0273-2289/94/4546—0453$03.00

Evaluation of Discrete Cellulase Enzyme Activities from Anaerobic Digester Sludge Fed a Municipal Solid Waste Feedstock

Scientific Note

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Index Entries: Anaerobic digestion; MSW; cellulase; electrophoresis; zymogram.

INTRODUCTION

Anaerobic digestion of municipal solid waste (MSW) represents a waste disposal option that results in the production of a gaseous fuel (methane) and an organic residue suitable for use as a soil amendment. The rate-limiting step in this process is the hydrolysis of polymeric substrates, such as cellulose (*1,2*). However, only limited information exists concerning the hydrolytic capacity of anaerobic digestion systems. We have previously reported methods for extracting active hydrolytic enzymes from digester sludge solids using a variety of detergents (*3*). Additionally, we used these protocols to quantitatively recover active digester-resident enzymes to determine their operational optima for activity (*4*).

In this study, we developed new protocols to partially purify endoglucanase enzymes from extracted anaerobic digester sludge fed a MSW feedstock. Fractions from preparative, native polyacrylamide gel electrophoresis (PAGE) were analyzed for endoglucanase activity using carboxymethyl cellulose (CMC) hydrolysis and zymogram activity staining. Finally,

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a fraction of the total endoglucanase enzyme from this system was evaluated for apparent mol wt using size exclusion chromatography, thus developing a preliminary picture of the cellulase system type.

MATERIALS AND METHODS

Feedstocks

The MSW feedstock used in this study was obtained from Future Fuels, Inc., Thief River Falls, MN. The MSW was processed using a combination of mechanical and manual separation. The MSW feedstock was obtained in two fractions: the food/yard waste fraction, and the paper and paperboard materials (also referred to as refuse-derived fuel [RDF] in the form of densified pellets). The food/yard waste fraction was stored at 4°C until it was blended with the RDF-MSW fraction. Most of the mixed MSW was stored at −20°C until it was used. The mixed MSW feedstock was analyzed and determined to be 72.7% total solids (TS), 12.5% ash, and 87.5% volatile solids (VS, as a percentage of the TS).

Previous investigations of the anaerobic bioconversion of MSW feedstocks identified the need for nutrient supplementation (*5*). Therefore, we added a nutrient solution as previously described (*5*), to adjust the moisture content of the feedstock, as well as to ensure sufficient nutrients for robust biological activity.

High Solids Digester Operation

The laboratory-scale high-solids reactors used in this preliminary study were described previously (*6*). The reactors consist of a cylindrical glass vessel positioned with a horizontal axis and capped at each end. The agitator shaft runs horizontally along the axis of the cylinder and mixing is obtained with a rod-type agitator (tines) attached to the shaft of 90° angles and in opposing orientation. Shaft rotation is provided by a low-speed, high-torque, hydraulic motor (Staffa, Inc., England). The glass vessel was modified with several ports, including two 3/4-in. ports for liquid introduction and gas removal and a 2-in ball valve (Harrington Plastics, Denver, CO) used for dry feed introduction and effluent removal.

The four high-solids reactors used in this study were maintained at 37°C in a temperature-controlled warm room. The reactors were batch fed daily by adding the relatively dry MSW feedstock and a liquid nutrient solution as previously described (*6*). Sludge was removed from the reactors on a biweekly basis and stored at 4°C until it was analyzed.

Low Solids Digester Operation

Four anaerobic digesters with 3.5-L working volumes and semi-continuous stirring (15 min of each 1/2 h) were constructed and operated as

previously described (*7,8*). The digesters were maintained in a 37°C constant temperature warm room. The anaerobic reactors were batch fed daily a volume of MSW plus nutrient supplement slurry to maintain a 14-d retention time. In the batch feeding protocol, a volume of effluent equivalent to the volume of feed added was removed daily to maintain the reactor sludge volume at 3.5 L. In the operation of the reactors, the solids retention time was equivalent to the hydraulic retention time.

Feedstock/Digester Effluent Analysis

The solids concentrations of both feedstocks and digester effluent samples were determined using 1-g aluminum weigh tins. A 20- to 30-g sample was loaded into preweighed tins and dried for 48 h at 45–50°C. The dried sample was then cooled to room temperature in a laboratory desiccator and weighed using a Sartorius balance (Model 1684MB). The percent TS was calculated on a wt/wt basis, and the percents VS and ash were determined by combustion of the dried samples at 550°C for 3 h in a laboratory-scale furnace.

Feedstock materials were analyzed for levels of carbon oxygen demand (COD) as previously described (*9*). The COD assay employed the microdetermination method with commercially available "twist tube" assay vials (Bioscience, Inc., Bethlehem, PA).

Levels of volatile organic acids (C_2-C_5 iso- and normal-acids) were determined by gas-liquid chromatography (GLC). A Hewlett-Packard Model 5840A gas chromatograph equipped with a flame ionization detector, a Model 7672A autosampler, and a Model 5840A integrator (all from Hewlett-Packard) were used. The chromatograph was equipped with a glass column packed with Supelco 60/80, Carbopack C/O.3%, Carbowax 20M/0.1% H_3PO_4 for separations.

The feedstocks were also analyzed with respect to specific polymer content as determined by the standard forage fiber analyses of acid detergent fiber (ADF) and neutral detergent fiber (NDF) as previously described (*10*).

Gas Analysis

Total biogas production in high-solids reactor systems was measured daily using precalibrated wet tip gas meters (Rebel Point Wet Tip Gas Meter Co., Nashville, TN). Total biogas production in low-solids systems was determined from calibrated water displacement reservoirs. The composition of the biogas produced was determined by gas chromatography as previously described (*11*). For this analysis, a Gow-Mac (Model 550) gas chromatograph equipped with a Porapak Q column and a thermal conductivity detector with integrating recorder was used.

Theoretical Methane Yield

The theoretical methane yield for the MSW feedstock was calculated as previously described (*12*) from the feedstock COD value. The ratio of actual methane yield for a given anaerobic fermentation system to the theoretical methane yield calculated from the feedstock COD value is a direct reflection of the organic carbon conversion of the substrate added.

Cellulase Enzyme Assay Methodology

Digester samples from both low-solids and high-solids systems fed the MSW feedstock were sampled on a weekly basis over a 4-wk period for analysis. Digester sludge samples were first diluted to 1–2% TS. The diluted samples were then split into two equal 30-mL samples. One of the samples was used for analysis of TS content. The second sample was used for enzyme assessment. For enzymatic assays, the diluted digester sludge sample was subjected to centrifugation at 15,000*g* for 20 min at room temperature to concentrate the particulate fraction. The supernatant was discarded and the pellet was resuspended in 15 mL of 100 m*M* Tris buffer at pH 7.0. Triton X-155 was then added to obtain a final concentration of 0.1% (v/v) and the sample was gently mixed for 16–20 h at 4°C. The samples were then centrifuged at 15,000*g* for 20 min at 4°C. The supernatant was removed and filtered using a 0.45-μm disposable Acrodisk syringe filter (Gelman Sciences, Ann Arbor, MI). The filtered supernatant was then assayed for various cellulase enzyme activities. As a control, the supernatant from the initial particulate concentration was assayed for enzyme activity and no activity above the assay background was determined.

The determination of β-D-glucosidase (EC 3.2.1.21), endoglucanase (EC 3.2.1.4), and exoglucanase (EC 3.2.1.74) in detergent extracts of digester sludge samples was measured as previously described (*13*). One modification to the assay protocol for "apparent" exoglucanase activity was the substitution of phosphate-swollen cellulose in 100 m*M* Tris, pH 7.0 instead of Whatman #1 filter paper strips.

Large-Scale Enzyme Extraction Protocol

Large amounts of active high-solids anaerobic digester sludge (1–2 kg) were extracted by dilution (1:10) with 20 m*M* Tris, pH 7.0. The Triton X-155 was added to a final concentration of 0.1% (v/v). The slurry was mixed for 24 h at 4°C using a reciprocal shaker to extract active hydrolytic enzymes from the sludge solids. Following incubation, the prep was centrifuged at 5,000*g* for 15 min at 4°C, after which the pellet was discarded and the supernatant again centrifuged at 17,000*g* for 5 min. The pellet was again discarded and the supernatant filtered through progressive Capsule filters including 3, 0.45, and 0.2 μm (Gelman Sciences, polyurethane membrane). The filtered supernatant was then concentrated 50-fold using an Amicon stirred cell with a polysulfone 10,000 mol wt (mw) cutoff (PM

10) membrane. The concentrated sample was stored at 4°C until it was analyzed.

Electrophoresis and Size Exclusion Chromatography

A 7.0% resolving polyacrylamide gel and a 3% polyacrylamide stacking gel were cast in the tube gel of the Bio-Rad (Richmond, CA) Model 491 Prep Cell System. Once the gel had polymerized, the apparatus was operated with a native buffer system, with included an upper buffer (37.6 m*M* Tris, 40 m*M* glycine, pH 8.9) and a lower buffer (63 m*M* Tris, 0.05*N* HCl, pH 7.5). The elution buffer was the same as the lower buffer.

Approximately 4 mL of 50X sludge extract was loaded onto the upper surface of the stacking gel and a constant current was applied for 8 h. Collection of fractions started just prior to the elution of the tracking dye. Ten-min fractions were collected and the fractions were subsequently assayed for endoglucanase (CMCase) activity as described above. To verify CMCase activity and to visualize the location of the endoglucanases, fractions were electrophoresed and visualized by zymograms. For this procedure, the fractions were electrophoresed on Pharmacia native gradient Phastgels and immediately placed on agar media containing CMC. The agar plates were incubated at 55°C for 60 min and endoglucanase activity was visualized after staining with Congo red dye. Identical gels were prepared and stained for total protein using either Coomassie blue or silver stain (depending on protein concentration).

Several fractions from the Bio-Rad Prep Cell demonstrating high CMCase activity were pooled, concentrated 3X, diafiltered in 20 m*M* acetate buffer, pH 5.0, and examined by size exclusion chromatography (SEC). For SEC analysis, a Superdex 200 column (Pharmacia, Uppsala, Sweden) was used. Five-milliliter fractions were collected at a flowrate of 0.5 mL/min. Resulting fractions were assayed for endoglucanase activity as described above.

RESULTS AND DISCUSSION

We have evaluated a variety of approaches to improving the overall economics of anaerobic bioconversion of MSW to methane. To this end, a high-solids digestion system, which provides mixing of relatively solid material, has been developed at NREL. The anaerobic bioconversion performance of the high-solids digestion system is compared to a conventional low-solids system fed an identical MSW feedstock. Fermentation parameters, such as pH, VS, and polymer conversion demonstrated a stable fermentation for both systems (data not shown). The data shown in Table 1 indicate that although similar levels of overall bioconversion occurred in the two systems, the high-solids system was functioning at 6.6 times the organic loading rate, with 4.4 times more sludge solids (less

Table 1
Comparison of Anaerobic Bioconversion of MSW Feedstock for Low- and High-Solids Digestion Systems

Digester	Organic loading rate g COD/L·d	Biogas productivity, mL/L·d	Methane content, percent	Methane yield, mL/L·d	Effluent total solids, percent	Percent bioconversion, based on COD
Low solids	2.3	918 ± 129	59.8	549 ± 77	4.9 ± 0.3	68.2
High solids	15.3	6225 ± 880	60.1	3741 ± 529	21.5 ± 0.3	69.9

Table 2
Comparison of Hydrolytic Enzyme Levels for Low- and High-Solids Digestion Systems

Cellulase activity[a]	Low solids	High solids
β-D-glucosidase, U/min	0.24 ± 0.04	0.34 ± 0.26
Endoglucanse, μmol glucose released/min	0.33 ± 0.12	1.11 ± 0.18
Exoglucanase, μmol glucose released/min	0.29 ± 0.04	0.27 ± 0.01

[a] Activities are calculated based on units of activity per gram digester sludge solids.

process water). Therefore, the high-solids system combines the advantages of decreased process water and increased organic loading rates, which serves to reduce the overall reactor size required 90–95% (i.e., as compared to the low-solids system). Additional process improvements require increasing the rate of feedstock polymer conversion.

The hydrolysis of cellulose, the major polymer of most biomass and MSW, has been demonstrated to be the rate-limiting step in anaerobic bioconversion of MSW (*1,2*). Therefore, we have developed protocols for quantitatively recovering these digester-resident enzymes for study in order to generate informed strategies for further enhancement. The comparison of discrete cellulase enzymes extracted from low- and high-solids systems are shown in Table 2. In general, similar levels of β-glucosidase, endoglucanase, and exoglucanase are present when compared on a per gram of sludge solids basis. However, it should be noted that the level of sludge solids is significantly greater in the high-solids system, and thus the overall level of hydrolytic enzyme activity per unit volume is far greater as compared to the low-solids system. This greater level of hydrolytic activity per unit volume allows for increased organic loading rates without loss of conversion efficiency. Still, little is known about the digester-resident cellulase enzymes present, especially in high-solids anaerobic digester systems. Additionally, the complex nature of sludge presents a significant problem to the purification of active enzyme for detailed study. Therefore, we began investigations into the use of preparative electrophoresis to partially purify cellulases from a representative high-solids anaerobic digester fed a MSW feedstock. The extraction of

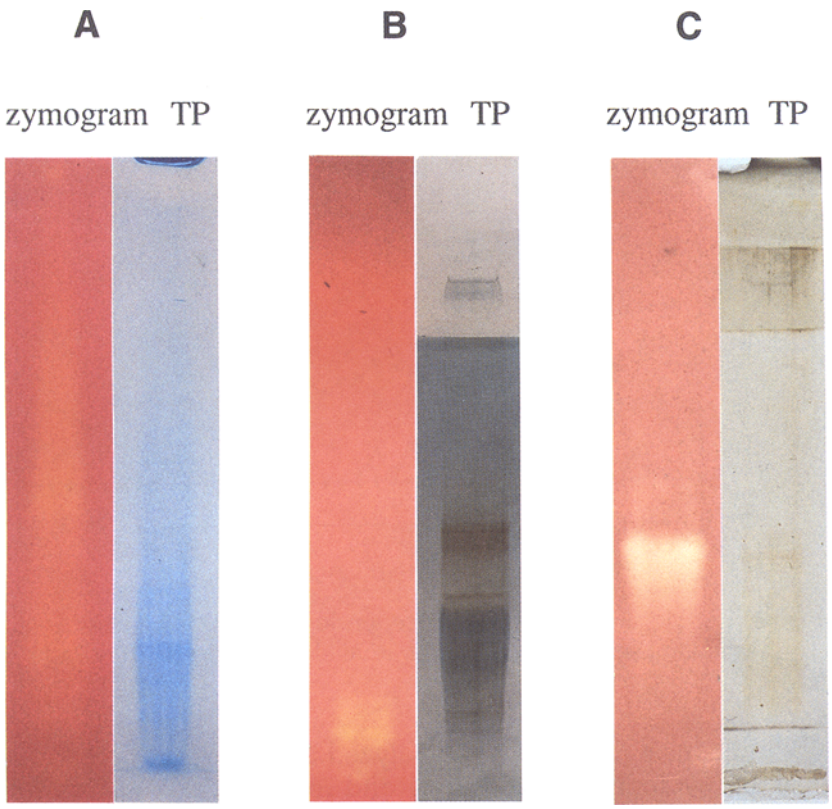

Fig. 1. Analysis of CMCase (endoglucanase) activity and total protein prepared in identical 12% native PAGE using Pharmacia Phastgels. Samples represent crude digester extract (A), pooled and concentrated fractions (9–12) from the Bio-Rad Prep Cell (B), and pooled and concentrated fractions from SEC (C).

large volumes of high-solids digester sludge using Triton X-155 was followed by removal of the solids by centrifugation and filtration and concentration of the protein using an Amicon stirred cell. Initial analysis of this crude extract by native gradient-gel electrophoresis is shown in Fig. 1A. Identical gels were examined for endoglucanase activity and total protein, both of which are not well separated and appear as a smear. This is most likely owing to the presence of detergents and microbe-derived lipids.

The crude protein preparation was then subjected to preparative electrophoresis using the Bio-Rad Prep Cell System. Broad activity profiles for the three discrete cellulase enzyme activities were determined (*see* Fig. 2). This result is expected, because of the diverse number of hydrolytic microorganisms that comprise the anaerobic consortia. Further analysis was performed using several of the endoglucanase fractions that demonstrated the highest level of activity (fractions 9–12). These fractions were

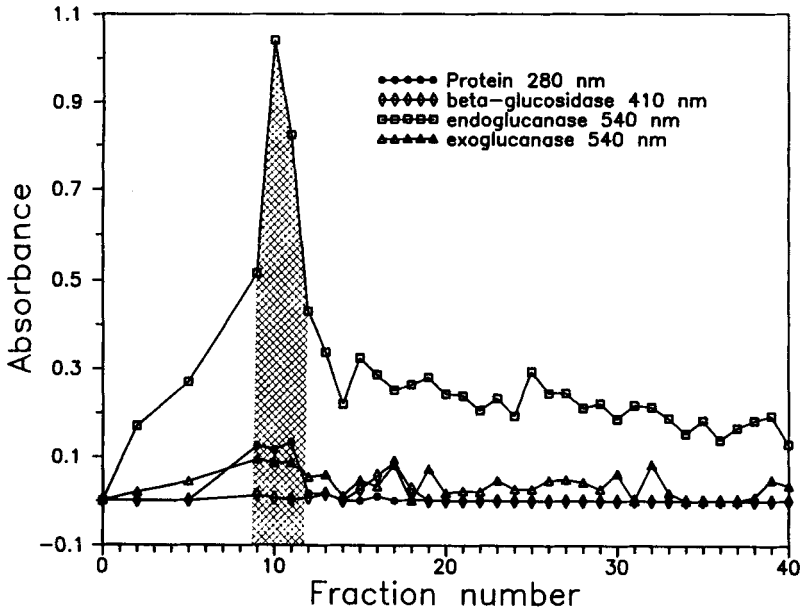

Fig. 2. Analysis of β-D-glucosidase, endoglucanse, exoglucanse, and total protein in fractions separated by preparative electrophoresis (Bio-Rad Prep Cell System).

pooled and concentrated 3X and analyzed by native gradient-gel electrophoresis for endoglucanase activity and total protein (*see* Fig. 1B). The zymogram indicates a significant, although broad, band of CMCase activity, whereas staining for total protein indicates that significant contaminating proteins are present that do not coincide with the migration of the CMCase activity.

The partially purified endoglucanase was further characterized by SEC. The sample was separated by molecular size (i.e., hydrodynamic radius) using a Superdex 200 column and the column calibrated using conventional mol-wt standards (*see* Fig. 3). Analysis of the fractions collected indicated a peak of endoglucanase activity that corresponded to an average mol wt of approx 32,000 daltons. The fractions demonstrating CMCase activity were subsequently pooled and concentrated 3X and analyzed by native gradient-gel electrophoresis. The data shown in Fig. 1C indicate a strong, relatively sharp band of CMCase activity by zymogram, which corresponds to a faint protein band determined by silver stain for total protein. Additional bands present in the total protein gel indicate that the endoglucanase is still only partially purified.

In summary, these preliminary data suggest that preparative PAGE may be a suitable technique to obtain partially purified cellulase enzymes from digester sludge for further study. Additionally, SEC of a fraction of the overall endoglucanases present in high-solids anaerobic digester systems reveals that these proteins may be of relatively low mol wt. Fur-

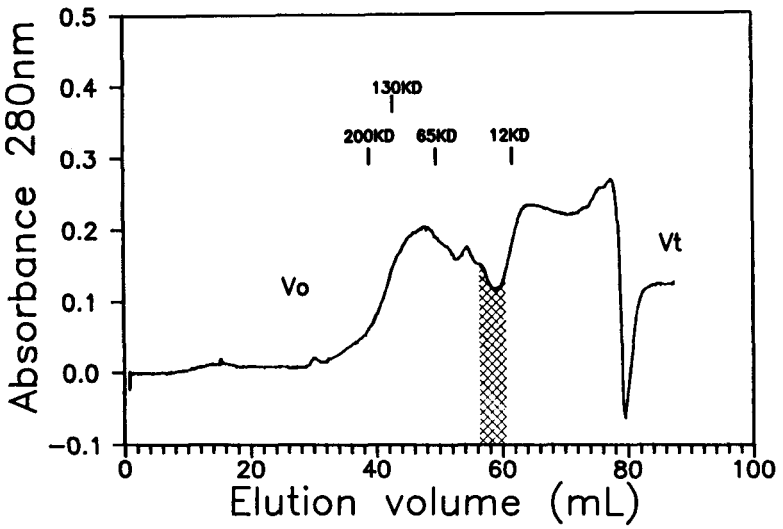

Fig. 3. Analysis of the endoglucanase-positive fractions from PAGE (Fig. 2) by size exclusion chromatography (SEC) on Superdex 200 in 20 m*M* acetate buffer, pH 5.0. Column fractions were tested for endoglucanase activity and total protein.

thermore, the absence of high mol wt, CMC-degrading fractions from the native PAGE (*see* Fig. 1) or the subsequent SEC indicates that no cellulosome-like structures exist in this detergent-extracted material. Such structures, measuring approx 2,000 and 250 kDa are readily visible in SEC fractions from *Clostridium thermocellum* and *Clostridium populeti* growth supernatant, respectively (*14*). This information, although presumptive, may indicate that methods that employ increased levels of shear for recovering cellulases from digester sludge may be appropriate because of the relatively small size of at least a portion of these endoglucanases.

ACKNOWLEDGMENTS

This work was funded by the Waste Management Program of the US Department of Energy, Office of Waste Reduction within the Office of Industrial Technologies.

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