Cellulose Degradation by Three Strains of Bacteria Found in the Gut of *Zootermopsis angusticollis*.

by

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Abstract

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Cellulose is the most abundant renewable organic molecule in the world, making it a huge resource of renewable energy. However, cellulose, along with lignin and hemicellulose, the other components of lignocellulosic materials, is very resistant to biological degradation. Lignocellulosic by-products constitute a large and growing waste stream during industrial conversion of plant materials into refined goods. This waste stream is currently disposed of by land filling, dilution, or combustion. With disposal costs rising, other strategies must be developed to handle the lignocellulosic materials.

Biological digestion of lignocellulosic materials can convert waste streams into valuable products. Most biological conversions are aimed at changing cellulose into ethanol to replace fuels currently supplied by petroleum. Cellulases, a family of enzymes, digest cellulose into glucose units. Cellulases are found in niches where lignocellulosic materials are abundant. The gut of wood-eating termites contain symbiotic organisms that digest cellulose. In this study, three different strains of bacteria were isolated from *Zootermopsis angusticollis*, the damp wood termite. These isolates were tested for their ability to digest cellulose using carboxymethylcellulose and crystalline cellulose as indicators.

These isolates, identified as members of the *Bacillus* genus, contained carboxymethylcellulase activity, as measured by a reduction in viscosity and an increase in reducing ends in media containing carboxymethylcellulose. Additionally, two of the isolates demonstrated the ability to digest crystalline cellulose as measured by dye release from dyed cellulose and metabolism of microfine crystals of cellulose. These assays demonstrated that two of the isolates had complete cellulolytic systems capable of digesting crystalline and modified cellulose. The results from these assays, which establish the capabilities of the cellulase systems, show potential industrial value for these two isolates.

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Acknowledgments

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Introduction

Cellulose. Cellulose is one of the most abundant organic macromolecules in the ecosystem (Ross, Mayer, & Benziman, 1991; Tewari, 1990). Technically, cellulose is a polymer of D-glucose units linked by β-1,4 glucosidic bonds that form large crystalline fibrils. In lay terms, cellulose is a long chain of carbon rings connected through an oxygen atom between each glucose molecule (Figure 1). When these chains are laid down next to each other, they form hydrogen bonds which hold the chains together to form large fibers (Figure 2).

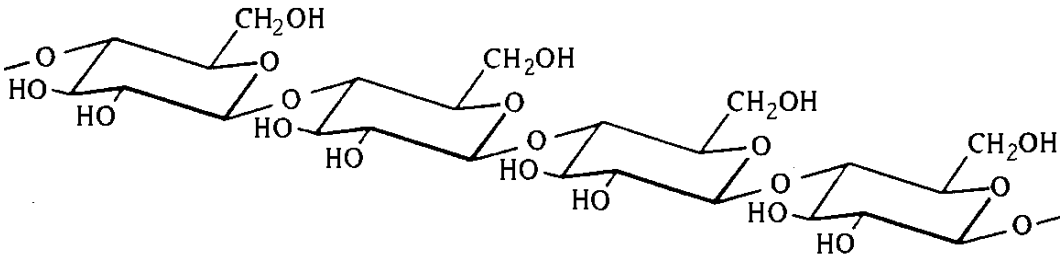

Figure 1. Line drawing of cellulose showing β 1-4 linkages between individual glucose units (Solomons, 1988).

Cellulose is produced by a variety of plants and bacteria, plus a few animals (Ross, Mayer & Benziman, 1991). It is found both as a homopolymer and in lignocellulosic material, a crystalline glucose polymer in a matrix with lignin and hemicellulose in an approximate mass ratio of 2:1:1 (OTA, 1995). Lignin is a polymer of three similar monolignol precursors, *p*-coumaryl, coniferyl, and sinapyl alcohols (Sarkanen *et al*., 1991). These alcohols include a benzene

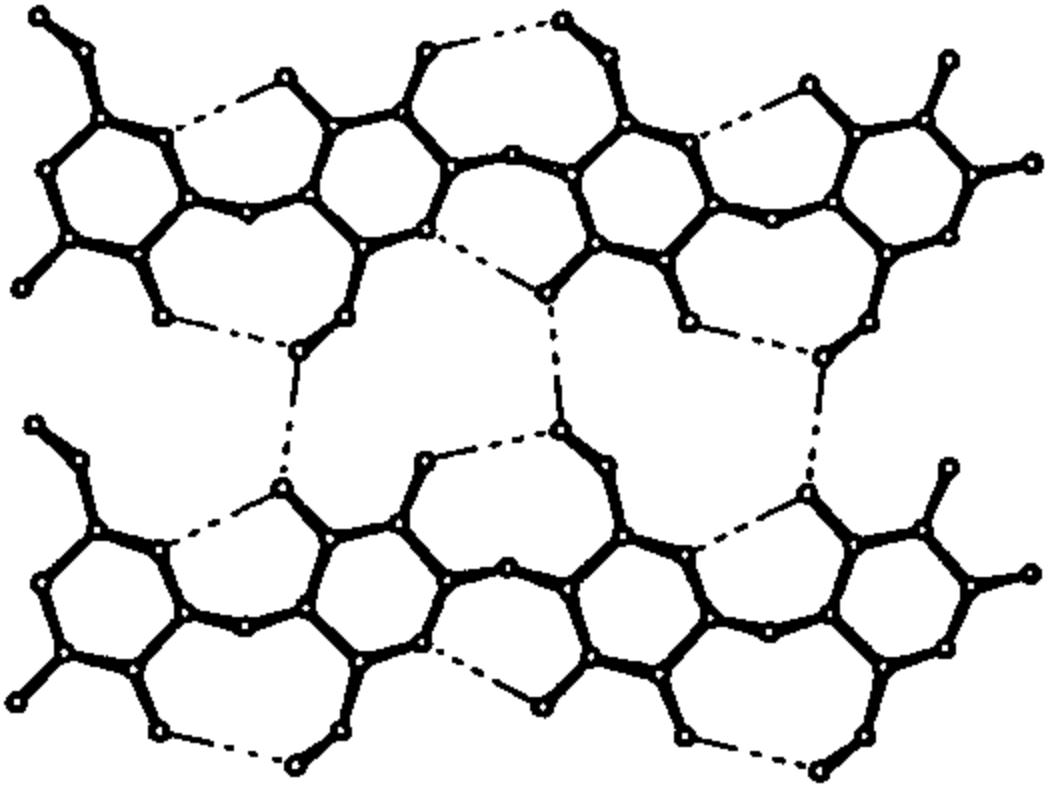

Figure 2. Line drawing showing potential hydrogen bonds (dashed lines) within and between cellulose molecules (Ross, 1991).

ring resistant to chemical and biological degradation. Hemicellulose is a group of non-glucose sugar polymers found in lignocellulosic material. The main components of hemicellulose are xylose, mannanose, and arabinose. This combination of cellulose, lignin, and hemicellulose is very resistant to enzymatic degradation (Wood & Kellogg, 1988a, 1988b). After products like sugars or gums are extracted from agricultural plants, cellulose, along with lignin and hemicellulose, remains a significant waste product, disposal of which poses an increasing cost to many manufacturers.

Microbial cellulases. Cellulase is a family of cellulolytic enzymes that degrade cellulose. One measure of how many cellulase systems are produced by organisms is the number of taxonomic families found in DNA sequences. There have been more than 30 different cellulase genes identified which have been categorized into nine families (Saddler, 1993). As more cellulose degrading systems are isolated and quantified, this number will grow. Complete and incomplete cellulose degradative systems are synthesized by many microorganisms, including fungi, actinomycetes, gliding bacteria, and true bacteria. Incomplete cellulase systems are those collections of proteins that are missing one enzyme, or have reduced functionality in cellulose digestion.

Cellulose degradation occurs in three general steps (Bisaria & Mishra, 1989; Klyosov, 1990). First, the long chain polymer is degraded into random lengths of 4 to 6 glucose units by an endoglucanase, which is often exported to the environment outside the cell. Second, an exoglucanase cleaves the shortened chains into dimers. This enzyme is exported or held in close association with the outer membrane. The final step is cleavage of the dimer into glucose by a β-glucosidase, most commonly located within the cell.

Microorganisms with cellulase systems inhabit many different niches, but thrive mainly in areas of higher concentrations of cellulose, such as soils, plants, and in the guts of plant-eating animals and insects. Termites host a large variety of symbiotic microorganisms in their gut that includes several cellulose degrading aerobic and anaerobic bacteria (Bignell, Oskarsson & Anderson, 1980; Eutick,

O'Brien & Slaytor, 1978; Thayer, 1976, 1978). The damp wood termite, *Zootermopsis angusticollis* (Banks & Snyder, 1920), is a common termite of the west coast of the United States (Thorne *et al*., 1993) and contains a diverse gut flora. However, little is known about that flora.

There is some controversy in the meager literature about termite symbionts as to the role bacteria play in digesting cellulose. Slaytor (1990) in his review claims that, "there is no evidence that bacteria are involved in cellulose digestion in termites and cockroaches." But many authors have found cellulolytic bacteria in both lower and higher Order termites. Indeed Breznak (1982) found that, "a number of bacterial isolates have been found to possess C_x-type[1] noncrystalline cellulase activity." The argument of whether bacteria produce cellulases necessary for termite survival could hinge on a semantical position of whether bacteria play a ancillary or insignificant role in cellulose digestion. Lower Order termites produce cellulases themselves and possess cellulolytic protozoa in the hindgut (Breznak, 1982; Slaytor, 1990). Studies have shown bacteria do not digest enough cellulose to supply all the termite's energy needs (Slaytor, 1990). But several studies have shown that bacteria fix nitrogen that may be critical to the termite's survival (Breznak, 1975, 1982; French, Turner & Bradbury, 1976; Protrikus & Breznak, 1977). This suggests that any cellulase digestion by bacteria would be beneficial to termites, although perhaps not life sustaining.

[1] A list of all abbreviations and acronyms can be found in Appendix A.

Industrial importance of cellulase. Cellulose occupies two fundamental industrial areas. First, as a resource in the form of pulp, fiber, and chemical or physical feedstocks. And second, as a waste product, after processing raw materials that contained cellulose. Both forms greatly impact industry. Resource cellulose funds the industry, while waste cellulose carries the cost of disposal. Industry is constantly searching for methods of improving the quality of their product. One area of research that shows promise in enhancing the value of cellulose, or lowering the cost of disposal, is biotechnology (Coughlan, 1990; Dhingra *et al*., 1993; Hartley *et al*., 1987; Kirk & Chang, 1990; Pommier *et al*., 1990; Wood & Ingram, 1992).

Biotechnology involves the use of isolated enzymes or fermentation conditions to alter natural or artificial substances. Cellulases, hemicellulases, and ligninases are enzymes designed to digest the various components of plant material. Currently cellulases and hemicellulases, mostly xylanases, are used to modify the structure of cellulose-containing substances. Commercial ligninases remain unavailable in quantity (Coughlan, 1990; Kirk & Chang, 1990). Even so, modification of pulp with cellulase and hemicellulase enhances lignin removal and lowers the amount of chlorine bleaching required to remove the remaining lignin (Kirk & Chang, 1990). The Weyerhaeuser Company, one of the world's largest pulp and paper manufacturers, currently uses several commercially-available cellulases to improve pulping of wood chips. Piles of wood chips are sprayed with a slurry of water and enzyme and allowed to sit for several hours

before processing. Partial digestion of the chips enhances the mechanical pulping process, reducing effluents and cost (Brooks, 1994).

Additional uses of cellulases include treatment of chemical and animal feedstocks. Uses of cellulosidic enzymes on straw can improve the digestibility of animal feedstocks by improving the ratio of carbohydrates to the undigestible fiber (Coughlan, 1990; Dhingra *et al*., 1993). Care needs to be exercised in the treatment of silage though, because excessive treatment leads to collapse of the cell walls and spoilage (Coughlan, 1990). Feedstocks used by the chemical industry benefit from pretreatment with cellulosidic enzymes by making sugars readily available for processing into ethanol or acetone while reducing the mechanical strength of the feedstock (Dhingra *et al*., 1993; Wood & Ingram, 1992). However, the ready supply of a low-cost competitor for chemical feedstock, petroleum, has limited the research and development of additional enzymatic manipulation schemes for lignocellulosic materials (Hartley *et al*., 1987; OTA, 1995; Saddler, 1993). Cellulose remains a moderate cost alternative to petroleum-based chemical feedstocks, that someday may replace chemicals derived from higher priced oil. In addition to resource cellulose, waste cellulose is becoming a valuable source of raw materials. Enzymatic digestion of the waste stream in many processes that involve cellulose improves the quality and reduces the quantity of by-products, allowing easier utilization of valuable raw materials.

Often the waste stream of cellulose is a water-saturated mass, ideal for microbial degradation but difficult to burn as fuel, a common disposal method.

Current industrial processes are aimed at developing enzymatic treatments of waste cellulose to produce sugars (glucose), gaseous fuels (methane and hydrogen), and liquid feedstocks (ethanol) (Coughlan, 1990; Dhingra *et al*., 1993; Hartley *et al*., 1987; Saddler, 1993). Although not available in quantity, ligninase shows promise in removing chlorinated compounds common in the bleaching process waste stream in several pilot projects (Kirk & Chang, 1990).

Another waste stream where cellulases are utilized is the recycling industry. For example, cellulases assist in deinking recycled paper by apparently degrading the ink-containing outermost fibers and allowing easier removal of the ink (Young, 1994; Zeyer *et al*., 1994). In addition to deinking, cellulases are used to enhance the freeness, the rate of water drainage in pulp (Sarkar, Cosper & Hartig, 1995), and upgrade the quality of recycled pulp fibers (Stork *et al*., 1995). Most of these treatments of recycled fibers by cellulases are designed to lower the energy input in the pulping process by providing more uniform, freer cellulose fibers that integrate into the virgin fiber mix to form a consistent product. Even with cellulase-driven enhancement of recycled fibers, limitations exist to the extent cellulose fibers can be recycled. In the pulping process, fiber shortening and degradation occur over time, limiting used fiber input to 35% by mass (Virtanen and Nilsson, 1993). Cellulases are used to homogenize the fiber length by degrading the shorter fibers preferentially over longer fibers.

Although purified enzymes allow more consistent control of the product, living organism enzyme systems lower the overall cost of utilizing a resource

(Hartley *et al*., 1987; Saddler, 1993). Bacteria containing cellulolytic systems that produce alcohol during sugar metabolism are very low cost producers of a valuable resource. Bacterial fermentation of lignocellulosic materials can produce ethanol at competitive rates with corn and sugar beets (Hartley *et al*., 1987; OTA, 1995; Saddler, 1993). The economic disadvantage in producing ethanol by enzymatic fermentation includes the current restriction of monoculture input, limiting sources to one type of material (OTA, 1995), and the low cost of petroleum alternatives (Hartley *et al*., 1987; OTA, 1995; Saddler, 1993).

Research goals. This research focused on isolating symbiotic termite microorganisms capable of digesting cellulose. Isolates were compared to known cellulase producers and additional experiments were developed to further characterize the cellulolytic abilities of each organism. Conclusions regarding the industrial value of these isolates are discussed where appropriate.

Materials and Methods

Termites

Collections. Several dozen termites identified as *Zootermopsis angusticollis*, the damp wood termite, were collected in September from a nest near Young's Cove, Section 30, Range 2W Township 19N in Thurston County, State of Washington. Identification of the termites was kindly provided by Dr. John Longino and Dr. Robert Sluss of The Evergreen State College in Olympia, Washington. The habitat surrounding the nest was second growth *Pseudotsuga menziesii* (Douglas-fir), coastal fringe. The nest was located in an overturned Douglas-fir stump that was partially buried. Specimens were collected from galleries that extended below ground but still were surrounded by woody material of the stump. These termites were kept in a 20 L container with Douglas-fir wood shavings for approximately 2 weeks before dissection. No nutritional additions, except sterilized deionized water, were provided during the 2 weeks.

Dissections. Dissections were carried out as described by Bignell, Oskarsson and Anderson, (1980); Breznak and Pankratz, (1977); Hendee (1933); and Thayer (1976). Termites were separated into two groups of fully developed workers and soldiers. Approximately half the collected termites were used in the dissection preparation. Prior to dissection each termite was exposed to a germicidal UV lamp for 15 minutes with agitation of the open glass dish to ensure complete

exposure of the dorsal and ventral areas. Additionally, each termite was then washed in a 70% ethanol solution. Termites were placed in four separate five-member groups for dissection. One termite from each pool was randomly selected and rolled over a tryptic soy broth (TSB) (Difco Co., Detroit, MI) agar plate to check for external contamination. No growth occurred after overnight incubation of the agar plates at 30°C. Each termite was decapitated using a scalpel under a dissecting microscope and the digestive tract, including the crop, was pulled through the opening. Each of these digestive tracts was carefully rinsed externally with sterilized deionized water before being added to the pool.

Isolations. After dissection, each group of digestive tracts was either coarsely chopped with a scalpel or homogenized with a sterile mortar and pestle in the presence of alumina. Four samples (100 µl) of the liquid material from each pool were then spread onto TSB plates and incubated at 30°C. Over several days each unique colony type, based on surface morphology or color was sampled and strains were isolated by repeated streaking on TSB plates. Twenty-four isolated strains were stored at -70°C according to Maniatis, Fritsch and Sambrook, (1982).

Assays

Inocula. Samples were taken from -70°C glycerol stocks and grown overnight on TSB agar plates at 30°C. Bacteria from these plates were either used directly to inoculate experiments, or grown overnight in liquid TSB media in a shaking water bath at 30°C and then used as inocula. Experiments which generated quantified data were inoculated with measured volumes of overnight cultures. Experiments that utilized cells but were not quantified, such as cell staining, were from cultures grown overnight on TSB agar plates. A list of standard tests and media preparations can be found in Appendix B.

Controls. *Bacillus subtilis*, wild type (*B. subtilis*, lab strain) and *Escherichia coli*, K803 (*E. coli*, lab strain) were selected as positive and negative controls in all of the following studies. *Bacillus subtilis* displays CMCase activity (Chan & Au, 1987; Lo *et al*., 1988) and limited cellulase activity (Chan & Au, 1987; Lo *et al*., 1988). *E. coli* possess neither enzyme activity (Balows *et al*., 1992), but was used to establish growing culture conditions, such as pH changes and depletion of nutrients, without utilizing the cellulose additions of interest. *E. coli* cultures were included in the experiment design to eliminate the possibility that changes in cellulose properties, such as viscosity reductions or increases in reducing ends, were due to changes in the media generated by the growth of cells and not the enzyme systems being tested.

Screening. Bacterial species were screened for cellulolytic activity by liquification of a 2.5% carboxymethylcellulose (CMC) solution (10 ml) containing medium viscosity CMC (Sigma Co., St Louis, MO) in minimal salts media (M9) as measured by visual inspection of the agitated test tubes containing each culture of interest. Bacteria were also screened for growth on M9 agar plates containing CMC or deionized water washed crystalline cellulose as the sole carbon source.

Assays. Presence of CMCase activity was determined by measuring changes in viscosity and reducing ends in media containing CMC (Chan & Au, 1987; Kricke *et al*., 1994; Thayer, 1976, 1978; Wood & Kellogg, 1988a). Cellulase activity was measured in test using both dyed and undyed crystalline cellulose in procedures described by Weimer, Lopez-Guisa and French (1990) and Wood and Kellogg (1988a). Three species that grew on cellulose or CMC-agar plates or modified a liquid CMC medium by reducing viscosity were tested in a quantified series of experiments for the ability to liquify CMC as measured by viscosity change using a slightly modified Thomas-Stormer viscometer. The viscometer was modified in the following manner. A Pasco Model 9260 photo-reflective sensor was attached to the face of the analog dial and the output from the sensor was connected to an Gerbrands Model G1270 electronic timer via a switching circuit seen in Figure 3. When the analog clock hand passed the sensor it triggered the timer to start. One full circuit of the analog

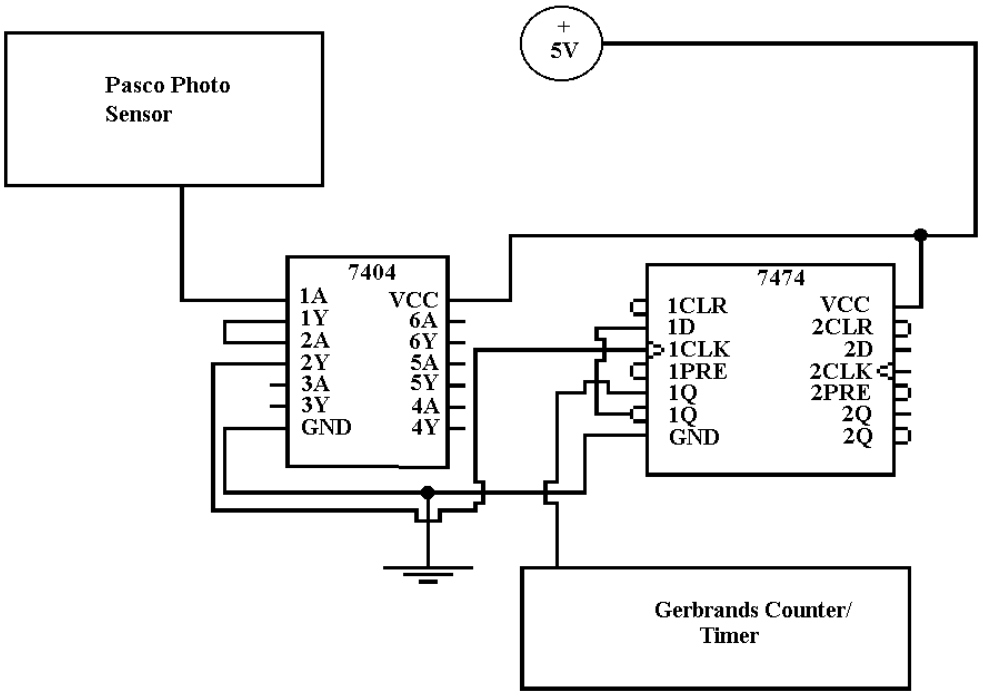

Figure 3. Schematic diagram of trigger circuit modification to Thomas-Stormer viscometer.

dial was chosen because it was a convenient time span of 9 to 13 seconds, allowing several repetitions of the experiment during a brief time period. Additionally, one full circuit minimized the complexity of the electronic circuitry required to trigger the timer by requiring only one pass of the analog clock hand. Upon completing a full circuit, the hand again triggered the timer to stop, yielding a time in seconds to four significant figures. This modification allowed repeatable time measurements to within 0.1%, approximately an order of magnitude improvement over hand timing with a stop watch.

Liquid media were inoculated with a 1000-fold dilution from a fresh overnight culture into M9 medium containing 2.5% CMC and 0.25% casamino acids (Difco Co., Detroit, MI), called CMCCA. Cultures were placed in a shaking water bath at 30°C. Samples (6 ml) were removed from the flasks at regular time intervals and subjected to centrifugation at 4,100g for 15 minutes. A sample (4 ml) of the supernatant was removed, taking care not to disturb the pellet, from these centrifuged samples, diluted 20-fold into water and tested for change in viscosity at 20°C. Results are given as percent change between samples and the ratio of uninoculated CMCCA and water.

Cultures containing CMC were also assayed for reducing ends using the 3,5- dinitrosalicylic acid method as described by Baum and Bowen (1972). A sample (100 µl) was removed from the centrifuged samples and added to 400 µl 3,5-dinitrosalicylic acid. The mixture was incubated 5 minutes in a water bath at 100°C. Sample absorbance was measured at 540 nm after dilution to a total volume of 2 ml. Results are given as micromoles of reducing ends per ml, based on a glucose dilution standard curve.

Crystalline cellulose assays were performed in two experiments. The first experiment examined the release of dye from cellulose azure (Sigma Co., St Louis, MO) according to methods described by Wood and Kellogg (1988a). Liquid media were inoculated with a 1000-fold dilution from a fresh overnight culture into M9 medium containing 0.5% cellulose azure and 0.25% casamino acids, called DCCA. Samples (750 µl) were removed, subject to centrifugation in

Eppendorf tubes at 14,000g for 10 minutes. Supernatant (600 µl) was removed taking care not to disturb the pellet and absorbance was measured against uninoculated DCCA media without dyed cellulose blank at 568 nm using a Hewlett Packard diode array spectrophotometer.

The second experiment, developed in this investigation, involved the apparent loss of microfine crystals of cellulose. Liquid media were inoculated with a 1000-fold dilution from a fresh overnight culture into M9 medium containing 1.0% undyed crystalline cellulose, EMCOCEL50M, (Mendel Co., Patterson, NY) and 0.25% casamino acids, called UCCA. Cultures were placed in a shaking water bath at 30°C. Samples (1 ml) were removed, subjected to centrifugation in Eppendorf tubes at 14,000g for 10 minutes and then most of the supernatant was carefully removed and discarded. One milliliter 95% ethanol was added to each tube, followed by vigorous agitation to completely resuspend the pellet. Samples were subjected to centrifugation at 14,000g for 10 minutes to remove water-soluble proteins, and 600 µl supernatant was carefully removed. Microfine crystals of cellulose remain suspended in solution for hours after centrifugation. These crystals displayed greatest light scattering at 204 nm as measured using a Hewlett Packard diode array spectrophotometer. Decrease in absorption of light at 204 nm indicated a decrease in microfine crystals of cellulose. For microscopic examination, a spot of each ethanol sample (0.5 µl) was placed on a stud, air dried, and examined using a scanning electron microscope.

Results and Discussion

Isolation of bacteria. Dissection of *Zootermopsis angusticollis* was accomplished under a dissecting microscope as previously described. One member of each pool of termites was checked for external contamination. Because no external contamination was found, all isolates were considered to originate in the stomachs of the dissected termites. Twenty-four different strains of bacteria determined by colony type were isolated. Fungi were discarded. Individual colonies were isolated on TSB plates and incubated overnight (30°C) to ensure that each species was monotypical. Next, the isolated species were characterized by a series of standard bacterial tests (Table 1), and screened for cellulolytic enzymes using CMC liquification assays (Thayer, 1976, 1978; Wood & Kellogg, 1988a) and agar plate growth utilizing CMC and crystalline cellulose as the sole carbon source (Thayer, 1976, 1978; Wood & Kellogg, 1988a).

Cellulolytic screening.

Identification of candidate organism genus. Initial cellulolytic screenings were conducted using plate growth on various crystalline cellulose and CMC sources. Of the 24 bacterial species isolated, only 3 displayed cellulolytic activity. Three isolates (HW25, HWM and HG5) which showed consistent cellulolytic ability were used in further studies. These isolates were further characterized by various

Table 1. Bacterial Characterizations of Gut Isolates[a]

	Gram	Catalase	Oxidase	Motility	Action	Cell shape	Colony morphology	Surface texture	Color	Length (μM)	Width (μM)	Cell Grouping
CW1	-	+	-	+	LT	R	R	S	G	2	0.5	SD
CW3	-	+	+	+	S	R	R	S	CB	1.5	0.5	S
CW9	+	+	+	+	TS	R	R	S	CB	2	0.5	MCS
CW12	+	+	-	+	TS	R	R	FR	C	2	0.75	SLC
CW14	-	+	+	+	FS	R	R	S	CB	1.5	0.5	S
CW16	+	+	-	-		R	SG	S	OR	0.5	0.5	CL
HW1	-	+	-	-		C	CC	S	CB	0.5	0.5	CL
HW3	-	-	+	+	FS	R	SG	S	C	1	0.5	S
HW7	+	+	+	-		R	R	S	C	2	0.5	SSC
HW9	+	+	-	+	LT	C	R	S	G	1	1	S
HW11	-	+	+	+	S	R	R	S	C	1	0.5	S
HW13	+	+	+	+	S	R	R	MR	C	1.5	0.5	MC
HW15	-	+	+	+	FS	R	R	S	C	1	0.5	SD
HW18	+	+	-	+	LT	C	R	S	G	0.5	0.5	SC
HW20	+	+	+	+	S	R	R	S	CB	3	0.75	SSC
HW22	+	+	-	+	LT	R	R	FR	C	2	0.75	SLC
HW25	+	+	-	+	S	R	R	MR	C	2.5	0.75	SSC
HWM	+	+	-	+	S	R	R	FR	WG	2	0.5	SSC
HG1	-	+	+	+	FS	R	R	S	CB	1	0.5	DS
HG3	-	-	+	+	LT	R	R	S	OB	2	0.5	S
HG5	+	+	-	+	LT	R	R	MR	CB	2.5	0.75	SD
HG12	-	+	+	+	LT	R	R	S	CB	1.5	0.5	SSC
HG14	-	-	+	+	LT	R	R	S	G	0.5	0.25	S
HG16	+	+	-	+	LT	C	R	S	W	0.5	0.5	SCL

Abbreviations:

Action	LT	Local twisting
	S	Swimming
	TS	Twisting swimming
	FS	Fast swimming
Cell shape	R	Rods
	C	Cocci
Colony morphology	R	Round
	SG	Stab growth
	CC	Concentric circles
Surface texture	S	Slick
	FR	Fine ripples
	MR	Medium ripples
+ Positive, - Negative		

Color	G	Golden
	CB	Creamy beige
	C	Cream
	WG	White gray
	OR	Orange red
	OB	Orange beige
	W	White
Grouping	S	Singles
Order indicates	SD	Singles & doubles
most prevalent	SSC	Singles & short chains(2-4)
grouping	SLC	Singles & long chains(2-10)
	SC	Short chains(2-4)
	SCL	Singles & clusters(4-10)
	DS	Doubles & singles
	CL	Clusters
	MCS	Medium chains(2-6) & singles
	MC	Medium chains(2-6)

[a]A brief description of each test listed in Tables 1, 2 and 3 can be found in Appendix B.

bacteriological tests (Tables 2 and 3). Tests showed that all 3 isolates were gram-positive facultative anaerobic rods capable of growth on a wide variety of carbon sources and under varying pH and temperature conditions. HW25, HWM and HG5 all grew best under slightly acidic conditions, pH between 7.0 and 6.0, and at 42°C. However, all experiments conducted were run at 30°C and pH 7.0 to enable comparisons between these experiments and those of Thayer (1976, 1978). Resistance was low to a variety of antibiotics except for ampicillin, which could be the result of low membrane permeability to the antibiotic. All isolates showed endospore formation after treatment in 80°C water for 30 minutes. Spore staining was used to determine the location and type of spore formation.

Table 2. Physical Characterizations of HW25, HWM and HG5

	HW25	HWM	HG5
Gram	+	+	+
Catalase	+	+	+
Oxidase	-	-	-
Motility	+	+	+
Cell shape	Rods	Rods	Rods
Colony color	Cream	White gray	Creamy beige
Length (μm)	2.5	2.0	2.5
Width (μm)	0.75	0.50	0.75
Spore tests	+	+	+

+ Positive, - Negative

Table 3. Growth Response of HW25, HWM and HG5 to Varied Conditions

	HW25	HWM	HG5
Carbon source			
Glucose	+	+	+
Lactose	+	+	+
Maltose	+	+	+
Glycerol	+	+	+
CMC	+	+	-
Fructose	+	+	+
Mannitol	+	+	+
Sucrose	+	+	+
Na Acetate	-	+	-
Galactose	+	+	+
MacConkey	-	-	-
EMB	-	-	+
Purple Broth	+	+	+
Mannitol salt	+	+	+
β hemolysis	+	+	-
TSI Slant	A	K	K
Butt	A	A	A
Gas	-	-	-
Hydrogen Sulfide	-	-	-
Anaerobic growth	+	+	+
Antibiotic resistance			
Ampicillin	+	+	+
Streptomycin	-	-	+
Chloramphenicol	-	-	+
Tetracycline	-	-	-
TSB plates			
pH conditions			
pH5.0	+	+	-
pH6.0	+	+	+
pH7.0	+	+	+
pH8.0	+	+	+
Temperature			
30°C	+	+	+
37°C	+	+	+
42°C	+	+	+

+ Growth, - No Growth, A Acid, K Alkaline

These test results suggest the isolates are in the genus *Bacillus*. According to Balows *et al*. (1992) *Bacillus* bacteria degrade cellulose or modified cellulose. HW25, HWM and HG5 fail to meet criteria for all genera of cellulose degraders listed in *The Prokaryotes* (Balows *et al*., 1992), except *Bacillus*. In addition, Breznak and Pankratz (1982) and Thayer (1976, 1978) report the presence of *Bacillus* bacteria in the gut of lower termites. Therefore the assumption was made that HW25, HWM and HG5 are in the *Bacillus* genus. No attempt was made to characterize these bacteria to species level.

Growth rates. As shown in Figure 4, *B. subtilis*, *E. coli*, HW25 and HWM all grow at approximately the same rate in liquid media at the stated conditions. HG5 lags slightly behind the other four bacterial strains but eventually grows to near the maximum density of approximately 10^{10} cells per ml. Plate counts of cells were hampered by the tendency of HW25, HWM and HG5 to chain in lengths of 2 to 5 cells. Cell counts were conducted using a hemacytometer once cultures reached stationary phase.

Quantitative assays. After initial screens for cellulolytic enzymes were successfully completed, more quantitative tests were developed using CMC and crystalline cellulose. All tests were carried out over several weeks using inocula from fresh overnight cultures grown on TSB media at 30°C. These test procedures were used to minimize differences in inoculum volumes and

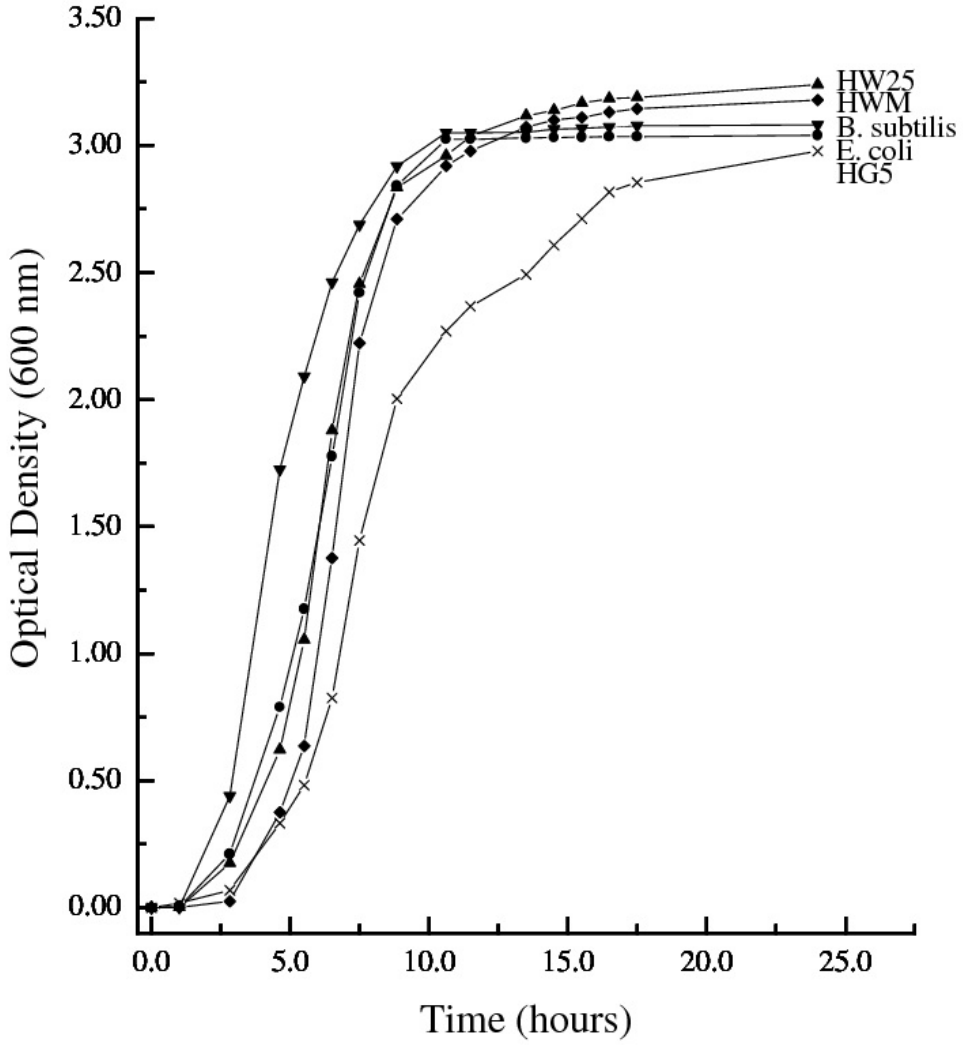

Figure 4. Growth curves for TSB cultures in 5 ml Corex centrifuge tubes in a shaking water bath at 30°C. ● E. coli, ▾ B. subtilis, ▴ HW25, ♦ HWM, × HG5

culture startup conditions. Most tests were conducted in triplicate, or were performed several times over a period of 2 years. Results note when multiple sampling was used.

Carboxymethylcellulose assays. CMCCA media (50 ml) were inoculated with fresh overnight culture (50 µl) and incubated at 30°C in a shaking water bath. Samples (6 ml) were taken from triplicate flasks, subject to centrifugation, and then viscosity of the supernatant was measured using a modified Thomas viscometer. Concentration of reducing ends in the supernatant was determined by 3,5-dinitrosalicylic acid assays.

Reduction in media viscosity was measured as described in materials and methods. Viscometer readings (in seconds) were converted to percent change to facilitate comparisons with other experiments (Thayer, 1976, 1978). Percent change in viscosity was calculated from the average value of all the measurements for the uninoculated CMCCA over the length of the experiment period versus an average value for water. These average values were 11.91 seconds ± 0.074 seconds ($P = 0.05$) for the uninoculated CMCCA and 9.93 seconds ± 0.047 seconds ($P = 0.05$) for water. The average difference between the uninoculated CMCCA and water was 1.98 seconds.

The bacteria (*B. subtilis*, HW25, HWM and HG5) displayed a decrease in viscosity of the liquid medium and an increase in reducing ends over time (Figure 5 and 6) that is consistent with digestion of CMC chain in a random manner. Digestion of CMC from the ends of the cellulose chain would not result in an observable increase in reducing ends and would slowly reduce viscosity. Random chain breakage would increase reducing ends and show rapid viscosity reduction (Thayer, 1976, 1978; Wood & Kellogg, 1988a). The *E. coli* culture displayed

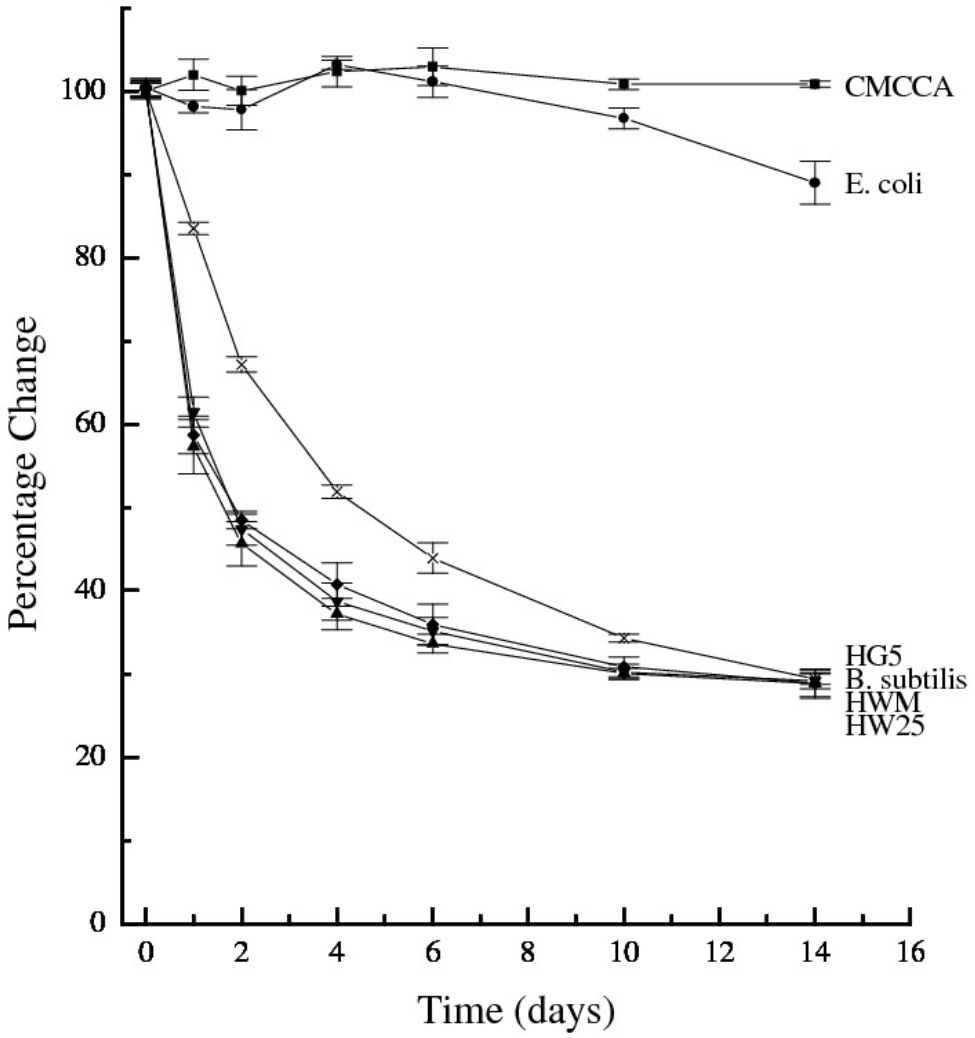

Figure 5. Change in viscosity in liquid culture over time. Liquid media (50 ml) containing 2.5% CMC and 0.25% casamino acids in M9 salts was inoculated with fresh overnight culture (50 µl) and grown at 30°C in a shaking water bath. Samples were taken from triplicate flasks, subjected to centrifugation at 4,100g for 15 minutes and viscosity of the supernatant was tested using a Thomas viscometer. ■ CMCCA, ● E. coli, ▾ B. subtilis, ▴ HW25, ♦ HWM, × HG5; bars represent ± one standard deviation.

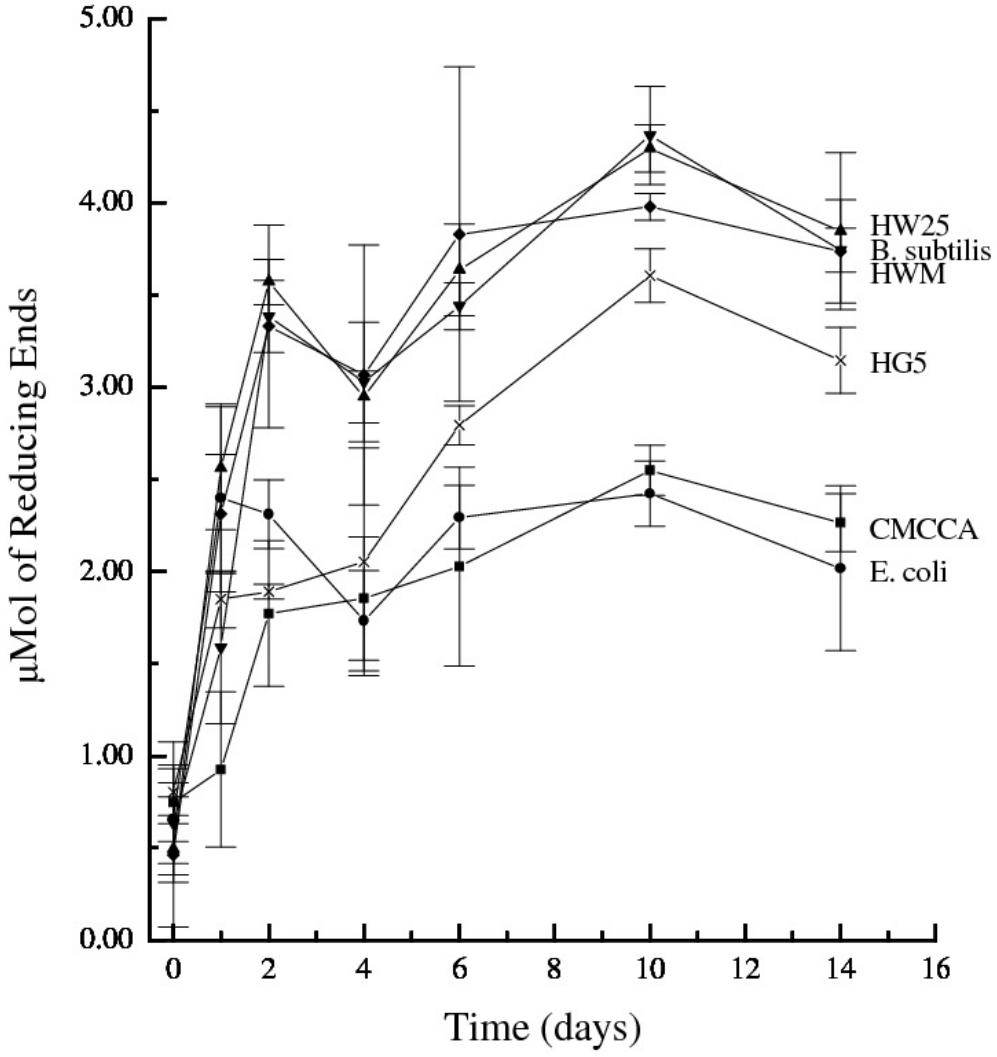

Figure 6. Change in reducing ends in liquid culture over time. Liquid media (50 ml) containing 2.5% CMC and 0.25% casamino acids in M9 salts was inoculated with fresh overnight culture (50 µl) and grown at 30°C in a shaking water bath. Samples (100 µl) were taken from triplicate flasks, subjected to centrifugation at 4,100g for 15 minutes and the supernatant was tested for reducing ends using 3,5-dinitrosalicylic acid assays. ■ CMCCA, ● E. coli, ▾ B. subtilis, ▴ HW25, ♦ HWM, × HG5; bars represent ± one standard deviation.

slightly lower viscosity and no significant change in reducing ends over the length of the experiment. This decrease in viscosity could result from slow digestion of the terminal glucose ends by β-glucosidase or some other undetermined enzyme system.

There were slight variations in values for the triplicate samples (Figure 5 and 6). Uninoculated CMCCA media viscosity was measured to determine the consistency of the viscometer readings, based on variations in sample volume, dilution factors and temperature readings. All viscosity measurements of identical samples varied less than 0.1% from the mean. Measurements were done in triplicate at each test point. Sample volumes were measured at 70, 75, 80 and 85 ml. One tenth of one percent variation in time measurements for sample volumes centered at 80 ml fell between 78.5 ml and over 90 ml. Dilution factors were measured at 1, 2, 3, 4 and 5 ml. One tenth of one percent variation in time measurements for dilution factors centered at 4 ml fell between 3.98 ml and 4.02 ml. Temperatures were measured at 19.0, 19.5, 20.0, 20.5 and 21.0 °C. One tenth of one percent variation in time measurements for temperature centered at 20.0°C fell between 19.9°C and 20.1°C. Because sample volume, dilution factor and temperature changes were held to under 0.1% and the modified viscometer gave repeatable measurements within 0.1%, variation of readings was due to conditions within the growing culture and not due to fluctuations in instrument readouts. Indeed, inocula induced changes in viscosity and reducing ends were consistent for all tests run over a period of 2 years.

B. subtilis, HW25 and HWM demonstrated similar results when grown in CMCCA. HG5 increased reducing ends and lowered viscosity more slowly than the other isolates. However after 14 days incubation viscosities of the supernatant were similar and reducing ends in HG5 samples were only slightly lower than the other isolates. This delay could be a slower growth rate or lower efficiency in cellulase activity. There were approximately 30% fewer cells in the HG5 cultures than in the other cultures, although consistent values were not obtained due to the viscous nature of the media and the chaining of the cells. In addition HG5 grew more slowly on the nutrient-rich nonselective media TSB (Figure 4). If HG5 cultures were indeed 30% less concentrated than those of other bacterial cultures, then the rate of hydrolysis of the CMC was approximately the same by HG5 as by *B. subtilis*, HW25 and HWM.

Crystalline cellulose assays. Cellulolytic activity was measured in liquid media containing either cellulose azure, a dyed crystalline cellulose, or undyed crystalline cellulose. Release of the dye indicated cellulolytic activity in the cellulose azure. Loss of microfine crystals in the undyed cellulose test was assumed to be due to cellulolytic activity (Weimer, Lopez-Guisa & French, 1990). Dyed cellulose assays are standard tests for measuring crystalline cellulose digestion. The undyed cellulose assay was developed in this report to eliminate the possibility that the dye in the dyed cellulose assay inhibited the digestion of crystalline cellulose.

Dye release from cellulose azure was measured by an increase in absorbance (568 nm) in the supernatant indicating cellulolytic activity (Wood and Kellogg, 1988a). Figure 7 shows the result of a 14 day experiment that was run concurrently with the CMC assays in Figures 5 and 6. HW25 cultures produced a marked release of dye into the supernatant. HWM cultures produced a small, but significant release of dye by day 14. *B. subtilis* and HG5 cultures showed slightly higher levels over uninoculated DCCA and *E. coli* samples. These results indicated that HW25 and HWM have complete cellulolytic enzyme systems, as defined as the ability to digest crystalline cellulose. *B. subtilis* and HG5 have a limited impact on the crystals of cellulose. Literature citations support this limited cellulase activity in *B. subtilis* (Balows *et al*., 1992; Chan & Au, 1987; Wood & Kellogg, 1988a).

Release of dye from the cellulose could be muted if the cells were able to digest the dye molecule. Bacterial growth experiments on dyes used in staining the crystalline cellulose were conducted on agar plates without additional nutrients and showed that none of the bacteria were able to significantly digest the dye. These controls also indicated that none of the test species had significant ligninolytic abilities. Dyes used to color cellulose closely resemble the lignin precursor molecule and are used in assays for ligninases (Wood & Kellogg, 1988b).

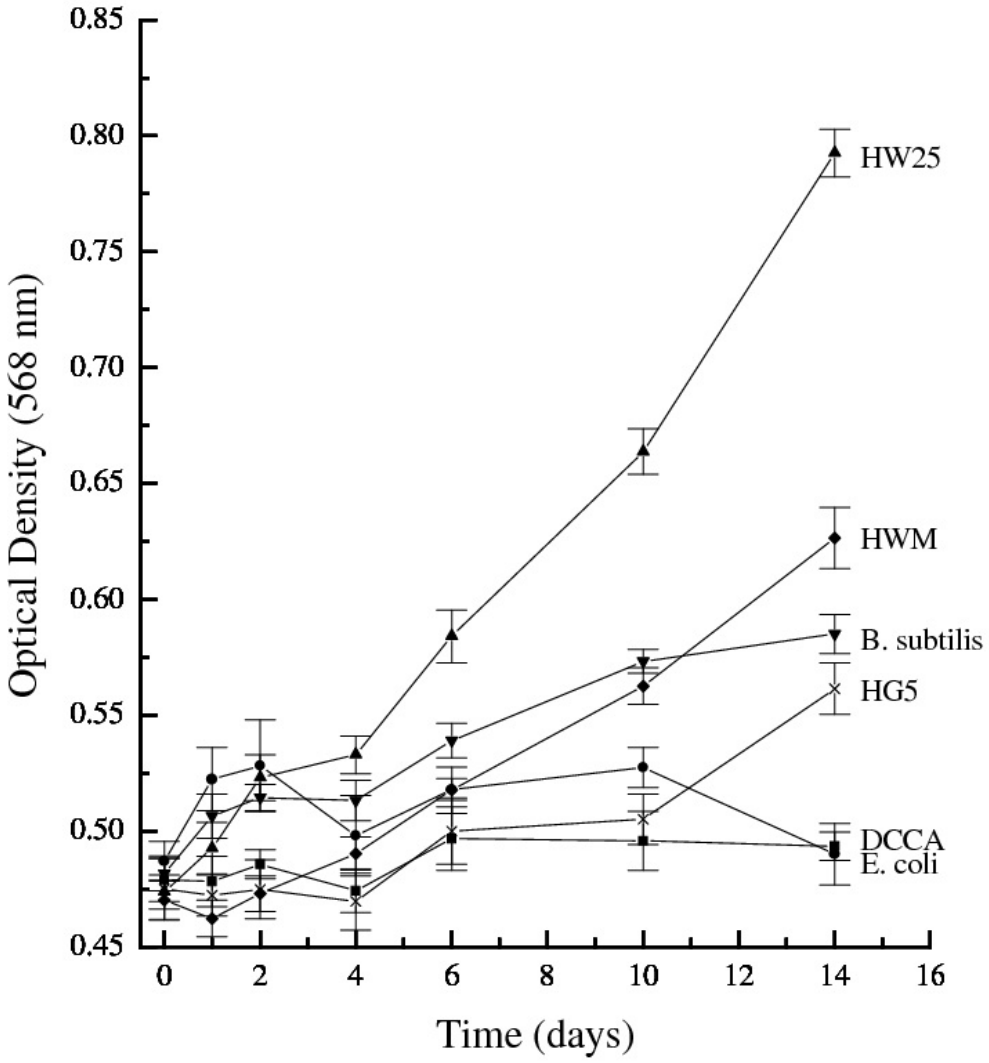

Figure 7. Digestion of crystalline cellulose as measured by release of dye. Triplicate samples of liquid media (5 ml) containing 1% cellulose azure and 0.25% casamino acids in M9 media were inoculated with fresh overnight culture (5 µl) and grown at 30°C in a shaking water bath. Samples were diluted two-fold, subjected to centrifugation at 14,000g for 10 minutes and absorbance was measured using a diode array at 568 nm. Results are given for undiluted samples of 1 ml. ■ DCCA, ● E. coli, ▾ B. subtilis, ▴ HW25, ♦ HWM, × HG5; bars represent ± one standard deviation.

Another crystalline cellulose assay utilized measurements of microfine undyed cellulose crystal loss. This loss of fibers was measured as described in materials and methods. These assays were conducted in addition to the dyed cellulose assays to eliminate the possibility that crystalline cellulose digestion was inhibited by the dye molecules. Microfine crystals of cellulose, less than 2 μm in length, remained suspended in the ethanol even after centrifugation. These tests were performed on cultures grown in UCCA at t = 14 days. When compared to the uninoculated UCCA HWM cultures showed a 25% decrease in absorption, and *E. coli*, *B. subtilis*, HW25 and HG5 showed a 10% decrease (results not shown). A spot of each sample (0.5 μl) was examined using a scanning electron microscope. The HWM spot was almost completely devoid of crystals. The uninoculated UCCA spot contained several dozen crystals approximately 1-2 μm in length. *E. coli*, *B. subtilis*, HW25 and HG5 spots had slightly lower numbers of crystals than the uninoculated UCCA but all had at least one to two dozen crystals.

Summary of results.

Both crystal cellulose and CMC assays indicated that *B. subtilis*, HW25, HWM and HG5 contained enzyme systems capable of degrading cellulose. HW25 and HWM could digest both crystalline cellulose and CMC. *B. subtilis* and HG5 could digest only CMC with any detectable efficiency. Tests designed to measure inhibition of by-products were performed, but were generally

unsuccessful due to the long duration of the experiments. Additions of glucose and cellobiose, by-products from cellulose degradation, to growing samples caused some inhibition in cellulose degradation initially (results not shown). But inhibition of cellulose degradation disappeared after a day and maintaining constant levels of inhibitors was difficult. Once a reasonable level of 0.25% for casamino acids was established early in the investigation of these isolates no further tests on supplemental nutrient optimization were conducted. Levels of nutrient additives were considered optimal if cell density reached 10^8 cells/ml within 24 hours, but cellulose degradation was not inhibited beyond the first day.

The undyed crystalline cellulose assay, the dyed cellulose assay, and the CMC assay displayed slightly different results from the isolates. All three isolates digested CMC, reducing viscosity and increasing reducing ends in the media. The dyed cellulose assay showed that HW25 contains an enzyme capable of releasing dye molecules from the crystalline cellulose. Finally, the undyed cellulose test demonstrated that HWM removed microfine crystals of cellulose from solution. The undyed cellulose assay was unique to this study. Although Weimer, Lopez-Guisa and French (1990) claimed microfine crystal loss was the result of digestion, they failed to develop an assay. The undyed cellulose assay provides a good alternative to dyed cellulose tests, where the presence of dye might inhibit digestion of the crystalline cellulose. HWM displayed cellulase activity on the undyed assay, but only marginal activity on the dyed assay. HW25 displayed significant dye release, but the culture showed only minimal levels of microfine

crystal digestion. This undyed cellulose assay demonstrated different results than the standard dyed assay used by many investigators.

Conclusions

Isolates. Of the 24 different strains isolated from the gut of *Zootermopsis angusticollis*, only three showed consistent cellulolytic activity. These isolates were selected for further study. *B. subtilis* and *E. coli* were selected to serve as positive and negative controls. Tests on HW25 and HWM indicated they contained both crystalline cellulose and CMC digesting enzyme systems. HG5 showed only CMCase activity. All three isolates grew under a variety of moderate conditions that lend themselves to many industrial processes.

Among the three isolates, HW25 and HWM displayed complete cellulase systems capable of digesting crystalline cellulose. In particular, HW25 released significant amounts of dye from the crystalline cellulose after 6 days of growth. None of the other isolates showed significantly elevated dye levels in the supernatant until day 10 and they never released as much dye as HW25, even after 14 days of incubation. HW25 grows as rapidly as *B. subtilis* and to a slightly higher titer. It grew under most conditions tested. This adaptability, cellulase production, and rapid growth, make HW25 the best candidate for further studies.

Assays. A number of reliable assays for measuring cellulose degradation exist, especially those of in Wood and Kellogg (1988a). The CMC assay used in these experiments was essentially that of Thayer (1978) and Wood and Kellogg (1988a), with slight modifications necessitated by the available equipment. The

major modification was the addition of the electronic trigger mechanism on the mechanical viscometer. This modification allowed reproducible measurements on samples over long periods of time in the experiments. The assay using dyed crystalline cellulose was adapted from that of Wood and Kellogg (1988a) to enable comparisons with other assays of cellulolytic activity. Adaptation of the assay consisted of changing the medium to match those used in CMC and undyed cellulose assays. The undyed cellulose assay was developed specifically for this project. Development of this assay stemmed from the observation that absorption in the UV range decreased when samples incubated for 14 days. This change in absorption was due to the loss of microfine crystals of cellulose. Loss of crystals was confirmed by electron microscopy. This undyed cellulose assay provided another crystalline cellulose test that showed slight differences between HWM and HW25, both of which contained cellulases capable of digesting crystalline cellulose. All three assays, CMC, dyed cellulose and undyed cellulose gave slightly different information, each unique to the specific nature of the assay. CMC assays were simple and rapid, useful to screening unknown isolates for CMCase activity. The dyed and undyed crystalline cellulose assays were useful in determining cellulase activity capable of digesting crystalline cellulose.

Dyed cellulose assays are used by many investigators to measure cellulolytic activity. The assay is inexpensive, easy to perform with a minimum of standard laboratory equipment and yields measurable results. However, the dye molecule used in the prepared cellulose may inhibit enzymatic digestion of the

crystals through competitive inhibition or lowered binding efficiency. The undyed cellulose assay developed in this investigation has many of the same advantages as the dyed assays. Low cost and ease of performance are the most appealing aspects of this assay. The undyed cellulose has the additional advantage of eliminating the potential inhibiting effects of the dye molecule. The disadvantages of this assay are the need for a scanning electron microscope to confirm that microfine crystals were digested and the difficulty in quantifying the results. The signal decrease in the assay was significant, but difficult to measure without additional treatments that increased the potential for false readings.

Comparisons to other cellulase producers. Since all the assays involved cell cultures, comparison with purified cellulolytic proteins were hampered. *B. subtilis* was chosen as a positive control because it possesses strong CMCase activity and shows slight crystalline cellulase activity. All three isolates showed similar CMCase activity to that of *B. subtilis*. HW25 and HWM surpassed the activity of *B. subtilis* in digesting crystalline cellulose. Commercially available enzymes were used to show that the assays were working; however rapid digestion of cellulose by purified enzymes was difficult to compare to the slow process in the growing cell cultures in which by-products of cellulose digestion were metabolized.

Another bacterial cellulase producer, *Bacillus stearothermophilus* (*B. stearothermophilus*) shows great promise in industrial application, because of its

rapid growth rate, wide range of temperature tolerance, and production of ethanol from lignocellulosic materials (Hartley, 1987). Isolates examined in this project should be tested for useful fermentation products and temperature tolerance. HW25 and HWM already show an ability to digest cellulose and grow rapidly on simple media. Mutation and selection of HW25 and HWM might produce a strain that produces ethanol from sugars released in the metabolism of cellulose. Combined with a high temperature tolerance and rapid growth, these strains could provide a viable alternative to *B. stearothermophilus* with yet unknown attractive properties such as higher efficiency in cellulose digestion or better batch growth response.

Additional tests on the isolates should measure their ability to fix nitrogen, metabolize unusual carbon sources, or grow at extreme conditions of temperature and pH. These tests could reveal important abilities helpful in industrial processes to provide a low cost, but valuable conversion of raw materials into refined goods.

Industrial value. Although almost all commercially available cellulases used in industry are purified from fungal sources, new and novel uses for cellulases are stimulating the search for enzymes capable of specific activity under special conditions. Purified enzymes are preferred over cell cultures in industrial settings because of the ease of controlling the reaction in a purified enzyme system (Brooks, 1994). If purified enzymes were available from the isolates described in

this report, extensive testing for optimal and extreme conditions would be warranted.

Protein sequencing of the purified enzymes would allow a better understanding of the protein's function, structural homologies to other proteins, and potentially, any inherent positive or negative traits. Using current biotechnology skills, genes could be mutated to produce proteins suited for specific applications. One or several of the proteins in these isolates could provide a novel solution to binding specificity or catalysis of cellulose, or other carbon sources. These isolates contain genes that code for cellulases. Transferring the cellulase genes into other hosts could increase the cellulase activity of a current cellulase producer through synergism. Alternatively gene transfer could add cellulase ability to a host that ferments glucose into a variety of useful products.

These isolates exhibited CMCase and crystalline cellulases capable of digesting cellulose under simple conditions. Although the cellulase systems were not tested for unusual activities such as temperature or pH tolerance, they may serve a specific function in the ever-changing landscape of biotechnology. Additional studies of interest would include testing a broader range of bacteria found in *Zootermopsis angusticollis*, and the isolation and purification of the enzyme systems found. With a better understanding of the capabilities of the isolated bacteria, industrial applications could be developed that are suitable for the isolates. Purification of enzymes from the isolates would allow more

meaningful comparisons with currently available cellulases. This understanding of the enzyme systems in the isolates would indicate where each of the enzymes would best serve industry as a digester of cellulose.

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Appendix A

List of Abbreviations and Acronyms

Abbreviated Term	Full Term
B. stearothermophilus	*Bacillus stearothermophilus*
B. subtilis	*Bacillus subtilis*
C_x-type	Noncrystalline cellulose
CMCase	Carboxymethylcellulase
CMC	Carboxymethylcellulose
CMCCA	M9 medium, 2.5% CMC and 0.25% casamino acids
DCCA	M9 medium, 0.5% cellulose azure and 0.25% casamino acids
EMB	Eosin Methylene blue
E. coli	*Escherichia coli*
M9	Minimal Salts Media
TSB	Tryptic Soy Broth
TSI	Triple Sugar Iron
UCCA	M9 medium, 1.0% undyed crystalline cellulose and 0.25% casamino acids

Appendix B

List of Microbiological Assays

Gram Stain:	Standard Gram stain utilizing log-phase cells. Gram stain tests for cell membrane structure (Balows *et al*., 1991, p. 1306).
Catalase:	Standard catalase assay utilizing cells grown overnight. Catalase assay tests for catalase enzyme presence (Balows *et al*., 1991, p. 1290).
Oxidase:	Standard oxidase assay utilizing cells grown for 3 days. Oxidase assay tests for oxidase enzyme presence (Balows *et al*., 1991, p. 1298).
Motility:	Hanging drop microscopic examination of log-phase cells. Hanging drop examination tests for motility in bacteria.
Cell Length & Width:	Determined by light microscopy of Gram stain cells using oil immersion magnification 1000X (Balows *et al*., 1991, p. 704).
Spore stain:	Standard spore stain (Wirtz-Conklin) utilizing heat-treated cells. Spore stain tests for spores within the cell (Balows *et al*., 1991, p. 1311).
Carbon source:	Agar and M9 salts, with appropriate carbon source at 1% by mass added, streaked with cells grown overnight. (Claus, 1989, p. 195-198).
MacConkey:	MacConkey agar streaked with cells grown overnight. MacConkey is a selective medium that selects for lactose fermentation (Balows *et al*., 1991, p. 1208).
EMB:	Eosin methylene-blue agar streaked with cells grown overnight. EMB agar is a selective medium that usually inhibits growth of gram-positive bacteria (Claus, 1989, p. 480).
Purple broth:	Purple broth agar with 2% galactose added streaked with cells grown overnight. Purple broth with galactose is a selective medium that selects for galactose fermentation (Balows *et al*., 1991, p. 1270).
Mannitol salt:	Mannitol salt agar streaked with cells grown overnight. Mannitol salt agar is a selective medium that usually inhibits growth of most bacteria, except staphylococci (Balows *et al*., 1991, p. 217).

β hemolysis:	Sheep's blood agar streaked with cells grown overnight. Blood agar is a selective medium that tests for growth on red blood cells. β hemolysis is the complete lysis of the erythrocytes (Claus, 1989, p. 422).
TSI slant:	Triple-sugar-iron slants inoculated with cells grown overnight. TSI slants test for sugar fermentation, gas production and anaerobic growth. Acid production shows fermentation of sugar. Alkaline production shows no sugar fermentation. Bubbles display gas production . Black color shows H_2S production (Claus, 1989, p. 286-288).
Antibiotic resistance:	Tryptic soy broth agar streaked with cells grown overnight onto plates with antibiotic added. Levels of antibiotic: ampicillin (100 μg/ml), streptomycin (30 μg/ml), chloramphenicol (20 μg/ml), tetracycline (15 μg/ml) (Claus, 1989, p. 397-399).
pH conditions:	Tryptic soy broth agar streaked with cells grown overnight. Phosphate buffers were used to adjust the pH of the plates before pouring. Additional pH measurements were conducted on the agar plates after several days of incubation at 30°C. The pH levels remained stable in the TSB agar plates.
Temperature conditions:	Tryptic soy broth agar streaked with cells grown overnight. Agar plates incubated at stated temperatures.