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Name of Principal Contact Person: **Dr. Daniel H. Zitomer**  
Organization: **Marquette University**  
Email Address: **Daniel.zitomer@mu.edu**  
WEB Address: **www.marquette.edu/wqc**  
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## **Abstract**

We developed new preservation methods for beneficial microorganisms that, when added to anaerobic digesters via bioaugmentation, increase methane production and stabilization of wastes. Our microorganisms produced methane after drying and storage. Culture conditions, drying methods, cryoprotectant presence, and biofilm development all influenced the post-storage methane production rate. We plan to continue to develop cultures to use for bioaugmentation of anaerobic digesters to increase the rate and extent of methane production. To commercially develop this enterprise, resources for pre-commercialization (scale-up) and beta testing are required. From the results of this study, the agricultural industry should consider drying and storage of methane-producing cultures as a possible method to deliver beneficial organisms that can improve anaerobic digestion processes.

## **Introduction**

Anaerobic digestion is a practical technology for renewable energy production and stabilization of agricultural residues in Wisconsin. The biogas produced contains methane and can be used to generate electricity and/or provide heat. The technology can be significantly improved by developing new commercial applications to digest wastes more completely. However, challenges must be overcome before the technology is used more widely. For example, of the 74 digesters in the US producing methane from animal manure in 1995, 40% operated poorly. But the prospects have been improving due to research and full-scale experience.

Lack of understanding regarding the identity and function of the most beneficial microorganisms in an anaerobic digester is a challenge to wider implementation. For too long, practitioners have regarded methanogenic cultures as “black boxes” that are difficult to characterize. The typical approach is that agricultural waste is pumped into a digester and whatever culture predominates is relied upon to produce methane. This has worked for many applications, but has been a failure for others.

A second major challenge involves lack of understanding regarding the benefits of “bioaugmentation”, defined as the addition of specific microorganisms to bioreactors to increase efficiency. As stated above, whatever culture predominates in a digester is now relied upon to make methane. Unfortunately, many practitioners are unaware of

bioaugmentation. However, the periodic introduction of beneficial microorganisms to an anaerobic digester (i.e., bioaugmentation) can lead to profound increases in methane production.

In the work described herein, methods to dry and preserve beneficial anaerobic microbes were developed so that bioaugmentation cultures can be produced and delivered to operating digesters.

## **Bioaugmentation and Microbe Preservation**

Bioaugmentation has been used to improve human health. Specific bacteria in food such as yogurt (*Lactobacillus bulgaricus*, *Bifidobacteria* sp., etc.) improve lactose digestion and, when present in the human digestive tract, support the immune system. Production of “probiotics” for the human and animal digestive tract is an active area of application and research.

Bioaugmentation for anaerobic digestion has been studied on a very limited basis and has not yet been applied in full scale. In the laboratory, researchers have decreased the start-up time of an anaerobic fluidized bed treating a pharmaceutical wastewater using bioaugmentation. Others have bioaugmented a stressed laboratory-scale anaerobic digester, and a 25% increase in methane production was observed in comparison to a non-bioaugmented digester. Also, a digester was bioaugmented to enhance biodegradation of polycyclic aromatic hydrocarbons (PAHs). A PAH-adapted microorganism was added and a significant increase in PAH removal was observed. We have developed cultures that improve the rate of methane production during anaerobic digestion. The addition of our bioaugmentation cultures to an anaerobic digester significantly increased biogas production in previous research. Four anaerobic digesters (50 mL each) were fed synthetic waste. Two digesters were bioaugmented with one of our cultures, whereas the other two digesters were not, and served as controls. Our results were more dramatic and beneficial than expected. Bioaugmented digesters continuously produced 36% more methane than controls, even after 100 days.

The heart of the work described herein is this: microorganisms for commercial application must be produced in a form that can be economically transported and stored. In addition, significant microorganism viability must be preserved during processing, storage, and reconstitution. Most commercial production methods rely on desiccation by liquid drying, freeze drying or spray drying. In these ways, water is removed, greatly reducing the mass for shipping and preserving the microorganism activity.

Desiccation is not a simple preservation method to implement. Not all microorganisms retain activity after drying under all conditions. Unfortunately, very little work has been reported regarding successful preservation/desiccation of anaerobic, methanogenic microorganisms. For many other types of organisms, however, survivability has been enhanced by adding protective agents (i.e., cryoprotectants) and altering growth media composition. Unfortunately, there is no general rule for optimum conditions, and different microorganisms have been found to retain maximum activity with different treatments/conditions.

## **Methods**

Research was performed to develop preservation methods for our existing anaerobic bioaugmentation cultures. We answered the following questions:

1. Do certain culture conditions help preserve methanogenic culture activity after drying and storage?
2. Do cryoprotectants improve survivability of methanogenic culture after drying and storage?
3. Do biofilms survive drying and storage better than aqueous suspended cultures?
4. Is preservation/storage of methanogenic cultures viable for bioaugmentation?

The following tasks were performed to answer these questions.

**Task 1: Determine activity of anaerobic cultures after drying.** We dried cultures presently existing in our laboratory that increase methane production when used to bioaugment laboratory anaerobic digesters. Three drying methods were employed and the results for the methods were compared. For Task 1, we also measured methanogenic activity after drying with and without cryoprotectants.

After drying, the cultures were held at 4° C for less than one week, reconstituted, and evaluated for methanogenic activity (how rapidly they converted substrate to methane). In addition, other dried culture samples were held using an accelerated storage protocol described below that simulates storage for 20 years. Subsequently, stored cultures were reconstituted and assayed for activity.

**Task 2: Determine if biofilms retain more activity than suspended cultures after drying/storage.** We postulated that anaerobic cultures grown as a biofilm on a surface would have superior activity after drying, storage and reconstitution as compared to cultures in aqueous suspension. We believed organisms in the inner region of a biofilm would be shielded from traces of oxygen that can be toxic, especially to obligate anaerobes that are already under stress due to desiccation. For this reason, we grew the bioaugmentation cultures on surfaces, dried the material, and measured culture activity after storage and reconstitution using drying Condition 1 with and without cryoprotectants.

## **Analytical Methods**

**Drying.** For drying, methanogenic cultures were centrifuged to concentrate biomass, the supernatant was discarded, and the resulting thickened biomass solids were amended with a solution of protective agents. The cultures were then dried.

**Accelerated and Short-Term Storage.** After drying, cultures were stored for three days or less at room temperature in a dessicator containing Drierite to provide low relative humidity (i.e., short-term storage). Subsequently, cultures were reconstituted, and evaluated for methanogenic activity. In addition, other dried samples were held using an accelerated storage protocol for estimating activity after prolonged storage. For this, dried cultures were held for 2 weeks at an elevated temperature (35° C). It has been found that storage at 37°C for two weeks simulates storage for approximately 20 years at 4°C for some microorganisms. Subsequently, the activity of reconstituted cultures were determined.

**Culture Activity Testing.** The activity of reconstituted cultures was determined using the specific methanogenic activity (SMA) assay as described by Coates et al. (1996). For this, dried cultures were reconstituted using reduced basal medium in 160-mL serum bottles. The bottles were sparged with oxygen-free gas and a substrate and carbon source for methane production was added. Methane production was determined over time, and the rate of methane production was calculated. In this way, the activity and percent change in activity due to the various treatments was compared.

**Other methods.** All other testing will be performed using standard methods (APHA et al., 2000).

## **Results**

Four existing methane-producing laboratory cultures (Cultures 1, 2, 3 and 4) were used for drying. Each culture was grown under different conditions and each demonstrated a relatively high rate of methane production before drying and storage. All cultures were dried using three different methods (Methods 1, 2 and 3) with and without cryoprotectants. In addition, all cultures were grown in suspension and as well as biofilms on solid carrier particles. The biofilms were only dried using drying Method 1. Therefore, a total of 32 drying treatments were performed. All results are averages of three replicates. Results are presented in Tables 1 through 13, below.

All cultures showed methanogenic activity after drying and storage. The average methanogenic activity loss for suspended cultures under all conditions after drying and storage was 54% (see Tables 1 through 6). Therefore, the suspended cultures, on average, maintained 46% of their methane-producing activity after drying and storage.

Culture conditions influenced activity loss after drying and storage (see Table 10). For suspended biomass, culture Conditions 2 and 3 resulted in the lower average methanogenic activity loss after drying and storage than culture Conditions 1 and 4.

The addition of a cryoprotectant significantly decreased the activity loss after drying and storage. For suspended biomass, the average activity loss after drying and storage without cryoprotectant addition under all drying methods utilized was greater than the activity loss with cryoprotectant addition (see Table 11).

The use of a biofilm versus suspended biomass led to increased activity after drying with a cryoprotectant (see Table 12).

Table 1: Results of Drying Method 1 - Suspended Biomass Without Cryoprotectant  
(Activity = mL CH<sub>4</sub>/g VSS-hr at 35°C, 1 atm)

Culture	Activity After Drying	Activity After Storage	Activity Loss After Storage (%)
1	196	75	61
2	52	29	56
3	80	57	71
4	32	19	41
Average	-	-	57

Table 2: Results of Drying Method 1 - Suspended Biomass With Cryoprotectant  
(Activity = mL CH<sub>4</sub>/g VSS-hr at 35°C, 1 atm)

Culture	Activity After Drying	Activity After Storage	Activity Loss After Storage (%)
1	163	101	38
2	52	36	31
3	228	172	25
4	44	27	39
Average	-	-	33

Table 3: Results of Drying Method 2 - Suspended Biomass Without Cryoprotectant  
(Activity = mL CH<sub>4</sub>/g VSS-hr at 35°C, 1 atm)

Culture	Activity After Drying	Activity After Storage	Activity Loss After Storage (%)
1	196	64	67
2	72	34	53
3	150	105	30
4	61	32	48
Average	-	-	50

Table 4: Results of Drying Method 2 - Suspended Biomass With Cryoprotectant  
(Activity = mL CH<sub>4</sub>/g VSS-hr at 35°C, 1 atm)

Culture	Activity After Drying	Activity After Storage	Activity Loss After Storage (%)
1	172	95	45
2	94	48	49
3	181	119	34
4	59	33	44
Average	-	-	43

Table 5: Results of Drying Method 3 - Suspended Biomass Without Cryoprotectant  
(Activity = mL CH<sub>4</sub>/g VSS-hr at 35°C, 1 atm)

Culture	Activity After Drying	Activity After Storage	Activity Loss After Storage (%)
1	165	13	92
2	151	51	66
3	303	19	94
4	87	39	55
Average	-	-	77

Table 6: Results of Drying Method 3- Suspended Biomass With Cryoprotectant  
(Activity = mL CH<sub>4</sub>/g VSS-hr at 35°C, 1 atm)

Culture	Activity After Drying	Activity After Storage	Activity Loss After Storage (%)
1	185	16	91
2	162	74	54
3	178	61	66
4	138	64	46
Average	-	-	64

Table 7: Results of Drying Method 1 - Biofilm With Cryoprotectant  
(Activity = mL CH<sub>4</sub>/g VSS-hr at 35°C, 1 atm)

Culture	Activity After Drying	Activity After Storage	Activity Loss After Storage (%)
1	136	94	31
2	127	93	27
3	150	123	18
4	108	70	35
Average	-	-	28

Table 8: Results of Drying Method 1 - Biofilm Without Cryoprotectant  
(Activity = mL CH<sub>4</sub>/g VSS-hr at 35°C, 1 atm)

Culture	Activity After Drying	Activity After Storage	Activity Loss After Storage (%)
1	244	68	72
2	84	56	33
3	205	161	21
4	72	47	35
Average			40

Table 9: Comparison of Activity Loss for Suspended Biomass Using Different Drying Methods Without Cryoprotectant

(Activity = mL CH<sub>4</sub>/g VSS-hr at 35°C, 1 atm)

Culture	Drying Method 1	Drying Method 2	Drying Method 3
1	61	67	92
2	56	53	66
3	71	30	94
4	41	48	55
Average	57	50	77

Table 10: Comparison of Activity Loss for Suspended Biomass Using Different Drying Methods With Cryoprotectant

(Activity = mL CH<sub>4</sub>/g VSS-hr at 35°C, 1 atm)

Culture	Drying Method 1	Drying Method 2	Drying Method 3	Average
1	38	45	91	58
2	31	49	54	31
3	25	34	66	42
4	39	44	46	43
Average	33	43	64	-

Table 11: Comparison of Suspended Biomass Activity Loss With and Without Cryoprotectant

(Activity = mL CH<sub>4</sub>/g VSS-hr at 35°C, 1 atm)

Drying Method	Average Activity Loss With Cryoprotectant	Average Activity Loss Without Cryoprotectant	% Decrease in Activity Loss using Cryoprotectant
1	33	57	42
2	43	50	14
3	64	77	17
Average	47	61	23

Table 12: Comparison of Activity Loss: Suspended Culture vs. Biofilm With Cryoprotectant (Drying Method 1)

(Activity = mL CH<sub>4</sub>/g VSS-hr at 35°C, 1 atm)

Culture	Average Activity Loss for Suspended Biomass	Average Activity Loss for Biofilm	% Decrease in Activity Loss Using Biofilm
1	38	31	18
2	31	27	13
3	25	18	28
4	39	35	10
Average	-	-	17

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Table 13: Comparison of Activity Loss: Suspended Culture vs. Biofilm With Cryoprotectant

(Activity = mL CH<sub>4</sub>/g VSS-hr at 35°C, 1 atm)

Culture	Average Activity Loss for Suspended Biomass	Average Activity Loss for Biofilm	% Decrease in Activity Loss Using Biofilm
1	58	31	47
2	31	27	13
3	42	18	57
4	43	35	19
Average	-	-	34

### Expected Economic Impact

There are 28 engineered anaerobic digesters existing or under construction for treating dairy manure in Wisconsin. These digesters treated the manure from over 23,730 dairy cows, producing approximately 3 million cubic feet of biogas per day containing approximately 1,800 million Btu per day of energy (enough to provide electricity for 7000 typical Wisconsin homes). In addition, Wisconsin has more than 35 anaerobic digesters processing dairy and cheese waste (e.g., Crave Brothers, Waterloo; Formost Farms USA, Baraboo; Alto Dairy, Waupun; Protient, Juda), food waste (e.g., United Wastes, Watertown) and other agriculture-related wastes. With new interest in renewable energy and environmental protection, more anaerobic digesters will be built in Wisconsin. It is estimated that these facilities produce approximately 9 million cubic feet of biogas per day containing 5,400 million Btu (5400 decatherms) of energy per day. It is conservatively estimated that bioaugmentation will increase biogas production by 5%, resulting in an extra 360 decatherms per day of biogas worth approximately \$780,000 per year assuming energy cost of \$6 per dekatherm.

More effective anaerobic digestion will also protect the Wisconsin environment. According to the Wisconsin Department of Natural Resources, there were 79 concentrated animal feeding operations (CAFO) dairy farms operating in the state in 2002, each having more than 700 dairy cattle. Agricultural non-point pollution in Wisconsin can be a contributor to surface water quality deterioration. In addition, methane produced during the storage of untreated manure is a greenhouse gas (GHG). Manure management may account for as much as 5% of US methane emissions in 1999 (US EPA, 2007). The results of this work can be used to produce a new bioaugmentation product to enhance anaerobic digestion of dairy manure and other agricultural residue. This will support reduction of surface water pollution and GHG emissions as well as generate renewable energy on Wisconsin farms.

The continued development of bioaugmentation products will support the Wisconsin bio-industry. Results can be used to support future development of other beneficial anaerobic bioaugmentation products.

## **Conclusions**

The following conclusions are made:

- (1) Culture conditions influence the extent to which methanogenic culture activity can be maintained after drying and storage.
- (2) Cryoprotectants improve the activity of methanogenic cultures after drying and storage.
- (3) Anaerobic biofilm activity is, on average, higher than suspended biomass activity after drying and storage.
- (4) Preservation and storage of methanogenic cultures is viable.

In the future, we plan to continue to develop cultures to use for bioaugmentation of anaerobic digesters to increase the rate and extent of methane production. To commercially develop this enterprise, resources for pre-commercialization (scale-up) and beta testing are required. From the results of this study, the agricultural industry should consider drying and storage of methane-producing cultures as a possible method to improve anaerobic digestion processes.