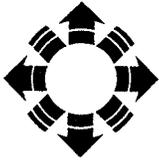


**Characterization and Anaerobic Digestion Analysis of  
Ethanol Process Samples**

**By**

**Pinnacle Biotechnologies Inc.**



**NREL**

National Renewable Energy Laboratory

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# PINNACLE

Biotechnologies International, Inc.

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July 1, 1998

Nick Nagle  
National Renewable Energy Laboratory  
1617 Cole Blvd.  
Golden, CO 80401

Dear Nick,

This letter report and the accompanying invoice serves as the conclusion of activities under NREL procurement P.O. #160809. Sample characterization data were summarized in a previous letter report. Presently, the anaerobic fermentation data and conclusions are described.

### **The Anaerobic Fermentation Bioassay**

It is important to note at the outset that the BMP assay may be useful in determining the potential level of bioconversion which may be possible for a test substrate. This assay may also give indications of a potential for a test substrate to cause inhibition of the anaerobic consortium which would limit or preclude conversion of the test substrate at least under anaerobic conditions. However, the BMP assay is always viewed as a rough cut analysis, with evaluation of continuous anaerobic digestion systems as a natural next step to provide better process data on rates and yields prior to engineering and costing commercial systems. The BMP assay may also be used to determine the effectiveness of treatments aimed at reducing sample toxicity or to improve the potential conversion rates and yields. Several important issues regarding the anaerobic fermentation studies (biochemical methane potential [BMP] assay) must be discussed prior to the interpretation of the data.

*The Anaerobic Culture.* A robust, diverse, anaerobic culture from a reliable, defined source is important to establishing the best fermentation analysis data. PINNACLE uses anaerobic cultures from anaerobic digesters at local municipal sewage treatment plants as assay and starter cultures as these cultures; 1) see a diverse mixture of organic wastes and therefore the microbial populations are diverse in biodegradative capabilities, 2) receive substantial macro and micro-nutrients and therefore are not operating under limiting or inhibitory conditions, and 3) are readily available and may be further obtained in large quantities for starting large scale applied systems once sufficient testing data is obtained.

The quantity of test culture used in the anaerobic fermentation assays is maximized to ensure rapid biodegradative results and to reduce the potential negative effects of dilution on the activity



of the culture.

*Negative Control.* A set of three negative controls were used during anaerobic fermentation studies to account for biogas production due to intrinsic organic matter contained in the anaerobic culture. It should be noted that any active culture used in fermentation tests will produce biogas from intrinsic organic matter unless the culture is first "washed" to remove this material first. For anaerobic fermentation studies, culture washing is detrimental to culture viability due to the potential to introduce oxygen or removal of complex macro and micro-nutrients. Without removing the intrinsic organics contained in the anaerobic culture, it is possible that an added test sample will negatively or positively affect the conversion of the intrinsic culture organics and therefore the background biogas production.

*Positive Control.* Generally, a positive control is selected which is similar to the composition of the test samples and which can serve as a check on the biodegradative capacity of the anaerobic culture used. The positive control is prepared at similar pH and organic loading to the test samples.

#### **Anaerobic Fermentation Studies**

*Test Samples.* Test sample characterizations were described in a previous letter report and indicated that samples MTX 7F, TiO<sub>2</sub>, and the Control Hydrolyzate were comparable in mass percent volatile solids (organic content) while sample BF 772014 was nearly 50% more dilute. The pH of all test samples were considerably below pH 7.0 and required adjustment with potassium hydroxide prior to fermentation studies. The analysis of chemical oxygen demand (COD), a measure of oxidizable carbon in the sample, indicated samples TiO<sub>2</sub> and the Control Hydrolyzate were similar and the highest of the samples while BF 772014 was the lowest.

*Positive Control.* For the positive control, a solution of protein hydrolyzate (BactoPeptone, Difco) was used. The use of a protein hydrolyzate sample was envisioned to be relatively close to the composition of the ethanol hydrolyzate samples. The mass percent volatile solids and COD values for the positive control sample were only slightly greater than samples TiO<sub>2</sub> and the Control Hydrolyzate.

*Pre-Incubation and Startup.* Anaerobic fermentation assays were initiated following incubation of the assay bottles for almost four days in order to reduce the background biogas production derived from the intrinsic organics in the anaerobic culture. A single volumetric loading was used (5%) which resulted in varying organic loadings for the different test samples from 1.41 to 2.87 grams of COD per liter of culture due to their individual concentrations.



**Results.** Immediate and strong biogas production was determined for all test samples as detailed in Figure 1. All samples also demonstrated the majority of the biogas production, hence the sample organic conversion, was complete within 5 to 10 days. The overall level of anaerobic bioconversion for each test sample is shown in Figure 2 based on the individual sample COD loading. A theoretical yield of 350 mL of methane per gram of COD added represents 100% conversion (Owen and McCarty, 1964). Anaerobic conversion data is shown in Table 1, below for the test samples after 26 days of incubation.

**Table 1. Anaerobic Fermentation Data and Final Analyses (26 d)**

Assay	BF 772014	MTX 7F	TiO2	Control Hyd.	Bacto Peptone
COD Loading (gCOD/bottle)	0.141	0.174	0.279	0.272	0.287
Theoretical CH <sub>4</sub> Yield (mL)	49.35	60.90	97.65	95.20	100.45
Actual CH <sub>4</sub> Yield (mL)	36.07	75.16	35.39	76.93	83.01
% Anaerobic Conversion	73.09	123.42	36.24	80.81	82.64
Final Biogas Methane (%)	61.40	61.86	64.56	61.43	64.98
Final pH	7.23	7.22	7.24	7.24	7.36

In general, the data indicates that the positive control (BactoPeptone), the Control Hydrolyzate, and BF 772014 resulted in similar levels of bioconversion (70% to 80%). If these samples were to be further incubated to 90 days, the final level of anaerobic conversion based on COD loading would most likely range from 90% to 100% of the theoretical. This slow approach to near complete digestion during the extended incubation period (final 60 days of a 90 day test) represents the adaptation of the anaerobic culture to minor, less common organics in the test samples.

The results found for the positive control, the Control Hydrolyzate and BF 772014 are characteristic of organic wastes which are eminently biodegradable. Test sample TiO<sub>2</sub> demonstrated limited biogas production indicating that organics in the sample were only partly biodegradable.

BMP data for NREL sample MTX 7F indicated greater than 100% conversion to the methane endproduct. This may be explained as either inaccurate COD analysis or active enzymes



contained in the sample which are effective in converting recalcitrant intrinsic organics (i.e., polymers) of the seed culture. Table 2. compares initial and final COD analysis for all four NREL test samples and validates the relative accuracy of the assay.

**Table 2. Re-Evaluation of NREL Test Sample COD Values**

Assay	BF 772014	MTX 7F	TiO2	Control Hyd.
Primary COD Assay (mg/L)	28,267	34,800	55,800	54,400
Secondary COD Assay (mg/L)	26,330	32,330	53,330	55,660
Difference (%)	-6.85	-7.10	-4.43	+2.32

As the accuracy of the test sample COD values are assured, the only plausible explanation is sample MTX 7F contained active hydrolytic enzymes which served to hydrolyze recalcitrant organics contained in the starter culture. Methods to test this theory and determine the true nature of the anaerobic biodegradation potential for this sample may include a thermal treatment of the sample to inactivate enzymes followed by conducting another BMP assay. In addition, the test sample could be analyzed by standard method for hydrolyzing enzyme activity.

### **Conclusion**

All samples tested demonstrated immediate and strong biogas production. None of the samples tested demonstrated toxicity to the anaerobic culture. The positive control demonstrated predicted effectiveness of the anaerobic starter culture. NREL samples BF 772014 and the Control Hydrolyzate demonstrated conversions similar to that of the positive control and may therefore be considered amenable to anaerobic treatment. NREL sample TiO2 demonstrated reduced conversion effectiveness which is likely due to some level of non-biodegradable organics in the sample. The excessive biogas production resulting in assays performed using NREL sample MTX 7F indicates that additional testing as described above is required to accurately predict the level of conversion possible.

While this data may be used to predict approximate fuel gas production which may result from treating large volumes of the respective organic streams using anaerobic digestion systems, in order to accurately engineer commercial-scale anaerobic systems, additional data from applied, longer-term operation of continuous anaerobic digestion systems should be obtained.



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Dear Nick,

The four NREL samples received from you were stored under refrigeration until being transferred by cooler to PINNACLE's Research, Development and Testing Center in Stanton, California for analysis and fermentation studies.

Rather than using Avecel as the positive control for these studies, a soluble substrate was used which more closely matches the NREL samples. The positive control substrate used was a Bacto Peptone solution at 4.5% w/v in distilled water. The NREL samples were analyzed on delivery PINNACLE's Testing Center. Total solids (%TS), volatile solids (%VS), and ash analyses were performed in triplicate. Analysis of sample pH were performed after a 2-point standardization of the combination pH probe.

**Table 1. Sample Analysis Upon Receipt**

Assay	BF 772014	MTX 7F	TiO2	Control Hyd.	Bacto Peptone
% Total Solids	2.73	4.49	5.56	5.20	5.67
% Volatile Solids	87.44	89.86	74.04	82.33	95.67
Mass % Volatile Solids	2.39	4.03	4.12	4.28	5.42
pH	5.39	4.93	5.24	5.36	7.08

As the NREL samples were considerably lower than the pH 7.0 necessary to perform the anaerobic digestibility analysis, they were adjusted to neutrality using a 5% w/v solution of KOH. A 50 mL aliquot of each sample was transferred to a small beaker. The sample was mixed using a magnetic stirrer and the pH monitored during KOH addition. The samples were then analyzed for Chemical Oxygen Demand (COD) using the HACH High Range Plus COD tube assay. All COD assays were performed in triplicate as detailed in Table 2.



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**Table 2. Sample pH Adjustment and COD Analysis**

Assay	BF 772014	MTX 7F	TiO2	Control Hyd.
Initial pH	5.39	4.93	5.24	5.36
mL KOH Added	0.56	1.22	1.11	0.8
Dilution Factor	0.9889	0.9762	0.9783	0.9843
Final pH	7.12	7.08	7.13	7.14
COD (mg/L)	28,267	34,800	55,800	54,400

For comparison, the COD level of the Bacto Peptone positive control was 57,400 mg/L.

## Anaerobic Digestibility Assays

The Biochemical Methane Potential (BMP) assay was used to address the biodegradability or toxicity of the NREL samples. The BMP assay employed a mesophilic anaerobic culture obtained from the Terminal Island Sewage Treatment Plant, Terminal Island, CA. This anaerobic culture was assayed prior to use as detailed in Table 3.

**Table 3. Analysis of the Terminal Island Anaerobic Culture Used in BMP Assays**

Assay	Value
Total Solids	3.11%
Volatile Solids	62.53%
Ash	37.47%
pH	7.43

The BMP assays were prepared in triplicate using serum bottles with a total volume of 162 mL. Using a 25 mL pipette, 100 mL ( $\pm$  1.8 mL) of active anaerobic culture was transferred to each serum bottle. The headspace of each serum bottle was then flushed with UHP nitrogen for 1-min. prior to closing the bottles with a rubber stopper and an aluminum crimp cap. The serum bottles were incubated at 37°C with shaking (200 rpm) using a Lab-Line Orbit Environ-Shaker.



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The serum bottles were incubated for a period of almost 4 days prior to commencing the BMP assay in order to reduce background biogas production from intrinsic organic matter contained in the anaerobic sludge culture. In order to reduce the negative effects of dilution on the BMP anaerobic culture, a standard 5 mL addition of each test substrate was used. This represented roughly a 5% dilution of the anaerobic culture. The actual organic loadings and theoretical methane potential for each substrate varied as per its relative composition as described below in Table 4.

**Table 4. BMP Organic Loadings**

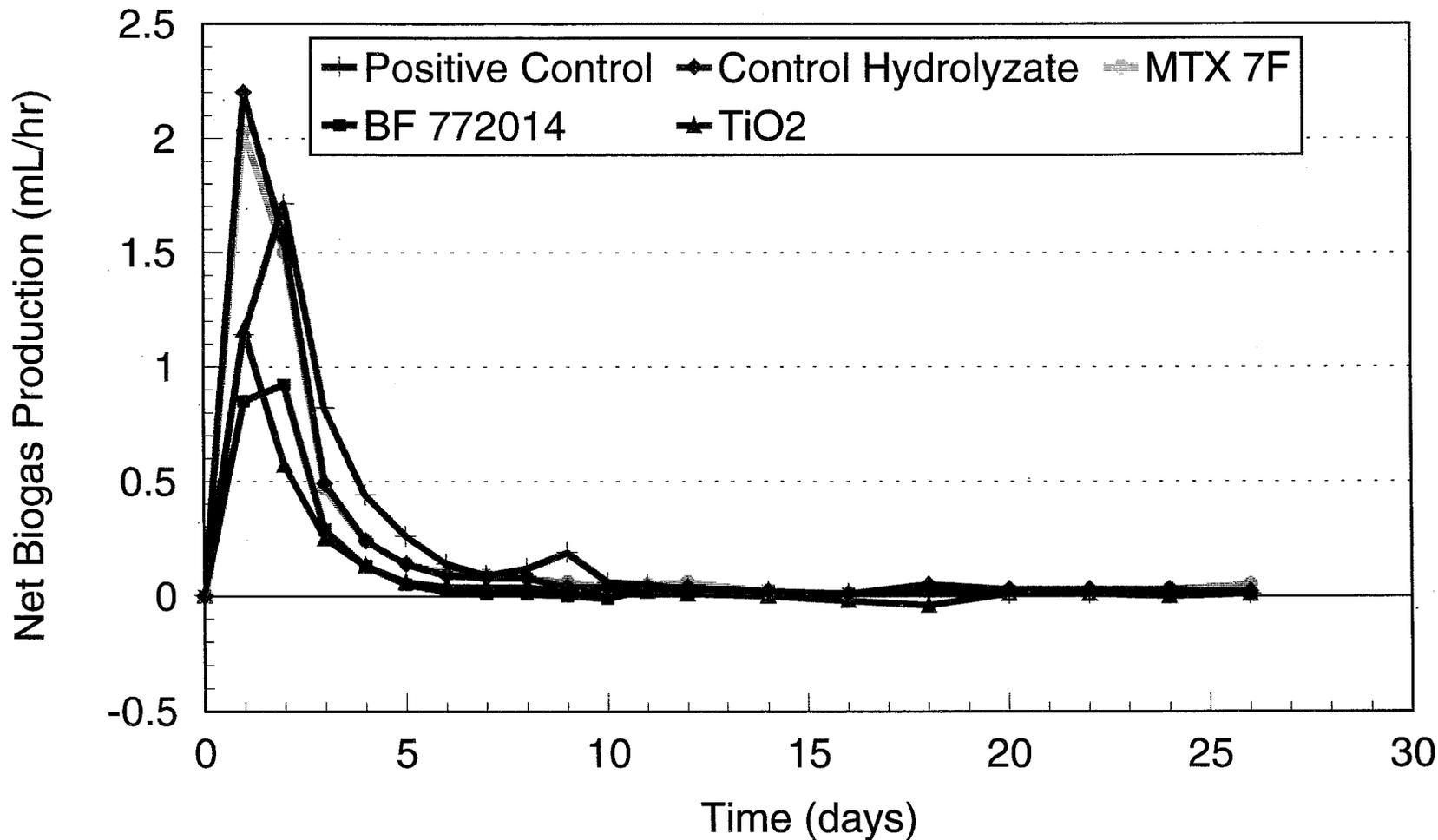
Sample	Volume Added	Organic Loading		Theoretical Methane Yield (mL)**
		gVS/bottle*	gCOD/bottle	
Bacto Peptone	5 mL	0.271	0.287	100.45
BF 772014	5 mL	0.118	0.141	49.35
MTX 7F	5 mL	0.197	0.174	60.90
TiO <sub>2</sub>	5 mL	0.202	0.279	97.65
Control Hydrolyz.	5 mL	0.211	0.272	95.20

\* Volatile solids loading corrected for sample dilution during pH adjustment.

\*\* Theoretical methane yields based on COD loading using a yield of 350 mL CH<sub>4</sub> per gram COD added (Owen and McCarty, 1964).

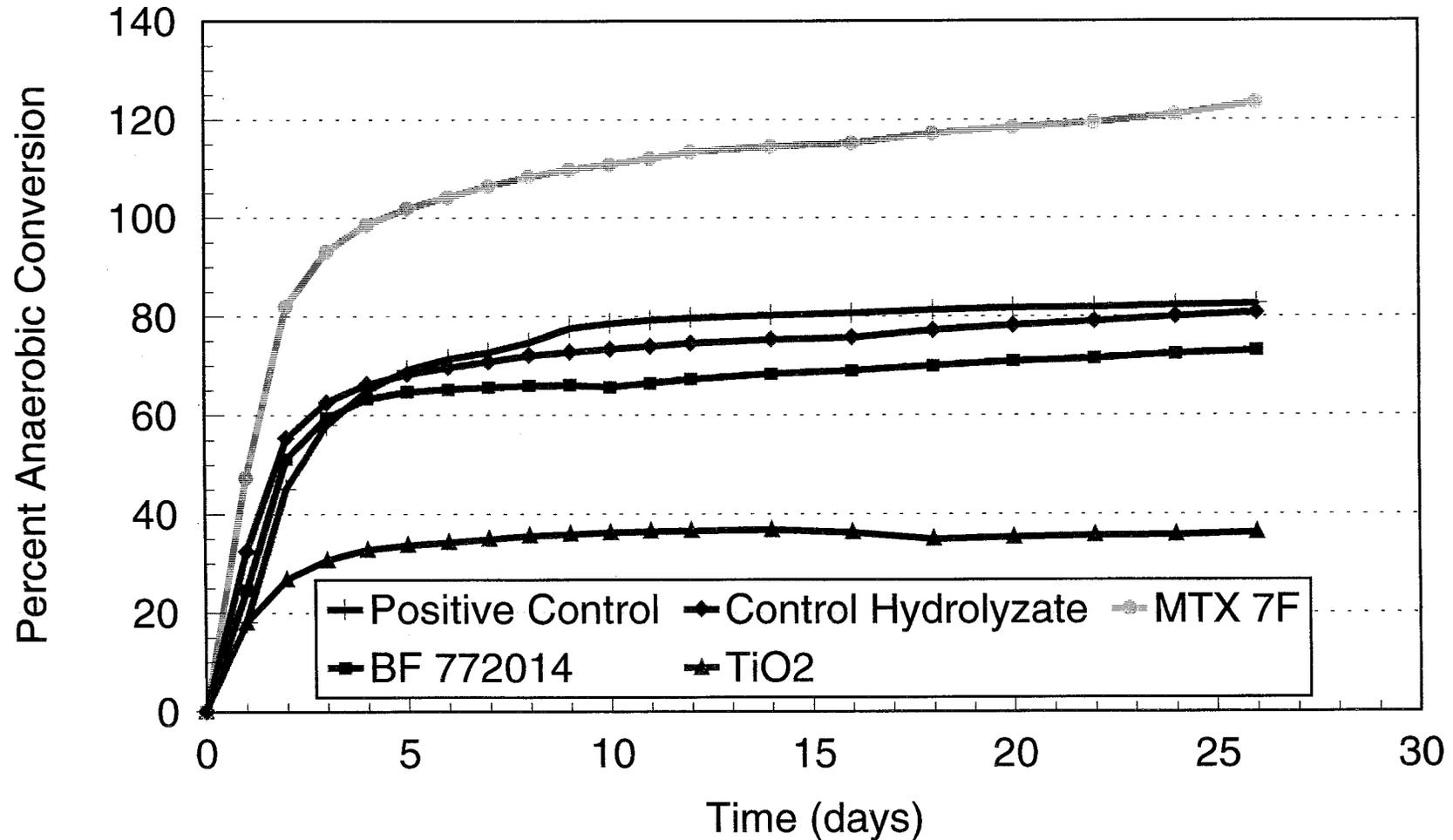
# Biochemical Methane Potential Assay

## NREL Ethanol Process Samples



# Biochemical Methane Potential Assay

## NREL Ethanol Process Samples



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