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# Novel thermophilic cellulolytic isolates belonging to the phylum Chloroflexi

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# Novel Thermophilic Cellulolytic Isolates Belonging to the Phylum *Chloroflexi*

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## Introduction

Current biofuel technologies utilize valuable foodstuffs, such as corn kernels and cane sugar, as sources of easily metabolized sugars. Microbes are used to ferment these sugars into bioethanol, a first-generation biofuel. However, in order to avoid diverting foodstuffs from the food supply, the development of second-generation biofuels technology is necessary. Second-generation biofuels are produced by converting structurally complex lignocellulosic biomass, such as agricultural and municipal wastes, to fermentable sugars or directly to biofuels (1).

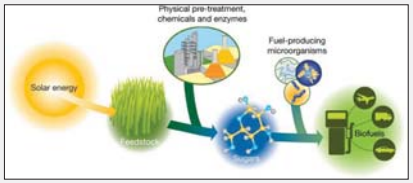



**Fig. 1.** Second generation biofuel processing. Solar energy is converted to lignocellulose by photosynthesis. Chemically and physically pre-treated plant material is de-polymerized into sugar monomers via microorganisms or cellulases. Simple sugars are then fermented into biofuels by microbes. (Illustration from Rubin, 2008).

The major technological hurdle limiting the mass production of second-generation biofuels is the difficulty in efficiently converting structurally complex lignocellulosic materials to fermentable sugars or directly to biofuels. The discovery of novel thermophilic microorganisms and enzymes that have high activities or broad substrate ranges on plant polymers addresses this challenge.

## Research Objectives

**Objective I:** To obtain pure cultures of thermophilic microbes capable of cellulose degradation

**Objective II:** To characterize the morphology and physiology of the isolates

**Objective III:** To identify isolates through DNA sequencing of the 16S rRNA gene and phylogenetic analysis

## Experimental Design

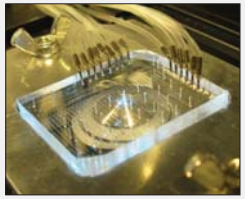
**Objective I:**

Corn stover and aspen shavings were incubated in Great Boiling Spring in Gerlach, Nevada (Fig. 2) and used as inocula for 72 enrichments designed to support the growth of strongly cellulolytic microorganisms. The enrichments were tubes containing Filter Paper Medium I (Castenholz Medium D with filter paper as the sole organic carbon source and additional vitamins and nitrogen).

**Fig. 2.** Photograph of Great Boiling Spring.

One enrichment generated a biofilm capable of degrading insoluble cellulose, and this consortium was found to contain a mixture of rods and filaments. Rods belonging to the genera *Geobacillus* and *Rhodothermus* were isolated by plating and found to have weak cellulolytic activity on insoluble cellulose. It was hypothesized that the filaments were responsible for the strong cellulolytic activity displayed by the community, but the filaments were not observed to grow on plates. Hence, optical tweezers and a microfluidic cell sorting device (Fig. 3) were employed to physically isolate the filaments from the community. 33 single filaments, six small groups of filaments, and one negative control were sorted into Filter Paper Medium I and incubated at 55 C.

**Fig. 3.** Photograph of a microfluidic cell sorting device.



**Objective II:**

Isolates were tested for growth on 12 different media known to support growth of related organisms (2-4). The media were prepared with different combinations of organic carbon sources, redox environments, pH values, and use of agitation to give a total of 28 different conditions. Gram staining was employed to characterize the cell wall of the isolates.

**Objective III:**

The DNA of two pure cultures, JKG1 and JKG2, was isolated, the 16S rRNA genes was amplified and the PCR products were sent for DNA sequencing. The resulting near full-length 16S rRNA genes sequences were submitted to NCBI BLAST to find and compare regions of local similarity to other microorganisms. These sequences were aligned in mothur, and then imported into ARB, where neighbor-joining and maximum likelihood trees were produced.

## Results and Discussions

**Objective I:**

Six cultures inoculated with single filaments grew to high cell density and efficiently degraded the filter paper at temperatures ranging from 45-65 C (Fig. 4).

**Fig. 4.** Photograph depicting the extent of filter paper degradation performed by isolates after 16 days of incubation in Filter Paper Medium I at 50 C.

**Objective II:**

All six isolates were unbranched multicellular filaments ranging from 20 to >600 µm in length and 0.7-0.8 µm in width (Fig. 5A,B). All isolates were capable of gliding motility but were not motile in liquid medium.

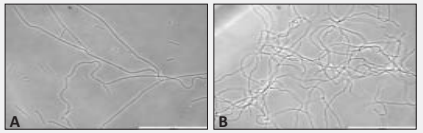



**Fig. 5.** Phase-contrast micrographs of the isolates in Filter Paper Medium I (A) and in R2A Medium (B).

Of the 28 different conditions tested, the best growth was observed in R2A Medium while shaking at 65 RPM. Filaments sometimes aggregated into spherical (Fig. 6A) or thread-like (Fig. 6B) structures and were observed to cling to glass surfaces. The isolates stained Gram-negative. In addition, some dense cultures were visibly pink or red and all cell pellets produced through centrifugation were pink or red.

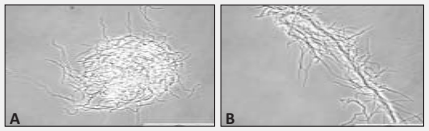



**Fig. 6** Phase-contrast micrographs displaying isolates aggregating into spherical (A) and thread-like (B) structures in R2A medium.

**Objective III:**

BLAST results showed that the isolates are members of the Phylum *Chloroflexi*, with 84% sequence identity similar to their nearest cultured relative, *Roseiflexus castenholzii*, indicating that the isolates are only distantly related to cultivated microorganisms. The evolutionary distance tree (Fig. 7) places the isolates within the Class *Chloroflexi*, branching between *Herpetosiphon aurantiacus* (non-photosynthetic) and *Roseiflexus castenholzii* (photosynthetic).

If photosynthetic, the isolates could be the most phylogenetically "ancient" phototrophs, while if not photosynthetic, the isolates could shed light on the evolution of photosynthesis.

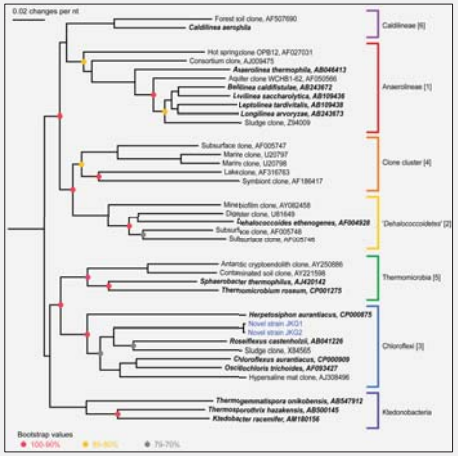



**Fig. 7.** Neighbor-joining tree of the Phylum *Chloroflexi*. 10,000 bootstrap replicates were performed and node support of at least 70% is displayed. Outgroups used were *Escherichia coli* (X80725), *Bacillus subtilis* (D26185), and *Corynebacterium diphtheriae* (X84248), after Hugenholtz et al (5).

## Future Directions

Detailed phylogenetic and phenotypic characterization is underway to determine whether the organisms represent a novel order or family within the *Chloroflexi*, further resolve the branching pattern of the phylogenetic tree, and to delineate isolate activities against specific biopolymers. Future enzymatic and genomic characterization will determine whether these organisms or their enzymes may be useful for increasing the efficiency of second-generation biofuels technology.

## Literature Cited

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## Acknowledgements

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