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MICROBIOLOGICAL STUDIES TOWARDS OPTIMIZATION OF METHANE FROM MARINE PLANT BIOMASS

ANNUAL REPORT

(1980 - 1981)

Gas Research Institute
8600 West Bryn Mawr Avenue
Chicago, Illinois 60631

MICROBIOLOGICAL STUDIES TOWARDS OPTIMIZATION OF METHANE FROM MARINE PLANT BIOMASS

ANNUAL REPORT FOR 1980 - 1981

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16. Abstract (Limit: 200 words)

The microbiological conversion of marine plant biomass was studied with stabilized kelp-degrading methane-producing enrichment cultures. Mannitol and alginate are used concurrently. Ethanol is produced shortly after feeding kelp and subsides rapidly. Dissolved hydrogen ranged from 5 nM to 1.2 uM. The appearance of ethanol correlates with increased hydrogen levels which is expected if interspecies hydrogen transfer functions to maintain low concentrations of the more reduced fermentation products. An improved method was developed for measurement of volatile fatty acids in sea water medium based on gas chromatography of the phenyl ester derivatives. Acetate and propionate were found in the greatest concentrations with formate, butyrate and isobutyrate in lower concentrations. The pool sizes will be used with turnover rate constants to determine total flux of each intermediate. A strain of Methanococcus mazei has been isolated that degrades acetate to methane. Also, a highly enriched culture of a previously unreported acetate-degrading methanogen was obtained. New strains of hydrogen and formate-utilizing methanogens were isolated. Mannitol and alginate degrading strains were isolated that resemble Cytophaga sp.

Formate dehydrogenase from Methanobacterium formicicum was purified 71-fold and initially characterized. The isolated enzyme contains a cofactor not previously reported in methanogens.

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RESEARCH SUMMARY

Title: "Microbiological Studies Towards Optimization of Methane from Marine Plant Biomass"

Contractor: Virginia Polytechnic Institute and State University

GRI Contract Number: 5014-363-0178

Principal Investigator: J. G. Ferry

Time span: July 1, 1980 to June 30, 1981

Annual Report

Major Achievements:

The research goals of this project are two-fold: to identify the most important substrates and products utilized by specialized types of bacteria that together convert marine biomass to methane and to isolate and study these bacteria with the view to improving the rate and reliability of the process. This research will decrease the cost and improve the reliability of biomass conversion to methane.

Achievements during the period reported were:

(1) Development of an improved technique for measurement of fermentation products and methanogenic precursors in sea water media. The technique is more sensitive and less variable than previously reported techniques.

(2) Identification of intermediates and their pool sizes in stabilized methanogenic kelp-degrading enrichment cultures.

(3) Development of culture conditions for and establishment of highly enriched methanogenic cultures that utilize hydrogen, formate, acetate and propionate.

(4) Isolation of a new acetate-degrading methanogen which constitutes a new genus.

(5) Isolation of new strains of hydrogen- and formate-utilizing methanogens from the marine environment.

(6) Characterization of formate dehydrogenase from Methanobacterium formicicum, the enzyme that initiates methanogenesis from formate.

(7) Identification of a new cofactor in methanogens not reported previously.

(8) Isolation and characterization of several alginate- and mannitol-degrading anaerobes.

Recommendations:

It is recommended that future research emphasize acetate-degrading methane-producing bacteria because acetate appears to be an important intermediate in the conversion of marine biomass to methane. Purification of the formate dehydrogenase enzyme provides an important starting point for further research on the pathways of methane formation and should be continued.

Description of work completed:

Conversion of all forms of biomass to methane is accomplished by an anaerobic microbial food chain comprised of at least three metabolic groups of organisms. The first two metabolic groups degrade biomass to hydrogen, formate and acetate, which are the only substrates utilized by the third metabolic group, the methanogens. Although acetate is the major substrate for methanogens, few of these organisms are in pure culture and little is known of the process.

Stabilized enrichment cultures were established that degrade kelp to methane for use in reaching the above objectives. Inocula from kelp enrichments were used to obtain highly enriched cultures of methanogens that utilize hydrogen, formate or acetate. From these cultures we have isolated a strain of Methanococcus mazei that utilized acetate and have obtained a highly enriched culture of a new methylotrophic methanogen not previously described. New marine strains of hydrogen and formate-utilizing methanogens were also isolated.

A new technique was developed to measure volatile fatty acids in sea water medium. Preliminary results suggest acetate and propionate are the major intermediates in the conversion of kelp to methane. Measurement of turnover rates will allow calculation of total flux for each intermediate to more accurately determine their relative importance in the conversion of this particular marine biomass to methane.

Another overall objective is the biochemical understanding of how methanogens convert substrates to methane. Characterization of formate dehydrogenase, the enzyme that initiates the conversion of formate to methane, is a contribution to this overall objective. The enzyme was purified 71-fold from M. formicicum and shown to have the following properties: (1) a molecular weight of 288,000 amu; (2) donates electrons from formate to FAD, FMN and coenzyme F_{420}; (3) is strongly inhibited by azide and cyanide; (4) contains molybdenum; and (5) contains a new cofactor not previously shown to be present in methanogens. Cytochemical staining of whole cells of M. formicicum have shown formate dehydrogenase is located within intracytoplasmic vesicles. These results begin to advance the understanding of formate conversion from the organism level to a more defined molecular level.

Project implications:

As part of GRI's Basic Research program in Biomass, this grant addresses the conversion of biomass to methane by methanogens, a special class of microorganisms. GRI is interested in biological conversion of biomass because it produces only methane and carbon dioxide, it is not adversely affected by high water content in the biomass, and the conversion can take place at ambient temperatures.

As is well known, methanogens occur in nature in highly complex communities of microorganisms whose physiology and biochemistry have been poorly understood. The rate of conversion and the characterization of the methanogens are areas which impact impending technology. GRI's basic research in biomass is aimed at understanding the physiology and biochemistry of methanogens.

This grant is providing a better understanding of the molecular processes involved in the biological conversion of biomass to methane. Next year's research will study paired cultures of the isolated methanogens in addition to continuing the present endeavors. This will help lay the foundation for constructing a defined microbial food chain capable of better converting biomass to methane.

GRI is pleased with accomplishments of this project and approves of the direction of the research.

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I. Research Summary

Stabilized enrichment culture were established that degrade kelp to methane for use in reaching the objectives. Inocula from kelp enrichments were used to obtain highly enriched cultures of methanogens that utilize hydrogen, formate or acetate. From these cultures we have isolated a strain of Methanococcus mazei that utilizes acetate and have obtained a highly enriched culture of a new methylotrophic methanogen not previously described. New marine strains of hydrogen and formate-utilizing methanogens were also isolated.

Several alginate- and mannitol-fermenting bacteria have been isolated and conditions for growth determined. Isolates are from the genera Bacteroides, Clostridium and Cytophaga. The low potential electron carrier, likely to be involved in acetate and hydrogen production, has been purified from Bacteroides ovatus and studied.

A new technique was developed to measure volatile fatty acids in sea water medium. Preliminary results suggest acetate and propionate are the major intermediates in the conversion of kelp to methane by stabilized enrichment cultures. Measurement of turnover rates will allow calculation of total flux for each intermediate to more accurately determine their relative importance in the conersion of this particular marine biomass to methane.

II. Overall Project Objective

Conversion of all forms of biomass to methane is accomplished by an anaerobic microbial food chain comprised of at least three metabolic groups of organisms. The first two metabolic groups degrade biomass to hydrogen, formate and acetate which are the only substrates utilized by the third metabolic group, the methanogens. Although acetate is the major substrate for methanogens, few of these organisms are in pure culture and little is known of the process. A primary objective of this reasearch is the isolation and study of acetate-degrading methanogens to improve our understanding of acetate conversion to methane which is of major importance in the conversion of all types of biomass. A second objective is the identification of important intermediates in the conversion of marine plant biomass to methane and isolation of organisms responsible for their conversion to methane.

III. Summary of Previous Work

A. Stabilized kelp-degrading methane-producing enrichment cultures were developed for use as a model system.

B. A new technique was developed to grow methanogens with volatile fatty acids as substrate. The method was developed using formate as substrate with the goal in mind to apply the technique to growth of acetate-degrading methanogens. In the studies with formate, new information was obtained regarding the conversion of this substrate to methane and was recently published.

C. Techniques available for measurement of volatile fatty acids in sea water medium were found to be unacceptable for our purposes. Plans were started to develop a more sensitive and accurate technique to measure pool sizes and turnover rates of intermediates in sea water medium.

D. Methods for purification of formate dehydrogenase from M. formicicum were developed and initial characterization of this enzyme was begun.

E. Several alginate- and mannitol-degrading anaerobes were isolated and identified.

IV. Specific Objectives

A. Develop a technique for measurement of low concentrations of volatile fatty acids in sea water medium.

B. Identification of intermediates and their pool sizes in stabilized kelp-degrading methanogenic enrichments.

C. Isolation of methanogenic bacteria from the marine environment with emphasis on acetate-degrading methanogens.

D. Purification and characterization of formate dehydrogenase from Methanobacterium formicicum.

E. Selection and characterization of carbohydrate-fermenting bacteria.

V. Work Performed

A. Conversion of organic acids to methane.

This past year we have focused on measurement of the pool sizes of intermediates in stabilized kelp-degrading methane-producing enrichment cultures. A senstive and reliable method for measurement of volatile fatty acids in sea water medium was unavailable and imposed a technological problem in the measurement of intermediates. Improvements in an existing gas chromatographic technique resulted in the identification of the intermediates and their pool sizes at various times after feeding kelp. The results are tabulated below:

Intermediate	Pool size (mM)		
	1 day	6 days	20 days
formate	0.1	0.05	0.06
acetate	1.3	6	0.1
propionate	0.4	2.5	2.2
butyrate	0.7	1.3	0.1
ethanol	0.2	-	-
succinate	4.5	4.5	4.2
dissolved H_2	1.2×10^{-3}	5×10^{-8}	5×10^{-8}

The times indicated are days after addition of frozen chopped kelp to the methanogenic kelp-degrading cultures.

Succinate appeared in the highest concentration and was last to subside as the conversion of kelp to methane progressed. Ethanol production peaked two days after the addition of kelp and decreased rapidly to below detectable levels. The appearance and disappearance of ethanol correlated with dissolved hydrogen concentrations which ranged from 50 nM to 1.2 uM. The relationship between ethanol and hydrogen suggests that interspecies hydrogen transfer functions to regulate the proportion of reduced fermentation products. The volatile fatty acids which appeared in the highest concentations were acetate and propionate. In the next year, the turnover rate of each intermediate will be determined. The pool sizes and turnover rate constants will then be used to calculate the total flux of each intermediate. This information will identify which intermediates are the most important and indirectly identify the most active organisms of the food chain. For example, the turnover of succinate may be very low and although it is in high concentrations it may be a relatively less important intermediate.

In additon to H_2-oxidizing strains, we have obtained a highly enriched culture of an acetate-degrading methanogen from kelp-degrading methane-producing enrichment cultures that is a strain of Methanococcus mazei. Plans are to isolate the organism from a minor contaminant and initially characterize it. The growth characteristics of this methanogen will be determined to provide a data base for the eventual coculture with acetate-producing kelp-degrading fermentatives. We also plan to study this organism in biochemical detail since it appears to function in a wide variety of methanogenic food chains and may be the most important acetate-degrading methanogen. We have also obtained in pure culture a methylotrophic methanogen not previously described. Isolates of marine alginate and mannitol degraders have been obtained from marine sediments situated below kelp beds. We have also obtained similar isolates from the stabilized kelp-degrading methanogenic enrichments of marine origin. Morphologically, the isolates resemble Cytophaga sp. The marine origins of these isolates are especially significant since they are most likely to degrade marine biomass efficiently and coculture with marine methanogens. In the next year, we plan to characterize these species to provide a data base for coculture with methanogens with the view to construct a defined microbial food chain that converts kelp to methane.

B. Formate dehydrogenase.

Another overall objective is the biochemical understnding of how methanogens convert substrates to methane. Characterization of formate dehydrogenase, the enzyme that initiates the conversion of formate to methane, is a contribution to this overall objective. The enzyme was purified 71-fold from M. formicicum and shown to have the following properties: (1) a molecular weight of 288,000; (2) donates

electrons from formate to FAD, FMN and coenzyme F_{420}; (3) is strongly inhibited by azide and cyanide; (4) contains molybdenum; and (5) contains a new cofactor not previously shown to be present in methanogens. Cytochemical staining of whole cells of M. formicicum have shown formate dehydrogenase is located within intracytoplasmic vesicles. These results begin to advance the understanding of formate conversion from the organism level to a more defined molecular level.

C. Graduate student progress.

Mr. Harold May has successfully completed his second quarter of studies toward the Ph.D. degree. He is very enthusiastic and shows good promise as a researcher. Hal has helped other graduate students with routine work, is learning techniques, and has begun a minor project of his own.

Mr. Kevin Sowers has completed his course work and has established a firm foundation on which he will be able to complete his Ph.D. thesis. All of the work in paragraph A (p.5) was accomplished by Mr. Sowers.

D. Selection and Characerization of Carbohydrate-Fermenting Bacteria (J. S. Chen).

We have further identified the alginate-fermenting bacteria isolated from our enrichment cultures (in collaboration with Drs. W. E. C. Moore and L. V. Holdeman of this Department). Two of the obligate anaerobes were identified as Bacteroides ovatus. Another obligate anaerobe was identified as Clostridium clostridiiforme, but this isolate no longer ferments alginate as a pure culture. Two additional isolates which efficiently convert alginate to acetate under anaerobic conditions turn out to be facultative anaerobes, and have been identified to be species within the genus Cytophaga. The Cytophaga isolate is not a described species according to the eighth edition of the Bergey's Manual of Determinative Bacteriology. B. ovatus (strain 1S2a2, formerly designated as 1S2a2-FA) is able to grow in the defined minimal medium of Varel and Bryant with either glucose or alginate as the carbon source and with phosphate as the buffer. Growth can be demonstrated in the test medium after five serial transfers. The growth rate was improved by the addition of peptiase or yeast extract.

The production of acetate, formate and hydrogen by a paired culture, consisting of an alginate-fermenting organism and a mannitol-fermenting organism, has been studied in medium containing both alginate and mannitol. Initial results showed better utilization of alginate than mannitol by the paired culture. We plan to test additional mannitol-fermenting organisms in the paired culture and also to adjust the population ratio between the two organisms in a paired culture in attempt to achieve an equally efficient utilization of both substrates.

A selected alginate-fermenting bacterium Bacteroides ovatus (1S2a2) has been mass-cultured. The low potential electron carrier, which is likely involved in acetate and hydrogen producton, has been partially purified from this organism. Both ion-exchange chromatography and gel filtration are effective for the purification. The electron carrier is assayed by a hydrogenase-linked reaction which measures the activity of ferredoxin and flavodoxin. The electron carrier is found to be a relatively oxygen-sensitive protein (the half-life in air is about six hours on ice) with a molecular weight about 15,000. Although the molecular weight is in the range of many bacterial flavodoxins, the low potential electron carrier isolated from B. ovatus cells (grown with alginate as the carbon source) appears to be a ferredoxin-like protein because of its characteristic UV-Vis absorption spectrum. Our earlier work showed that B. fragilis, a similar organism, contained a flavodoxin-like electron carrier when the cells were grown with glucose as the carbon source. We also found that fermentation pattern of our isolate of B. ovatus, when grown with alginate as the carbon source, is distinctly different from that with glucose as the carbon source. We are studying the low potential electron carrier under both growth conditions to see if it undergoes any change when the metabolic pathway changes.

VI. Major achievement during the year.

Major achievements during the past year are: (1) Development of an improved technique for measurement of fermentation products and methanogenic precursors in sea water media. The technique is more sensitive and less variable than previously reported techniques. (2) Identification of intermediates and their pool sizes in stabilized methanogenic kelp-degrading enrichment cultures. (3) Development of culture conditions for and establishment of highly enrichred methanogenic cultures methylotrophic methanogen. (5) Isolation of new strains of hydrogen- and formate-utilizing methanogens from the marine environment. (6) Characterization of formate dehydrogenase from Methanobacterium formicicum, the enzyme that initiates methanogenesis from formate. (7) Identification of a new cofactor in methanogens not reported previously. (8) Growth of alginate- and mannitol-degrading organism in defined media. (9) The simultaneous utilization of alginate and mannitol by a mixed culture. (10) Isolation and study of the low potential electron carrier of an alginate-fermenting organism.

VII. Conclusions

A. Stabilization of a naturally occurring marine microbial food chain and its characterization has provided a starting point from which the process can be further understood and improved.

B. Organisms have been isolated from a stabilized kelp-degrading methanogenic enrichment culture of marine origin. The isolates are respresentative of two metabolic groups that comprise the microbial food chain. The isolation of acetate-degrading methanogens is most significant. This research has established a basis for study of the individual components of the food chain and interactions between individual components.

C. The isolation of formate dehydrogenase, the enzyme that initiates formate conversion to methane, has provided techniques applicable to the study of methanogens on a molecular basis and revealed information on a general class of enzymes which function in methanogenesis.

VIII. Work Planned

This next year it is planned to determine turnover rates of intermediates in the kelp-degrading cultures and with pool sizes calculate the total flux of intermediates. Isolation of organisms will continue and it is planned to characterize selected strains for coculture work. Emphasis will be placed on acetate-degrading methanogens. Work will also continue on purification of formate dehydrogenase to homogeneity and its characterization. Low potential electron carriers present in alginate- and mannitol-fermenting bacteria will be further investigated.