Simultaneous biotic and abiotic degradation of lignocellulose under extreme alkaline conditions

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Introduction

Exposure of plant material to high pH hastens delignification, thereby freeing up structural carbohydrates for biofuel-yielding reactions. We hypothesized that natural alkaline springs would possess microorganisms capable of catabolizing lignocellulosic substrates, such as switchgrass, under the alkaline conditions that permit simultaneous chemical delignification. Therefore, we sought to characterize the biotic and abiotic transformations that occur within alkaline switchgrass enrichment cultures that had been inoculated with decaying plant material obtained from serpentine calcium hydroxide springs (pH ~11.9) and isolate cellulose-degrading microorganisms from these cultures.

Fig. 1. Sampling of decaying calcium carbonate-trapped plant material from within alkaline springs at *The Cedars*.

Materials and Methods

Enrichment cultures: Two switchgrass enrichment cultures were established, one termed 'CL' with inocula from *The Cedars,* Sonoma County, California (Fig. 1) and one with switchgrass only (termed 'NA'). The cultures were maintained by transferring 0.5 ml of previous culture to 0.125 g ground switchgrass in 25 ml of a synthetic CBM medium mimicking the composition of water at Barnes Springs in *The Cedars* (*Science,* 1967, **156**:830-2) [2.2 mM NaCl, 12.3 µM $MgCl_2$, 30.7 µM KOH, and 1.32 mM $Ca(OH)_2$ (final pH 11.5)] and incubated at 37°C with 50 rpm shaking. The medium was replaced once to twice weekly. Microorganisms were enumerated and isolated by serial dilution and culturing onto various alkaline media (Fig. 2).

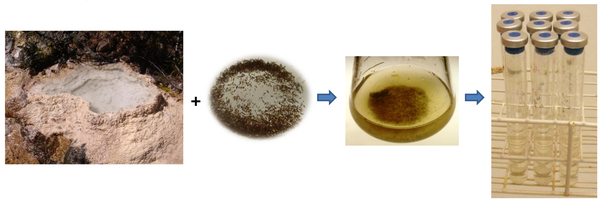

Fig. 2. Procedure for isolating facultative anaerobic strains FA1 and FA2. The 12th generation of the CL enrichment culture was serial diluted under anoxic conditions in pH 10.3 medium containing cellulose as the sole carbon source.

Analytical procedures: To monitor chemical and microbiological changes in the enrichment cultures we used a combination of light microscopy, synchrotron radiation-based Fourier Transform Infrared spectromicroscopy and scanning electron microscopy. Isolated microorganisms were subject to biochemical analysis and 16S rRNA gene sequencing.

Results

- Both the NA switchgrass-only control and *The Cedars*-inoculated CL switchgrass serial batch enrichment cultures degraded swithchgrass at a similar rate in an alkaline medium (Fig. 3A & B). Degradation of switchgrass in the CL culture was enhanced by supplementation with nitrate (Fig. 3A) but not phosphate (Fig. 3B). There is a progressive increase in phenolics (Fig. 3C) in the incubation medium and decrease in pH (Fig. 3D).
- Both the NA and CL cultures contain a diverse assemblage of eukaryotic and prokaryotic microbes (Fig. 4); the diversity of culturable carboxymethylcellulose degrading bacteria (Fig. 5) and fungi (not shown) was greater in the CL culture.
- Strain FA1, the only bacterium isolated from the CL enrichment culture under anoxic conditions (Fig. 2), identified as a *Cellulomonas* sp., most closely related to *C. flavigena* (Fig. 5, Table 1), exhibited extensive colonization of autoclave-sterilized switchgrass (Fig. 6).

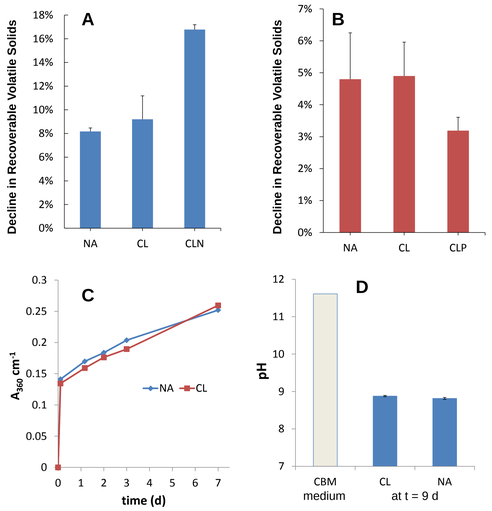

Fig. 3: Changes in NA and CL cultures during incubation in CBM medium. **A** and **B,** Decline in recoverable switchgrasss volatile solids after 9-day incubation of switchgrass inoculated with 15th generation NA culture, 15th generation CL culture and 15th generation CL culture with 5 mM KNO3 (CLN; **A**) or K_2HPO_4 (CLP; **B**) (means ± SE, n = 3). **C,** Release of lignin-derived phenolics into the media. **D,** Change in medium pH (means ± SE, n = 6). .

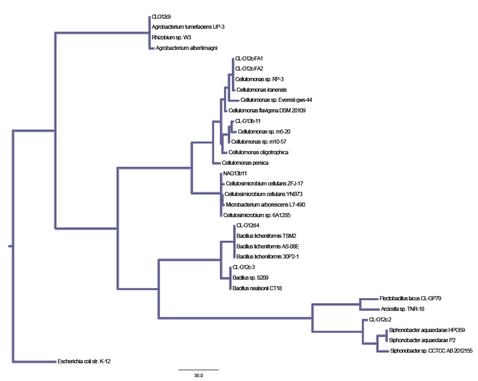

Fig. 5: Phylogenic tree of 16S rRNA gene sequences of CMC-degrading isolates from the NA and CL switchgrass enrichment cultures compared to known strains.

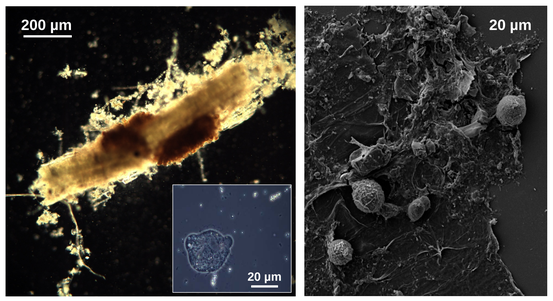

Fig. 4: Light (*left*) and scanning electron (*right*) micrographs of colonized switchgrass fragments in CL cultures. *Inset,* mineral fecal pellet formation by an unidentified ciliate.

Table 1. Characteristics of strain FA1 relative to type strains of *Cellulomonas flavigena.*

Test	Result	
	strain FA1	*C. flavigena*[§]
Gram stain	+*	+*
Thioglycolate medium	facultative anaerobic	facultative anaerobic
Catalase (aerobic-grown)	+	+[1]
Catalase (anaerobic-grown)	–	n.d.
Oxidase	–	v[1]
Urease	–	–[2]
Nitrate reduction	+	+[2]
β-glucosidase	+	+[1]
Amylase	+	+[1]
Cellulase	+	+[2]
Ethanol tolerance	5.5%	n.d.
Growth on[†]:		
D-Glucose	+	+[2]
Maltose	+	+[2]
Sucrose	+	+[2]
Mannitol	+	–[1],–[2]
Xylose	+	+[2]
Dextrin	+	+[2]
Ribose	+	+[2]
Gluconate	–	–[1], +[2]
N-acetyl-D-glucosamine	–	–[1]

*Readily destains.[3]
†Growth on carbon sources assessed with Phenotype MicroArrays (Biolog Inc., CA).
Abbreviations: n.d., not determined; v, variable.
§References:
1 Pourcher, A.-M. et al. *FEMS Microbiol. Ecol.* 34 (2001).
2 Stackebrandt, E., Schumann, P. & Prauser, H. *Prokaryotes* 3, 983-1001 (2006).
3 Stackebrandt, E. & Kandler, O. *Int. J. Syst. Bacteriol.* 29, 273-282 (1979).

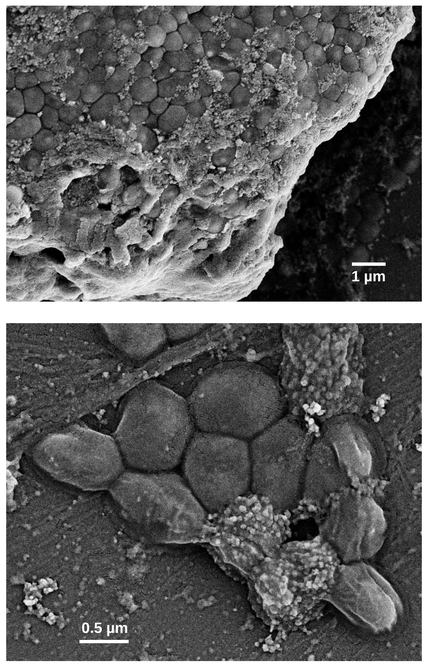

Fig. 6: Scanning electron micrographs of switchgrass colonized with *Cellulomonas* sp. strain FA1 in alkaline medium (pH 10.3).

Conclusions and Future Prospects

- At high pH abiotic lignin dissolution can occur simultaneous with biological cellulose degradation thereby potentially allowing for consolidation of chemical lime pretreatment with biological treatment processes.
- Strain FA1 possesses desirable characteristics for potentially exploiting its cellulose-degrading capacity including, facultative anaerobic growth, utilization of sugars found in hemicelluloses (e.g. mannose, arabiniose, galactose, xylose), and ethanol tolerance.
- We are currently cloning a cellulase from strain FA1 to investigate potential alkaline tolerance adaptations of the enzyme.
- We will be investigating the capacity of strain FA1 to ferment sugars to ethanol and potentially engineering the organism to preferentially express this fermentation pathway.

Acknowledgements

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