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Anaerobic Digestion of Lignocellulosic Biomass and Wastes

Cellulases and Related Enzymes

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ABSTRACT

Anaerobic digestion represents one of several commercially viable processes to convert woody biomass, agricultural wastes, and municipal solid wastes to methane gas, a useful energy source. This process occurs in the absence of oxygen, and is substantially less energy intensive than aerobic biological processes designed for disposal purposes. The anaerobic conversion process is a result of the synergistic effects of various microorganisms, which serve as a consortium. The rate-limiting step of this conversion process has been identified as the hydrolysis of cellulose, the major polymeric component of most biomass and waste feedstocks. Improvements in process economics therefore rely on improving the kinetic and physicochemical characteristics of cellulose degrading enzymes. The most thoroughly studied cellulase enzymes are produced by aerobic fungi, namely *Trichoderma reesei*. However, the pH and temperature optima of fungal cellulases make them incompatible for use in anaerobic digestion systems, and the major populations of microorganisms involved in cellulase enzyme production under anaerobic digestion conditions are various bacterial producers. The current state of understanding of the major groups of bacterial cellulase producers is reviewed in this paper. Also addressed

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in this review are recently developed methods for the assessment of actual cellulase activity levels, reflective of the digester "hydrolytic potential," using a series of detergent extractive procedures.

Index Entries: Anaerobic digestion; cellulase; biomass conversion; methane production.

ANAEROBIC DIGESTION

An Overview

The anaerobic digestion process takes place through the synergistic action of four different types of microorganisms: hydrolytic, fermentative, acidogenic, and methanogenic bacteria (Fig. 1). Numerous reviews have been published describing in detail the level of understanding of the anaerobic digestion process and the terminal steps of methane production (*1–10*). The hydrolytic bacteria in these consortia use cellulase enzymes to depolymerize cellulose to simple sugars. Proteins, pectins, hemicellulose, and starches (if present in the feedstock) are also degraded enzymatically. The fermentative bacteria convert these monomers to organic acids, primarily propionic and acetic acid. The acidogenic bacteria convert these acids to hydrogen, carbon dioxide, and acetate, which the methanogens utilize by two major pathways to produce methane and carbon dioxide.

The anaerobic digestion of lignocellulosic materials, such as municipal solid waste and woody biomass, is limited by the rate of hydrolysis (*9,11*). The primary biodegradable polymer in biomass, cellulose, is shielded by lignin, a relatively inert, of polyphenylpropane three-dimensional polymer (*12*), and by hemicellulose (*13*). This complex structure dictates that natural biodegradation occurs on a time-scale of months or years, rather than hours or days. Such slow rates of polymer degradation require large retention times, reactor volumes, and thus, capital costs in large-scale application.

Extracellular hydrolytic enzymes, such as cellulases, amylases, lipases, and proteases, have been shown to be present in anaerobic digester effluents (*14*). Also, preliminary experiments indicate that hydrolytic enzymes added to the anaerobic digestion process *in situ* increase rates of conversion (*15*). Yet, the types, activities, stabilities, and relative concentrations of these enzymes have not been examined rigorously. Although similar in some characteristics to the rumen systems, little information is available specifically on the characteristics of most of the cellulolytic enzymes produced by anaerobic bacteria found in digesters fed lignocellulosic substrates. Grohmann et al. demonstrated that cellulase preparations from three known cellulolytic/hemicellulolytic microorganisms fail to reach the same extent of cellulose hydrolysis as do anaerobic consortia on biomass substrates (*16*), indicating that additional enzymatic activities are important. Dramatic improvements in digester performance may eventually be

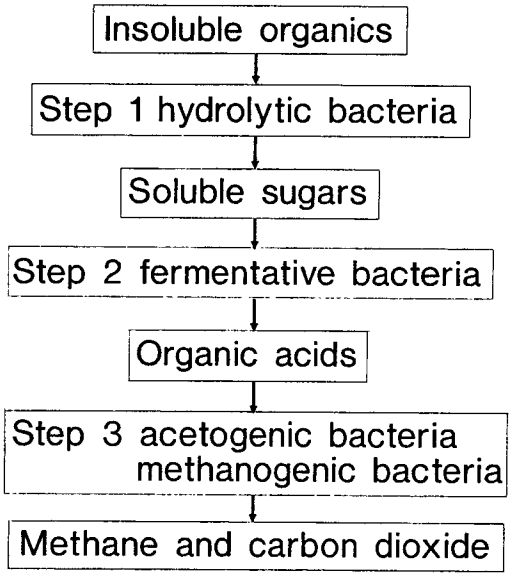

Fig. 1. Biological pathway for anaerobic digestion.

possible by considering the types and levels of enzyme activities, both naturally occurring and from augmentation (*15*), in the anaerobic digestion system. Clearly, new candidates for the production of cellulase enzymes useful in anaerobic digestion are yet to be discovered in ecosystems such as the rumen and fertile soil.

ANAEROBIC BACTERIAL CELLULASES

The degradation of cellulose by bacterial systems occurs both aerobically and anaerobically. The well-studied anaerobic bacteria include members in the genera *Bacteroides, Clostridium, Ruminococcus, Micromonospora,* and *Acetivibrio cellulolyticus.* For the sake of completeness, we will mention that the well-characterized aerobic bacterial systems include species within the genera *Cellulomonas, Bacillus, Pseudomonas, Cellvibrio, Cytophaga, Microbispora, Thermomonospora,* and *Acidothermus cellulolyticus.* These microorganisms, although some may be potentially microaerophilic, have not been shown to play a major role in anaerobic digestion processes, and thus, will not be reviewed here. This review will address both mesophilic and thermophilic cellulolytic bacteria, some of which have been extensively reviewed by Duong et al. (*17*), Ljungdahl and Eriksson (*18*), and most recently, by Coughlan and Ljungdahl (*19*).

According to studies on fungal cellulolytic enzymes, the model of crystalline cellulose hydrolysis requires synergistic action of at least three cellulase components: endo-1,4-β-D-glucanase (EC 3.2.1.4, carboxymethyl

cellulase or CMCase), exo-1,4-β-D-glucanase (EC 3.2.1.91, often called a cellobiohydrolase), and β-D-glucosidase (EC 3.2.1.21, cellobiase) (*20,21*). There has been a tendency to compare bacterial cellulase systems with fungi; thus, the bacterial cellulolytic enzymes are often characterized in the same terms. All cellulolytic bacteria synthesize endoglucanase and either β-glucosidase (*22,23*) or cellobiose phosphorylase (*24*), or a combination of the two (*25*). Both aerobic and anaerobic bacteria, including *Ruminococcus flavefaciens* (*24*), *Cellvibrio gilvus* (*26*), *Cellulomonas fimi* (*27*), *Clostridium thermocellum* (*28*), and possibly *Bacteriodes cellulosolvens* (*29*), synthesize cellobiose phosphorylase that catalyzes cellobiose to glucose and glucose-1-phosphate. As some of these organisms do not produce a β-D-glucosidase, it seems reasonable to assume that the phosphorylases provide a means of metabolizing cellulose degradation products and of diminishing the inhibitory effect of cellobiose on cellulase activity (*30*). The existence of exocellulase in bacteria has been questionable for a long time. However, exoglucanases were purified from a number of bacterial species, including *Micromonospora bispora* (*31*), *Cytophaga* sp. (*32*), *Streptomyces flavogriseus* (*33*), *Cellulomonas uda* (*34*), *Clostridium stercorarium* (*35*), *Acetivibrio cellulolyticus* (*36*), and *Ruminococcus albus* (*37*).

Cellulolytic enzymes from *C. thermocellum* are organized into a distinct multiunit complex, which has been termed "cellulosome" by Lamed et al. (*38*). Scanning electron microscopic evidence by Lamed et al. (*39,40*) demonstrated a clear correlation between cellulolytic activity and the appearance of protuberance-like structures on the bacterial cell surface. This correlation extended over a wide range of physiological and evolutionary boundaries, including *Acetivibrio cellulolyticus, Bacteroides cellulosolvens, Cellulomonas* sp., *Clostridium* sp., and *Ruminococcus albus;* they all exhibited these surface structures under conditions in which cellulases were formed whether gram-positive or gram-negative, aerobic or anaerobic, mesophilic or thermophilic. Lamed et al. concluded that the cell surface cellulase containing structures might be of general consequence to the bacterial interaction with, and degradation of, cellulose (*40*). Moreover, the immunochemical properties of the cellulolytic bacteria examined were compared with those of *C. thermocellum* by using a specific anticellulosome antibody preparation. These studies indicated that the cellulosome concept might be a more general feature of cellulolytic microorganisms (*40*). On the contrary, Kauri et al. showed that bacterial degradation of cellulose did not depend on cell-to-fiber contact, and suggested that when cellulose was at a greater distance from the cell, the removal of end products reduced catabolite repression of cellulase formation (*41*).

Cellulolytic bacteria can be found that produce only cell-bound cellulase, such as *Cytophaga* (*32*); only cell-free cellulase, such as *Cellvibrio vulgaris* (*42*), *Bacillus* sp. (*43*), *Clostridium* sp. (*44*), *Acetivibrio cellulolyticus* (*45*), *Acidothermus cellulolyticus,* and *Thermoactinomyces* (*46,47*); and both cell-bound and cell-free cellulase, such as *Pseudomonas* (*48*), *Bacteroides*

succinogenes (*49*), and *Cellvibrio fulvus* (*50,51*). However, the location of cellulase in bacteria also depends on the environments in which the bacteria are grown and the age of the culture (*50,52*). Efficient hydrolysis of insoluble materials by bacteria generally requires close bacterial-substrate interfaces. In the case of cellulose, the location of the enzymes on the cell surface may be required for the hydrolysis of the cellulose crystalline structure. This characteristic creates a challenge in measuring the total cellulolytic activity present in anaerobic digesters, since the supernatant activity only represents a fraction of the total activity present.

The synthesis of cellulases is regulated by induction and catabolite repression mechanisms. Cellulases are induced by soluble derivatives from cellulose or several other low-mol wt carbohydrates, but enzyme synthesis is repressed by the presence of glucose or other readily metabolized sugars. Evidence supporting this conclusion has been obtained for bacteria, e.g., *Acetivibrio cellulolyticus* (*53*), *Acidothermus cellulolyticus* (*54*), *Cellulomonas uda* (*55*), and *Thermomonospora* (*56–58*). The known inducers for bacterial cellulase synthesis are cellulose, cellulose derivatives, cellobiose, sophorose, and lactose.

In fungal systems, cellobiose induces cellulase synthesis by *Trichoderma reesei* (*59,60*), *Sporotrichum pulverulentum* (*61*), *Sporotrichum thermophile* (*62*), *Aspergillus terreus* (*63*), and *Penicillium janthinellum* (*64*), at either high or low concentrations. Lactose is also an inducer in some species, including *T. reesei*. Moreover, sophorose is a powerful inducer in *T. reesei* (*65*), *Trichoderma pseudokoningii* (*66*), and bacterial *Pseudomonas fluorescens* var. *cellulosa* (*67*), whereas in *Acetivibrio cellulolyticus*, it repressed endoglucanase production as efficiently as glucose (*53*). In summary, the effects of cellobiose, lactose, and sophorose on cellulase synthesis of bacterial systems are significantly different from fungal systems.

Anaerobic Bacterial Cellulase Producers

The cellulolytic systems of anaerobic bacteria have received in-depth study of late. They are similar in many respects. Coughlan and Ljungdahl made some generalizations:

1. Cellulase complexes located on the cell surface mediate adherence of anaerobic cellulolytic bacteria to the substrate;
2. As cultivation proceeds, the complexes are released from the cell and from the substrate, and although they may remain for a time as functional complexes in the culture fluid, they ultimately decompose to free polypeptides;
3. The activity of enzyme complexes against crystalline cellulose depends on calcium (or magnesium) and dithiothreitol; and
4. Definitive proof of the existence in complexes of an enzyme with exoglucanase activity is not available (*3*).

Acetivibrio Cellulolyticus

This bacterium is an obligate anaerobe, and grows at pH 6.5–7.7 at temperatures from 20 to 40°C (*68*). Of about 30 polysaccharides and sugars tested, *A. cellulolyticus* differs from most cellulolytic organisms in its ability to utilize only cellulose, cellobiose, and salicin for growth (*68*). The major fermentation products of cellobiose or cellulose degradation reported were acetic acid, hydrogen, and carbon dioxide (*69*). The organism showed maximum cellulase activities (5.9 U/mL of exoglucanase and 12.5 U/mL of endoglucanase) in a medium containing 1 g/L cellulose powder CF-11 (Whatman) after 3 d of incubation (*45*). The cellulase activity increased progressively during the exponential growth phase and corresponded to the rate of growth (*45*). The specific filter paper activity (40 U/mg protein) of this enzyme was higher than those from commercial preparations of cellulases from *T. viride* (Sigma Chemical Co.) and *Aspergillus niger*.

Polyacrylamide gel electrophoresis of the cellulase from culture supernatants of this microorganism showed the presence of four major enzymes: a β-glucosidase, an exoglucanase, and two endoglucanases. The endo- and exoglucanase activities were mainly detected extracellularly, whereas cellobiase activity was apparently cell associated. The mol wt of the cellulolytic enzymes were β-D-glucosidase, 81,000 dalton; exoglucanase, 38,000 dalton; endoglucanase C2, 33,000 dalton; and endoglucanase C3, 10,400 dalton (*36*). Both exo- and endoglucanase activities were shown to be regulated by induction and catabolite repression (*53*). Only cellobiose and salicin stimulated endoglucanase synthesis; salicin was approx one-half as effective as cellobiose when cells were grown on cellobiose or salicin media supplemented with various disaccharides and glucose. The addition of cellobiose to the cellulose substrated did not affect the endoglucanase activity; however, exoglucanase activity was inhibited. When cellulose was supplemented with glucose, endoglucanase activity was inversely related to increasing glucose concentration (*53*).

Although the cellulase system of *A. cellulolyticus* was not inactivated by air, it has been shown to function optimally in a reducing environment (*70*). Ca^{2+} and a reducing agent, such as dithiothreitol, were required for maximum cellulose hydrolysis (*71,72*).

Clostridium Thermocellum

C. thermocellum is a thermophilic, cellulolytic bacteria that can grow at 60°C (*46*). There has been interest since the turn of the century in using thermophilic, cellulolytic clostridia commercially because they ferment cellulose directly to ethanol and organic acids. All *C. thermocellum* and *Clostridium thermocelluloasaeum* strains are able to ferment cellulose and cellobiose. Although *C. thermocellum* degrades xylan, the resulting xylose and xylobiose are not fermented and accumulate in the broth (*73*). McBee reported that no growth of *C. thermocellum* occurred on glucose, fructose,

arabinose, lactose, salicin, sorbitol, or mannitol (*74*). Ng et al. reported growth on cellulose and cellobiose but not on glucose or xylose (*75*). Johnson et al. proved that *C. thermocellum* can utilize cellulose, cellobiose, glucose, fructose, and sorbitol as carbon sources (*76*). Gomez et al. found that serial culture of *C. thermocellum* on glucose resulted in adapted cultures that no longer had a lag, and preferred glucose over cellobiose as a carbon source (*77*). However, Ng and Zeikus demonstrated that *C. thermohydrosulfuricum*, which is noncellulolytic, consumed glucose in preference to cellobiose for growth, and *C. thermocellum* metabolized cellobiose in preference to glucose (*78*). The two species also differed significantly in the enzyme activities responsible for cellobiose metabolism, in that *C. thermohydrosulfuricum* utilized cellobiase and hexokinase, but *C. thermocellum* utilized cellobiose phosphorylase and phosphoglucomutase (*78*). A cellodextrin phosphorylase was also present in *C. thermocellum*, which phosphorolyzes β-1,4-oligoglucans with formation of α-D-glucose-1-phosphate and preservation of the glucosidic bond energy (*79*).

During the earlier stages of growth, most of the cellulolytic activity of *C. thermocellum* was bound to the cellulose (*80–83*). Moreover, this culture produced a yellow affinity substance (YAS) that bound to the cellulose fibers (YAS-cellulose) and facilitated the binding of the cellulolytic enzyme system to the substrate (*82,84*). As cellulose was consumed, the bound enzyme was released as free enzyme to the culture fluid. The bound enzyme contained two major components, a large complex termed originally-bound large, OBL (100×10^6 dalton); and a small complex termed originally-bound small, OBS (4,500,000 dalton) (*85*). The free enzyme was composed of two major fractions; one can rebind to YAS-cellulose, the other cannot (*81*). All four fractions from the bound and the free enzymes exhibited CMCase activity, but only the bound and the free rebindable complexes extensively solubilized crystalline cellulose (*86*). These complexes, consisting of at least 14 different types of polypeptide, have been termed cellulosomes for the cell-associated, cellulose-binding, multicellulase complex (*38*). The mechanism of cellulose degradation by bacteria was derived from the ultrastructural details of the cellulolytic enzyme complexes of *C. thermocellum* using electron microscopy (*87*). It was postulated by Mayer et al. that on binding of a cellulose chain, a simultaneous multicutting event occurs that leads to the release of cellooligosaccharides of four units in length (C4; cellooctaose). Rows of smaller-sized subunits with lower center-to-center distances, which are also present in the cellulosome, subsequently cleave the C4 fragments (or cellulose) to C2 (cellotetraose) or C1 (cellobiose). In this way, the cellulosome can catalyze the complete hydrolysis of cellulose (*87*).

The cellulolytic enzymes and β-glucosidase have been found to be constitutive and extracellular enzymes in *C. thermocellum*. The crude enzyme was oxidatively inactivated by air and inhibited by sulfhydryl reagents

(*88*). Cellulase of this culture had the ability to solubilize native and derived forms of crystalline cellulose (cotton, filter paper, and Avicel) in the presence of Ca^{2+} and DDT (*88,89*). By contrast, the complexes required neither calcium nor thiol for activity against amorphous or soluble derivatives of cellulose. Both agents were not needed for the activity of the free endoglucanases against carboxymethyl cellulose (*65,90*). *C. thermocellum* also produced a cell-associated β-D-glucosidase, which is capable of hydrolyzing cellobiose to glucose but may play a minor role in vivo relative to cellobiose phosphorylase because β-glucosidase K_m for cellobiose is about 10 times greater than that of the cellobiose phosphorylase (*73*).

Clostridium populeti

Anaerobic, mesophilic, spore forming, cellulolytic bacteria isolated from digester systems include *C. populeti* isolated by Sleat and Mah (*91*), and *C. cellulovorans* isolated by Sleat, Mah, and Robinson (*92*). Both species are similar but differ in the production of formate from cellobiose by *C. cellulovorans* and endospore morphology. Both of the organisms were isolated from a mesophilic digester fed a high cellulose content feedstock (popular wood). *C. populeti* fermentation products from glucose include acetate (16 mol/100 mol of glucose), butyrate (72 mol/100 mol of glucose), lactate (50 mol/100 mol of glucose), H_2 (98 mol/100 mol of glucose), CO_2 (111 mol/100 mol of glucose), and trace amounts of ethanol and succinate (*91*). A yellow pigment, similar in respect to cellulose binding to the YAS complex produced by *C. thermocellum,* is produced when *C. populeti* is grown in liquid media on pebble-milled cellulose. The yellow pigment is bound to the cellulose fibers, which tend to become viscous and form clumps. The maximum rate of cellulose degradation in carbonate-buffered pH 7 medium was reported for pebble-milled cellulose as 29 mg/L·h, and 8.7 mg/L·h for Avicel (*91*). Using high performance size exclusion chromatography, Grohmann et al. (*16*) showed that cellulase activiaty of *C. populeti* culture concentrate from supernatant (measured by CMC hydrolysis) elutes as a single peak corresponding to approximately 250,000 dalton.

The maximum rate of pebble-milled cellulose degradation by *C. cellulovorans* in carbonate-buffered medium at pH 7 was reported to be 53 mg/L·h, and the maximum rate for Avicel as 19 mg/L·h (*92*). These rates of hydrolysis are comparable to *C. thermocellum* supporting observations that rates of cellulolysis at mesophilic temperatures can approach thermophilic rates.

Bacterial Cellulase Producers of the Rumen

Bacteroides cellulosolvens *and* Bacteroides succinogenes

The predominant rumen cellulolytic bacteria are reported to be *B. succinogenes, Butyrivibrio fibrisolvens, R. albus,* and *R. flavefaciens* (*93,94*). In

addition, *Clostridium sp., Eubacterium cellulosolvens, Micromonospora ruminantium,* and *Micromonospora propionici,* have also been isolated from rumen fluid. Another *Bacteroides* species, *B. cellulosolvens,* was isolated from sewage sludge (*95*). This culture is a nonspore-forming, mesophilic anaerobe, capable of growing in a synthetic medium containing cellulose or cellobiose as the sole carbon source. It produces large amounts of acetic acid, CO_2, and H_2, along with small amounts of ethanol and lactic acid, as the end products. It does not require yeast extract for growth and does not utilize glucose or xylose as a carbon source (*95*).

It was reported that *B. cellulosolvens* produced extracellular and cell-associated cellulases that possessed endoglucanase, exoglucanase, and xylanase activities and were able to degrade filter paper *in situ*. However, this enzyme system lacked β-glucosidase activity (*29*). Growth of *B. cellulosolvens* on Solka Floc caused the accumulation of cellobiose, glucose, and xylose. The accumulation of sugars, particularly glucose, was not brought about by the extracellular enzyme system produced by *B. cellulosolvens,* but by the bacterial cells with the help of cell-associated enzymes. Cellobiose can be converted by glucose-1-phosphate and glucose by the action of cellobiose phosphorylase (*29*). Cellulase activities were inhibited by ethylenediamine tetraacetate (EDTA), whereas ascorbic acid and dithiothreitol increased these activities (*29*).

B. succinogenes can ferment cellulose, cellobiose, glucose, starch, dextrin, maltose, and trehalose to succinate and acetate as the main products; carbon dioxide is fixed (*96,97*) during growth of *B. succinogenes* S-85; the cells appeared to adhere to the cellulose and produced both extracellular and cell-bound carboxymethyl cellulase (CMCase) (*49*). Cell-free culture supernatants and cell extracts from cellulose-grown cultures had very low hydrolytic activity. Cells grown on either cellobiose or glucose exhibited cell-bound CMCase and cellobiase activities. Cultures grown on cellulose had seven to eight times more CMCase activity than cellobiose- or glucose-grown cultures. Seventy percent of the CMCase activity was present in the supernatant, of which 50–62% was associated with sedimentable membranous fragments, 9–13% with nonsedimentable material with a mol wt greater than 4,000,000 dalton, and 28–38% with molecules having a mol wt of approx 45,000 dalton (*98*). The cellobiase, which was largely cell associated, appeared to be constitutive. This enzyme was sensitive to air, but the activity was maintained in an atmosphere of nitrogen in the presence of dithiothreitol. However, the CMCase activity was not oxygen sensitive (*99*). The only hydrolysis product was glucose. Therefore, the enzyme cleaving cellobiose appeared to be a β-1,4-glucosidase rather than a cellobiose phosphorylase. *B. succinogenes* was the first bacterium reported to possess a CMCase associated with membrane fragments.

An enzyme, cellodextrinase, that released the cellobiose group from *p*-nitrophenyl cellobioside was also isolated from the periplasmic space of *B. succinogenes* grown on Avicel crystalline cellulose (*100*). The mol wt of the enzyme was approx 40,000 dalton and the isoelectric point was 4.9.

The function of cellodextrinase presumably is to hydrolyze cellodextrins, which enter the periplasmic space, to cellobiose and glucose for transport into the cell. This enzyme exhibited low hydrolytic activity on acid-swollen cellulose and practically no activity of carboxymethyl cellulose, Avicel cellulose, and cellobiose. However, it hydrolyzed *p*-nitrophenyl lactoside as well as *p*-nitrophenyl cellobioside and released cellobiose from cellotriose and from higher cellooligosaccharides (*100*). The endoglucanase and cellobiosidase activities coded for by the Cel gene of *B. succinogenes* were expressed and secreted into the periplasmic space in *E. coli* (*39*). Nondenaturing gel electrophoresis of the periplasmic Cel endoglucanase revealed the presence of three components, two with different charges each having a mol wt of 43,000 dalton, and one with a mol wt of 55,000 dalton.

Ruminococcus albus *and* Ruminococcus flavefaciens

R. albus, R. flavefaciens, and *B. succinogenes* are important cellulose-degraders found in the rumen of cattle and sheep. Most isolated strains ferment cellulose and xylan, and all ferment cellobiose. Fermentation of glucose and some other carbohydrates depends on the particular strain. The major products of cellobiose fermentation are acetic acid, formic acid, ethanol, succinic acid, and hydrogen. The formation of succinate involves fixation of CO_2 (*101*). *R. flavefaciens* and *B. succinogenes* can ferment the highly ordered crystalline cellulosic substrates, but *R. albus* cannot. Wood and Wilson found no evidence for exocellulase production by *R. albus* (*102*), but Ohmiya et al. purified cellobiosidase from this culture (*37*). Numerous strains of *B. succinogenes, R. albus,* and *R. flavefaciens* require one or more of the following acids: isobutyric, isovaleric, 2-methylbutyric, and *n*-valeric (*103*).

R. albus has been shown to adhere to cellulose fibers (*104,105*). Moreover, several workers have identified a large cellulase complex on the surface of this microorganism (*106*). Such a complex might correspond to the cellulosome of *C. thermocellum* and could be expected to contain the full complement of enzyme and adhesive activities (*107*). The morphological and enzymatic studies from many workers (*108–110*) indicated that different cellulolytic rumen bacteria differ in their physical association with the surface of highly crystalline cellulose. Cells of *B. succinogenes* were very intimately adherent to the cellulose, but *R. flavefaciens* appeared to be less intimately associated with the cellulose surface. Stack and Hungate (*103,111*) showed that 3-phenylpropanic acid (PPA), which is present in rumen fluid, did not directly affect the growth of *R. albus.* PPA increased the production of an extracellular glycocalyx, which was known to affect cell adhesion to cellulose (*112*), and resulted increased rates of cellulose digestion (*113,114*).

In PPA-grown, cultures of *R. albus* produced substantial quantities of cell bound cellulase, as well as a very high-mol wt extracellular enzyme and lesser amounts of two low mol wt enzymes. PPA-deprived bacteria produced greater total amounts of cellulase, which all existed in soluble, low-mol wt forms (*111*). Gel electrophoresis showed that the availability of PPA did not affect the kinds of proteins produced, but the distribution of two major proteins, 102,000 and 85,000 dalton, between cells and supernatant was PPA dependent. These two proteins predominated in the cell pellets of PPA-grown cultures, whereas they were chiefly extracellular constituents of PPA-deprived cultures (*111*). Cell-associated cellulase activity was correlated to the presence of lobed capsular structures in *R. albus*. The secreted enzymes appeared to be associated with the presence of large vesicular structures, the formation of which required PPA (*111*).

The rates of cellulose digestion in the culture of *R. albus* 8 supplemented with phenylacetic acid (PAA), butyric acid, and rumen fluid were compared by Stack et al. (*114*). PAA and rumen fluid were equally effective, but PAA was effective only when PPA was present. Omission of PAA increased the time for cellulose digestion to almost 2 days, whereas omission of PPA almost stopped growth, with all of the cellulose being digested only after 2 weeks. It was found that *R. albus* 8 utilizes PAA for phenylalanine biosynthesis (*114*).

R. flavefaciens cells, during growth in pure culture, released cellulase, endoglucanase, and xylanase into the culture fluid. The xylanase had a mol wt in excess of 800,000 dalton (*115*). This microorganism hydrolyzed cellulose to yield only cellobiose as a product (*115*). The addition of PPA had little effect on the growth of *R. flavefaciens* (*113*). *R. flavefaciens* was shown to possess a prominent glycoprotein coat, which contained rhamnose, glucose, and galactose as principal carbohydrates. The culture adhered strongly by means of this coat to cotton cellulose and to cell walls in leaves (*116*). It had been reported that a cellobiose phosphorylase and glucokinase were present in *R. flavefaciens* (*24*). The *Ruminococcus* cellulase system was repressed by the presence of disaccharides, such as cellobiose, sucrose, and lactose. In addition, cellobiose, if present, must first be used before cellulose was degraded (*117*).

LEVELS OF CELLULASES AND OTHER HYDROLYTIC ENZYMES IN ANAEROBIC DIGESTERS

Assay Protocols

Historically, information concerning cellulose degradation in anaerobic digesters has been mostly limited to studies that measure cellulose disappearance from the system. Little information on the mechanisms

and biocatalysts involved is available, in part because of the complexity of the system. Frequently, the characteristics of microorganisms isolated from anaerobic sludge have been used to infer the performance of cellulolytic enzymes in anaerobic digesters. This extrapolation of data from single organisms to a complex consortium, as occurs in anaerobic digestion, must be considered with caution. Understanding the interrelatedness of cellulose degradation in anaerobic digesters requires assay protocols that measure the resident digester enzymes accurately.

The bound cellulolytic activity of *Clostridium thermocellum* in culture fluid is easily eluted from cellulosic substrates with distilled water or 5 m*M* salt or buffer solutions when the pH is below 4 or above 9 (*82*). Other agents reported to elute *C. thermocellum* cellulase from cellulose include 50% ethanol (*19*) and 100 m*M* triethylamine-maleate buffer, pH 6.85 (*39*). However, the conditions for elution of most bacterial cellulase enzymes have not been reported.

Recently, digester supernatants have been shown not to contain appreciable levels of hydrolytic enzymes. The resident enzymes are located primarily in the particulate fraction (*118*). Elution of these enzymes, therefore, is necessary for accurate determination of the hydrolytic capacity of an anaerobic digester. Extraction protocols similar to those used to elute bound enzymes from *C. thermocellum* have been shown not to be effective for the extraction of digester sludge, indicating the hydrolytic enzymes in anaerobic digesters are more tightly bound (*118*), or simply lose activity under these conditions. A series of detergent extractions developed by Adney et al. were designed to extract active enzyme from anaerobic digesters (*118*). Digester solids were prepared and analyzed as follows: the particulates from a 30-mL sample were removed by centrifugation (15,000g) at 4°C for 20 min. The particulates were washed three times with 100 m*M* Tris buffer pH 7, and resuspended in 15 mL of buffer. The extraction procedure consisted of agitating the sample with a Fisher model 346 rotator at 25°C in the presence of either 0.1% SDS, 1% Triton, or 0.1% Zwittergent for 1 h. The particulate material was then removed by centrifugation at 15,000g at 4°C for 20 min, and the supernatant was used to perform the enzyme assays. Extractions with Tris buffer alone were also performed for comparison. Although there was some detergent-specific tendency for optimal recovery of specific enzymes, SDS was chosen for routine work because it produced the highest recoveries of all three cellulase-class activities (*see* Table 1). Experiments using "spike and recovery" tests with commercial cellulases were used to show that all cellulase activity added to digester sludge could be recovered using SDS extraction (*119*).

These extraction procedures utilizing low detergent concentrations were used to extract active enzymes from the particulate fraction of sludges taken from continuously stirred tank reactors (CSTR) fed municipal solid waste (MSW). Specific enzyme activities were found to correlate with

Table 1
Enzyme Activity Analysis of Extracted Sludges from Laboratory- and Pilot-Scale Mesophilic Anaerobic Digesters Fed Processed MSW

Activity, substrate	Laboratory-scale[a] U/mL digester sludge	Pilot-scale[b] U/mL digester sludge
Exoglucanase (cellulose-azure)	0.030±0.003	-0-
Endoglucanase (CMC)	0.198±0.046	0.1070±0.096
β-D-glucosidase (pNP-β-D-glucoside)	0.150±0.002	0.0410±0.017

[a]3.5-L CSTRs.
[b]ETU (Solcon), 1200-gallon digester at Disney World, Orlando, FL. Exoglucanase activities were estimated using cellulose-azure substrate as described earlier (*118*). Here, standard curves were developed for the hydrolysis of each azure-linked substrate by cellulases of known activity. By this method, one cellulose-azure hydrolysis unit corresponds to one filter paper unit. Endo-1,4-β-glucanase activities were defined as CMCase activities. Here, one unit of activity was that amount of enzyme required to liberate one microgram glucose from CMC in 60 min. β-D-Glucosidase was determined according to the method of Wood (*120*) as aryl-β-glucosidase activity by the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside. One unit of activity was defined as that amount of enzyme that catalyzes the cleavage of 1.0 μmol substrate per min. All assays were performed at 37°C in triplicate.

conventional methods for determining cellulose removal (i.e., acid detergent fiber analysis) in seven different reactors (*119*). The accurate determination of the levels of specific cellulase activities may be a good indicator of the hydrolytic capacity of a biomass-fed digester.

Detectable Levels of Hydrolytic Enzymes in Bench- and Large-Scale Anaerobic Digesters

Although the classical approach to the characterization of cellulose degradation in anaerobic digestion systems is by analysis of feedstock cellulose loss, a more rapid and accurate determination of hydrolytic potential must target assays of resident cellulase enzymes. Table 1 shows a comparison of the hydrolytic enzyme activities found in both bench-and pilot-scale digesters fed a processed MSW feedstock (*119*).

Although many of the operational parameters of the two digester systems studied were similar, important differences in digester mixing design (i.e., laboratory-scale digesters were operated as CSTRs and the Solcon reactor was operated as an unmixed/settling-bed reactor) and feedstock source, storage, and handling may explain the differences in enzyme levels shown in Table 1. In general, the unmixed Solcon reactor resulted in substantial variations in specific enzyme activities in digester sludge,

Table 2
Comparison of Cellulase Activities
Found in Selected Natural and Commercial Systems

System	Filter paper activity	CMCase activity
Digester detergent extracts[a]	0.0006 U/g dry sludge	0.15 U/g dry sludge
Moist pasture soil[a]	NA	0.006–0.012 U/g dry soil
Rumen contents[b]	NA	1.4 U/g solids
Industrial saccharifications of woody biomass[c]	20 U/g cellulose	600 U/g cellulose

[a]From (*118*).
[b]Calculated from (*121*).
[c]Loading necessary to achieve complete hydrolysis in 1-2 d (*122*).

depending on sample depth within the reactor. Because of the unmixed design, significant variability was found for all of the activities examined in the Solcon digester. However, in comparison with the laboratory-scale CSTR systems (operated under optimum nutrient levels), the Solcon reactor was similar with the exception of exoglucanase titers. Unfortunately, the detergent assay protocols described here and by Adney et al. have not, as yet, been used routinely at the site of a large-scale anaerobic digester (*119*). In such a scenario, immediate information concerning the disposition of the cellulose-degrading capability of the digester could allow operators to adjust feed rate, feed type, and the overall digester operational protocol.

However, both digester systems exhibit extremely low levels of cellulase activities (exoglucanase, endoglucanase, and β-glucosidase) when compared to industrial saccharifying processes in which the hydrolysis of cellulose in the feedstock is optimized with respect to enzyme loading (*see* Table 2). Therefore, these data indicate the significant level of improvement possible for the attainment of the maximum rates of cellulose hydrolysis in the anaerobic reactor system.

SUMMARY

For reasons of commercial expediency, the majority of research efforts regarding cellulase characterization has focused on fungal enzymes, with *T. reesei* receiving the most attention. Because anaerobic digesters are operated at approx pH 7 and 37°C, these *T. reesei* enzymes, normally most active at pH 5 and 45°C, are considerably suboptimal for this application. Recent detergent-extraction assays of digester sludges have shown that the digester resident cellulase titers are quite low and, more importantly, exhibit activity characteristics (i.e., pH and temperature optima)

that are *also* suboptimal for commercial digester operation (*14*). This discovery strongly suggests that the consortia commonly used to "seed" large digesters is not capable of adapting optimally to the operational conditions resulting in the best methane production. An examination of the cellulase enzymes produced by anaerobic, cellulase producing bacteria discussed earlier in this paper reveal that, although several display suitable activity characteristics (pH and temperature), they suffer from the bane of bacterial cellulases—low productivities. Again, fungal systems have received the benefit of a long development history, which has also led to cellulose productivities (filter paper units/L culture broth·h) between 10 and 100 times that of the best bacterial system. This case is well illustrated by a comparision of the maximum obtainable cellulase concentration and productivity from batch fermentation of *C. thermocellum* (i.e., 140 FPU/L and 1.9 FPU/L·h, respectively) with values from the best commercial preparation of *T. reesei* cellulases (i.e., 18,000 FPU/L and 94 FPU/L·h, respectively) (*54*). However, it should be mentioned that most bacterial cellulase producers reach maximum cellulase titers, low as they are, in less time than do fungi. With this condition in mind, new sources of cellulase enzymes must be identified.

Recent work indicates that the analysis of cellulase enzyme activities may be the best method for determining the projected cellulose conversion of the overall anaerobic digestion system, and therefore, the hydrolytic power of the system under evaluation. With this development, the analysis of enzyme activities may also serve as a "real time" method of monitoring the stability of the system, with radical changes in enzyme activities indicative of potential process upset. Information concerning the effects of system operation parameters, such as temperature and pH, and their effects on the optimum activity of digester-resident hydrolytic enzyme systems, may provide for the development of strategies permitting enhanced process rates.

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