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THE EFFECTS OF OXYGEN AND CHLOROFORM ON MICROBIAL ACTIVITIES IN A HIGH-SOLIDS, HIGH-PRODUCTIVITY ANAEROBIC BIOMASS REACTOR

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Abstract—The effects of oxygen and chloroform addition on microbial metabolic activities were measured on a unique thermophilic high-solids high-productivity methanogenic reactor using a combination of radiotracer, lipid analysis, and microcosm techniques. Methane production and the incorporation of ^{14}C-2-acetate into methane were decreased by both oxygen and chloroform treatment. Oxygen increased and chloroform decreased total CO_2 production and CO_2 production from labeled acetate. Introduction of oxygen decreased methanogenesis by facultative anaerobes competing for reducing equivalents. Chloroform was directly toxic to methanogens at the low application level, and inhibited all metabolic activities at the high application level. Increased diether lipid synthesis by methanogens was observed with oxygen addition, and methanogen lipid synthesis continued at a low level in the absence of detectable methanogenesis with chloroform addition. These responses may be due to previously unknown adaptations to toxic compounds by methanogens.

Keywords—Archaebacterial lipids, inhibitors, lipid analysis, methanogenesis, poly-β-hydroxyalkanoate, radiotracer analysis, triglyceride.

1. INTRODUCTION

Anaerobic degradation of municipal solid waste and organic industrial solid wastes is limited by the resistance of some biopolymers, such as lignins, to anaerobic microbes. The introduction of an oxidant such as oxygen, sulfate, or nitrate has been suggested[1] to initiate their consumption. This could be used to affect the bulk or texture of the solids produced. Addition of oxidants could also be used to counteract reactor failure due to over-feeding, volatile fatty acids being consumed by facultative aerobes present in the reactor community, and raising the pH. This benefit would have to be balanced against possible oxidative damage to the methanogens.

Another major problem in the anaerobic degradation of solid waste is reactor failure due to toxic compounds in the feed material. The methanogens are one of the key physiological groups in these reactors due to their crucial role in removing the products of fermentation. They are also extremely sensitive to a number of compounds commonly found in municipal and industrial waste streams including chlorinated organics[2] and heavy metals.[3] Inhibition of the methanogens leads to accumulation of volatile fatty acids, acidification, and reactor failure. Knowledge of the process of reactor failure would be useful in designing strategies for alleviation or prevention.

In this study, radiotracer techniques[4] and lipid biomarker analysis[5] were used to determine the effects of oxygen and chloroform addition on the microbial activities in microcosms of a high-solids, high-productivity bioreactor.

2. MATERIALS AND METHODS

Chloroform, acetone, methanol, hexane, diethyl ether, and ammonium hydroxide were analytical grade (Baxter Scientific, McGaw Park, IL). Liquid scintillation counting (LSC) was done with a LKB 1212 Rackbeta (Gaithersburg, MD) using the channels ratio correction method and the scintillation fluid Ecolume (ICN Biomedicals, Irvine, CA).

2.1. *High-solids reactor*

The reactor consisted of a 20 l polypropylene carboy with 5 kg of reactor material maintained

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at 55°C. It was fed every Monday, Wednesday, and Friday by removing 5 to 15% of the reactor contents and replacing it with a 1:1 mixture of α-cellulose (Sigma Chemical Co.) and ground sorghum (*Sorghum bicolor*, Stanford Seeds variety X9204) moistened with a trace mineral solution.[6] Reactor contents were maintained between 25 and 32% total solids, an organic loading rate of 18 $gVS \cdot kg^{-1}d^{-1}$, with a volatile solids removal efficiency of 74.8% and a methane production rate of 5.7 $l \cdot kg^{-1}d^{-1}$. (Since reactor contents were a moist solid, it was measured by weight rather than the more common volume units.) Ammonia was supplemented at from 32 to 40 mg $N\text{-}kg^{-1}d^{-1}$. A description of the reactor's performance,[6] and studies of its metabolic activities[7] and its biomass and community structure[5] have been published.

2.2. *Pressure tube microcosms*

An approximately 100 g sample of the reactor contents was placed in a small plastic container under a headspace of nitrogen. Approximately 2 ml of the material was gently packed into the open end of a 3 ml plastic syringe that had the Luer-lock end cut off. The sample was immediately placed in a pre-weighed pressure tube (Bellco Glass Inc., Vineland, NJ) with 0.74 μCi of ^{14}C-2-acetate (New England Nuclear, Boston, MA) in 1 ml sterile, anaerobic, deionized water, and vigorously shaken. The precise weight of sample taken was determined by difference. Pressure tube microcosms were incubated for 0 or 24 h at 55°C before biological activity was stopped with 5 ml formalin. Tubes to be inhibited at 0 h had the formalin added before the sample. The treatment compounds (oxygen and chloroform) were also added to the pressure tubes before the reactor sample. After addition of formalin, the tubes were stored inverted and frozen until analysis. Two samplings were done, the first for the high level chloroform treatment and the second for the oxygen and low level chloroform treatment. Each included controls inhibited at the time of sampling, controls incubated without chloroform or oxygen, and treatments incubated with either chloroform or oxygen. All controls and treatments were performed in triplicate.

2.3. *Treatments*

Oxygen addition was performed by equilibration of the pressure tubes with room air for 30 min, inoculation, injection of 30 ml air, and 5 h later injecting 10 additional ml room air. This gave 308 μmol O_2 per gram reactor material. Chloroform treatments were with either 4 or 100 μl chloroform, to yield 26 or 630 μmol chloroform per gram, respectively.

2.4. *Headspace analysis*

For the determination of total and labeled methane and carbon dioxide, the tubes were warmed to room temperature and 0.2 ml aliquots of the headspace injected into a Shimadzu GC-8A gas chromatograph equipped with a carbosieve 8000 column and a thermal conductivity detector. Radiolabeled methane and carbon dioxide were determined by a Packard Model 894 gas proportional counter connected to the gas chromatograph effluent. Standards for methane, carbon dioxide, and ^{14}C-carbon dioxide were made by diluting the gas with nitrogen in sealed serum bottles. The calibration curves were linear over the range used. The counting efficiency for ^{14}C determined for carbon dioxide (65%) was used for methane as well. Immediately after gas chromatographic analysis, the pressure tubes were opened and the pH measured in order to determine the partition coefficient for carbon dioxide between aqueous and gas phases. The pressure tubes were then refrozen until analysis of labeled lipids.

2.5. *Lipid extraction*

For analysis of the label incorporated into microbial lipids, the tubes were thawed and the contents extracted by the method of Bligh and Dyer[8] as modified.[9] The sample was transferred from the pressure tube into a 250 ml separatory funnel with 37.5 ml chloroform, 75 ml methanol, and 30 ml phosphate buffer (50 mM PO_4, pH = 7.4). The single-phase solvent was allowed to extract for at least 2 h with occasional shaking. Additional chloroform and water were then added (37.5 ml each), separating the organic and aqueous phases. The organic phase containing the total lipid fraction (TL) was collected and the solvent removed with a rotary evaporator at 37°C. Ten percent of the TL was reserved for liquid scintillation counting.

2.6. *Silicic acid column chromatography*

The lipid extract was separated into neutral lipid, glycolipid, and polar lipid fractions by silicic acid column chromatography.[10] One g of silicic acid (Unisil, 100–200 mesh, Clarkson Chem., Williamsport, PA) was slurried in chloroform and loaded into a 1 cm wide disposable

pipet plugged with glass wool. Neutral lipids were eluted with 10 ml chloroform, glycolipids with 10 ml acetone, and polar lipids with 10 ml methanol. Solvent was removed from each fraction with a stream of dry nitrogen and a 37°C water bath.

2.7. *Polar lipid components—fatty acids, diether, tetraether*

The polar lipid fraction was further separated into the eubacterial and eukaryote fatty acids (FA), and the archaebacterial diether (DE) and tetraether (TE). Treating the polar lipid with a strong acid hydrolysis (10% HCl, 100°C, 1 h) freed the phospholipid ethers as ether-alcohols[11] and the ester-linked fatty acids as free fatty acids.[12] Two ml of water and 2 ml of hexane/chloroform (4:1) were added, the test tube vigorously mixed, centrifuged, and the upper organic layer transferred to another test tube. The aqueous phase was extracted twice more with hexane/chloroform, the extracts pooled, and the solvent removed with a stream of dry nitrogen and a 37°C water bath.

The FA, DE, and TE components of the polar lipid methanolyzate were separated by thin layer chromatography on Whatman Linear K plates with hexane/diether/ammonia (80:20:2) as the mobile phase. The bands were visualized by exposure to iodine vapors. Stearic acid, 1,2-di-*O*-hexadecylglycerol, the TE from *Methanobacterium formicicum* were used as retention standards for FA, DE, and TE, respectively. The bands were scraped from the TLC plate, eluted into liquid scintillation vials with chloroform/methanol/acetic acid (50:50:1), and the solvent removed with a stream of dry nitrogen at 37°C.

2.8. *Glycolipid components—poly-β-hydroxyalkanoate*

The amount of radiolabel incorporated into poly-β-hydroxy-alkanoate (PHA) from acetate was determined by the filter paper method.[13] The glycolipid fraction was dissolved in a minimum volume of chloroform and deposited on 1 cm × 5 cm strips of filter paper. PHA was fixed to the filter paper in an 80°C oven for 30 min, and interfering lipids removed by alternating rinses with absolute ethanol and diethyl ether and the amount of radiolabel determined by liquid scintillation counting.

2.9. *Neutral lipid components—triglyceride*

Triglyceride was recovered from the neutral lipid fraction by TLC on Whatman linear K plates with hexane/ethyl ether/acetic acid (80:20:2) as the mobile phase. Tristearin was used as a retention standard. Lipid was recovered from the TLC plate and counted as for the polar lipid components.

3. RESULTS

3.1. *Pressure tube microcosms*

The methane and carbon dioxide production rates and the pHs observed in the control pressure tubes were the same as those measured in the reactor itself,[7] indicating that the anaerobic pressure tube microcosms were a suitable model system for evaluation of reactor activities *in situ*. Of the 10 measures of microbial activity

Table 1. Effects of oxygen and low and high concentrations of chloroform on microbial activities in the high-solids anaerobic biomass reactor, expressed as percent difference from control and the standard deviation, ((control − treatment)/control) × 100.

	Oxygen		Chloroform Low		Chloroform High	
Gas production						
Methane	−43	(47)	−99	(41)↓	−99	(44)↓
Carbon dioxide	87	(42)↑	−69	(39)↓	−82	(33)↓
Radiolabeled acetate utilization						
Acetate	2	(3)	−96	(4)↓	−99	(1)↓
Radiolabeled acetate incorporation						
Methane	−7	(5)↓	−100	(3)↓	−100	(1)↓
Carbon dioxide	100	(14)↑	−54	(35)↓	−92	(10)↓
Fatty acids	−28	(37)	−45	(53)	−99	(31)↓
Diether	166	(132)↑	−89	(58)↓	−97	(57)↓
Tetraether	−45	(34)↓	−96	(27)↓	−100	(26)↓
Poly-β-hydroxyalkanoate	92	(80)↑	75	(92)	−63	(61)
Triglyceride	−83	(56)↓	−84	(48)↓	−99	(55)↓

Number of replicates = 3. Statistical significance was tested by the Student's *t*-test at the 95% confidence level.
↑ = Significantly greater than control.
↓ = Significantly less than control.

reported in Table 1, introduction of oxygen statistically significantly increased 4 and decreased 3, low levels of chloroform significantly decreased 8, and high levels of chloroform significantly decreased 9. Therefore, the treatments affected metabolic activities in the microcosms.

3.2. *Oxygen treatment*

The introduction of oxygen decreased the production of total methane by 43% versus the controls, but it was not a statistically significant difference due to the high variability in measurement (Table 1). The percent of label incorporated into methane only decreased by 7%, significant at the 95% confidence level. The production of carbon dioxide was stimulated 87%, while the percent of label incorporated into carbon dioxide was increased by 100%, both differences are highly significant. The label incorporated into DE increased significantly (166%), into TE decreased significantly (45%), and was not significantly different for FA (Table 1). Oxygen treatment significantly increased the fraction of label incorporated into PHA and significantly decreased the fraction incorporated into triglyceride.

3.3. *Chloroform treatment*

The effects of chloroform were very different from that of oxygen. The amount of methane found in the chloroform poisoned tubes was of the same order as those tubes inhibited at zero hours, the methane entrapped in the material at sampling. The production of methane and incorporation of label into methane was entirely prevented by treatment with chloroform, compared to being decreased with oxygen. The production of carbon dioxide and the incorporation of label into it were decreased relative to controls, rather than increased as with oxygen treatment. The differences in all 4 of the biogas measures at both levels of chloroform addition were found to be statistically significant. The percent of label incorporated into both DE and TE decreased significantly at both levels of chloroform addition. The fraction of label incorporated into FA was not significantly changed by the low level of chloroform addition, and was significantly decreased by the high level chloroform addition. Label incorporation into PHA was not significantly affected, and into triglyceride was significantly decreased by both chloroform treatments.

4. DISCUSSION

4.1. *The effects of oxygen addition*

Oxygen addition decreased methane production (Table 1), for which there are at least 2 possible mechanisms: oxygen could have directly inhibited the methanogens, or the facultative anaerobes could have out-competed the methanogens for reducing equivalents when provided with a high-energy terminal electron acceptor. Both of these mechanisms predict a decrease in methane production, an increase in carbon dioxide production, and an increase in labeled acetate incorporation into carbon dioxide, as was observed. However, the oxygen toxicity mechanism predicts that label incorporation into methane should decrease by approximately the same amount as methane production. Label incorporation into methane was only decreased by 7%, while methane production was decreased by 43% (Table 1). Radiolabeled acetate consumption increased with oxygen addition, but not enough to account for the only 7% decrease in labeled methane by a change in the acetate pool size. Kiener and Leisinger[15] found that the plating efficiencies of *Methanobacterium thermoautotrophicum*, *Methanobrevibacter arboriphilus*, and *Methanosarcina barkeri* were not affected by 10–30 h of exposure to air-equilibrated media, so viable methanogens are possible under these conditions.

Further evidence for the viability of the methanogens is seen in radiolabeled acetate incorporation into the archaebacterial diether (DE) and tetraether (TE) (Table 1). The increase in label incorporated into DE and the decrease into TE may be due to the differences in oxygen susceptibility between sub-groups of methanogens,[15] or to an unknown adaptive reaction to oxygen toxicity. In a different thermophilic anaerobic biomass reactor system Henson *et al.*[14] found that supplementation with nitrate increased viable microbial biomass and decreased methane production. Rivard *et al.*[1] observed that the decrease in methane production in a nitrate-amended digester was due to the demand for electrons for nitrate reduction competing with carbon dioxide reduction to methane.

Addition of oxygen greatly increased the amount of carbon dioxide produced and the incorporation of radiolabeled acetate into carbon dioxide, but did not significantly change the proportion of label incorporated into fatty acids

or the amount of acetate consumed. The facultative anaerobes in the high-solids reactor were able to take advantage of the availability of oxygen for growth. Bacteria accumulate PHA under conditions of unbalanced growth—plentiful carbon and energy substrates but with cell division blocked by lack of a nutrient or an environmental factor.[13] The 92% increase in the amount of labeled acetate incorporated into PHA could be due to the facultative aerobic bacteria rapidly growing by oxygen respiration, and becoming limited by the lack of some nutrient. Similarly, in a study of the effect of nitrate on a thermophilic digester's performance, the amount of PHA produced increased.[14]

Triglycerides are synthesized by eukaryotes for carbon and energy storage.[16] The 83% decrease in acetate incorporated into triglyceride indicates that the eukaryotic components of the microbial community were adversely affected by the presence of oxygen. The fungi[17] and the protozoans with methanogen symbionts[18] found in methanogenic systems could have been poisoned by oxygen toxicity or overgrown by the aerobic bacterial bloom.

4.2. *The effects of chloroform*

Chloroform is specifically toxic to methanogenesis[2] and has non-specific membrane effects on all cells.[19] The addition of the low and high concentrations of chloroform decreased total and labeled methane production by from 99% to 100% (Table 1), and the proportion of label into DE and TE by from 89 to 100%. There were many differences between the 2 treatments, however. In the low chloroform treatment, total and labeled carbon dioxide production was decreased relative to controls, but not as much as in the high chloroform treatment, indicating that degradation of feedstock and labeled acetate continued with the low treatment. The incorporation of label into FA and PHA was not significantly changed by the low chloroform treatment, indicating that there was still significant eubacterial metabolic activity, while the high chloroform treatment reduced incorporation to nearly zero. The percent of label incorporated into archaebacterial DE and TE was significantly less than control for the low and the high chloroform treatments, indicating a specific effect on the methanogens. Radiolabel was still incorporated into DE in the low chloroform treatment in the complete absence of methanogenesis.

The aqueous concentration of chloroform used in the low level treatment, 26 μmol·g^{-1}, accounting for the ~29% total solids, was 4.4 mg·l^{-1}. Due to the extremely high solids content tieing up the water and lowering the amount of free liquid, the effective solution concentration of chloroform was much higher than that. Yang and Speece[20] found gas production from an acetate-fed methanogenic culture (CSTR, 50 day SRT) completely inhibited by 2.5 mg·l^{-1} chloroform. The lower sensitivity to chloroform of this high-solids system than the completely liquid one of Yang and Speece may have been due to the protective effect of digester solids, as reported for heavy metals.[3]

4.3. *The use of the microcosm to monitor metabolic activities*

The use of pressure tube microcosms, radiolabel tracers, and lipid analysis to determine the effects of added oxygen or chloroform gave detailed information on shifts in the activities of specific populations within the reactor. One of the most interesting results was the increase in radiolabeled acetate incorporation into archaebacterial DE with exposure to oxygen. Further experiments with pure cultures and samples from bioreactors are planned to determine whether this is due to a differential effect on different groups of methanogens, or an adaptive response to the presence of oxygen.

The effects of a possibly toxic compound on a municipal solid waste treatment plant cannot be tested on the plant itself. The detailed analysis of the effects of the toxicant on an appropriate microcosm system can be used to determine its mechanism of action and the possible efficacy of prophylactic measures.

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REFERENCES

1. C. J. Rivard, F. Bordeaux, J. M. Henson and P. H. Smith, Effects of addition of soluble oxidants on the thermophilic anaerobic digestion of biomass to methane. *Appl. Biochem. Biotech.* **17,** 245 (1988).
2. T. Bauchop, Inhibition of rumen methanogenesis by methane analogues. *J. Bact.* **94,** 171–175 (1967); and P. G. Thiel, The effects of methane analogues on methanogenesis in anaerobic digesters. *Water Res.* **3,** 215–223 (1969).
3. K. F. Jarrell, M. Saulnier and A. Ley, Inhibition of methanogenesis in pure cultures by ammonia, fatty acids, and heavy metals, and protection against heavy metals toxicity by sewage sludge. *Can. J. Microbiol.* **33,** 551–554 (1987).

4. J. S. Jerris and P. L. McCarty, The biochemistry of methane fermentations using ^{14}C tracers. *J. Water Poll. Control Fed.* **39,** 178–192 (1965).
5. D. B. Hedrick, B. Richards, W. Jewell, J. B. Guckert and D. C. White, Disturbance, starvation, and overfeeding stresses detected by microbial biomarkers in high-solids high yield methanogenic bioreactors. *J. Ind. Microbiol.* (in press).
6. B. K. Richards, R. J. Cummings, W. J. Jewell and F. G. Herndon, High solids anaerobic fermentation of sorghum and cellulose. *Biomass and Bioenergy* **1,** 47–53 (1991).
7. D. B. Hendrick, A. Vass, B. Richards, W. Jewell, J. B. Guckert and D. C. White, Starvation and overfeeding stress on microbial activities in high-solids high-yield methanogenic digesters. *Biomass and Bioenergy*, **1,** 75–82 (1991).
8. E. G. Bligh and W. J. Dyer, A rapid method of lipid extraction and purification. *Can. J. biochem. Physiol.* **35,** 911–917 (1959).
9. D. C. White, W. M. Davis, J. S. Nickels, J. D. King and R. J. Bobbie, Determination of the sedimentary microbial biomass by extractible lipid phosphate. *Oecologia* **40,** 51–62 (1979).
10. D. C. White, G. A. Smith, M. J. Gehron, J. H. Parker, R. H. Findlay, R. F. Martz and H. L. Fredrickson, The groundwater aquifer microbiota: biomas, community structure, and nutritional status. *Dev. Ind. Microbiol.* **24,** 201–211 (1983).
11. C. A. Mancuso, G. Odham, G. Westerdahl, J. N. Reeve and D. C. White, C_{15}, C_{20} and C_{25} isoprenoid homologues in glycerol diether phospholipids of methanogenic archaebacteria. *J. Lipid Res.* **26,** 1120–1125 (1985).
12. M. J. Gehron and D. C. White, Quantitative determination of the nutritional status of detrital microbiota and the grazing fauna by triglyceride glycerol analysis. *J. Exp. Mar. Biol.* **64,** 145–158 (1982).
13. R. H. Findlay, P. C. Pollard, D. J. W. Moriarty and D. C. White, Quantitative determination of microbial activity and community nutritional status in estuarine sediments: evidence for a disturbance artifact. *Can. J. Microbiol.* **31,** 493–498 (1985).
14. J. M. Henson, P. H. Smith and D. C. White, Examination of thermophilic methane-producing digesters by analysis of bacterial lipids. *Appl. Env. Microbiol.* **50,** 1428–1433 (1985).
15. A. Kiener and T. Leisinger, Oxygen sensitivity of methanogenic bacteria. *System. Appl. Microbiol.* **4,** 305–312 (1983).
16. M. J. Gehron and D. C. White, Sensitive assay of phospholipid glycerol in environmental samples. *J. Microbiol. Meth.* **1,** 23–32 (1983).
17. D. O. Mountfort, Role of anaerobic fungi in the rumen ecosystem. In *Perspectives in Microbial Ecology. Proceedings of the 4th International Symposium on Microbial Ecology, Ljubljana, Yugoslavia* (F. Megušar and M. Gantor, Eds) pp. 176–179. Slovene Soc. for Microbiology, Ljubljana (1986).
18. C. K. Stumm, K. B. Zwart, J. J. A. Von Bruggen and G. D. Vogels, Methanogenic bacteria as endosymbionts of anaerobic protozoa. In *Perspectives in Microbial Ecology. Proceedings of the 4th International Symposium on Microbial Ecology, Ljubljana, Yugoslavia* (F. Megušar and M. Gantor, Eds) pp. 169–175. Slovene Soc. for Microbiology, Ljubljana (1986).
19. T. A. Loomis, *Essentials of Toxicology*. Lee and Febiger, Philadelphia. p. 46 (1978).
20. J. Yang and R. E. Speece, The effects of chloroform toxicity on methane fermentation. *Water Res.* **20,** 1273–1279 (1986).