yield of 16–20 mmol/L, and addition of this culture to biogas reactors, operated on low-strength biomass substrate, resulted in augmentation of methane yield (unpublished data). Concentration of F_{420} routinely monitored correlated well with high-methanating situations. Mixed cultures are characterized by different substrate specificities for the different individual organisms of the consortia. It is therefore usual that mixed-culture population degrades various substrates, sequentially effected by different type(s) of organisms. The simple one-carbon substrate, methanol, could have been a possible substrate of significance in the early stages of evolution of life forms, and hence, a study of the metabolic interaction in this culture is considered ecologically significant.

The growth of the enrichment culture in mineral medium in the dark, with methanol as substrate, was predominantly methanogenic, giving 20–25 mmol of methane/L. In contrast, growth in the PS(m) medium under light revealed mainly a phototrophic growth as indicated by the presence of Bchl a and the morphology of the cells under these conditions. The initial H_2 formed by the substrate oxidation under light-grown condition may contribute to the phototrophic growth in the PS(m) medium, whereas in the dark, it may be diverted toward low-rate methanation. Cell growth, as well as the extent of pigment synthesis in mineral and basal media, exposed to light was much less, and it is significant that these media supported low-rate methanation even in the light, indicative of the requirement of other nutrients for the growth of the phototroph. The growth studies, when considered together, indicate that enhanced methanation in the dark ensues in the presence of minerals, whereas subdued growth of phototrophs under light conditions (owing to lack of nutrients, viz., peptone and yeast extract) is accompanied by low-rate methanation possibly contributed by the presence of minerals. Based on the reduced pigment levels, as well as the morphology of the light-grown cells in mineral and basal media, it is likely that a very similar bacterial population existed under these conditions.

Phototrophic purple bacteria are found in nearly all aquatic environments, including anaerobic digester sludge, where their population was

enhanced only in the presence of light (*33*). Phototrophic bacteria belonging to the genus *Rhodospirillacae* have been observed in small deep lakes, which receive a significant input of organic matter, such as sewage and waste water from slaughter houses (*34*) and canneries (*35*). The feasibility of waste-water purification using photosynthetic bacteria was shown by Kobayashi (*36,37*). Besides their natural occurrence, growth of purple nonsulfur bacteria in defined cocultures has also been reported, such as the symbiotic relationship between *R. capsulatus* and *A. vinelandii* shown by Okuda and Kobayashi (*38*). Recently, Kimmel et al. have used a defined triculture consisting of methanogens and purple nonsulfur bacteria to derive methane from synthesis gas (*39*), signifying the application potential of photrophic mixed cultures. Uffen suggested (*40*) an ecological role for carbon monoxide (CO) utilizing phototrophic bacteria in natural anaerobic niches in providing H_2 and CO_2, thus enabling the growth of the methanogens. In the present study, attempts to understand the growth and preservation of a mixed methylotrophic methanogenic population reveal the significant influence of the culture conditions in supporting the growth of one or more types of bacterial population. The methanol-grown enrichment culture was able to utilize succinate, malate, and pyruvate, in the presence and absence of methanol both in the light and dark. Though growth in the dark was less, the culture produced methane, which was in the decreasing order: basal $\simeq$ mineral $>$ PS + 5% CO_2 $>$ PS. Although most of the substrates tested support the dark growth of the mixed population in the basal and mineral media, methane formation was particularly augmented in the presence of methanol. It is likely that under these conditions, methanol is converted to methane, whereas the reducing equivalents flow from the oxidation of succinate or malate. Predominantly phototrophic growth in the light was decreased in the order, PS $\simeq$ PS + 5% CO_2 $>$ mineral $>$ basal. In general, methane formation under light-grown conditions was inversely correlated to the phototrophic growth. From the observations on the inclusion of CO_2 to the head space gas of the PS medium supporting low-rate methanation of succinate, and malate even in the light, it can be speculated that the reduction of CO_2 proceeds in the methanogenic pathway.

The study underscores the importance of culture conditions in maintaining the mutual interactions in a mixed methanogenic culture. The results indicate the stable coexistence of a methylotrophic methanogenic consortium consisting of purple nonsulfur bacteria. When the medium was devoid of nutrients, the growth of various species was retarded leading to an enforced nutritional dependence of the organisms for their essential requirements. Thus, it emerges from the present study that viable long-term storage and maintenance of the mixed methylotrophic methanognic population can be achieved only in the basal medium under room temperature conditions (*41*). In methanogens utilizing methanol, it

has been suggested that methanol oxidation to CO_2 occurs via a dismutation reaction (*41,42*). Under mixed-culture condition, the methylotrophic consortia may bring about the necessary oxidation of methanol enabling hydrogen and other metabolites to be shared with the methanogens. Further studies are being carried out in understanding the metabolic partners in this methylotrophic enrichment and to assess the methanol metabolism under different conditions.

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