

ABSTRACT

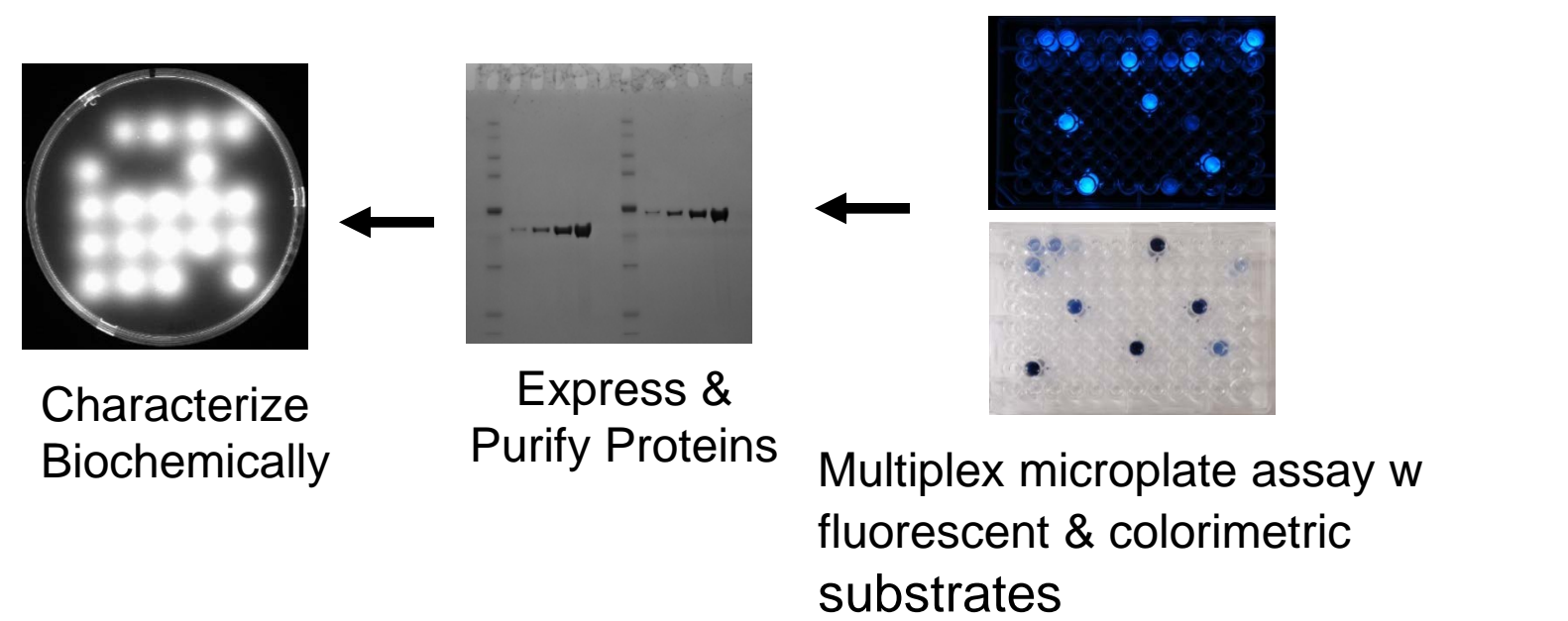
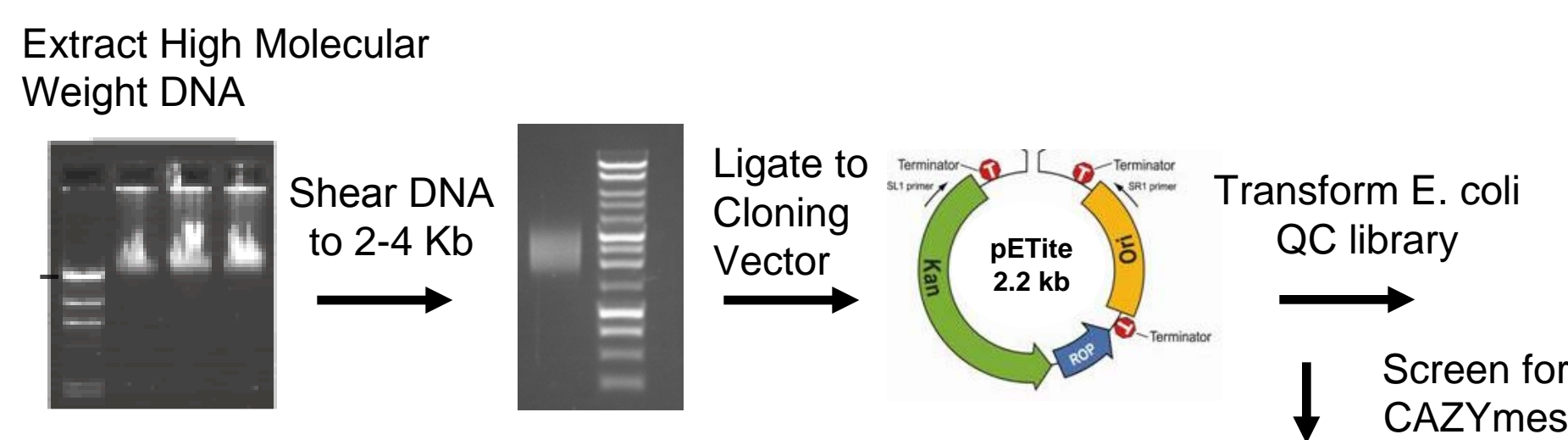
Much of what is known about the function of cellulolytic enzymes is based on work done using the cellulase system derived from *T. reesei*, a combination of cellobiohydrolases I and II, an endoglucanase, and a beta-glucosidase. Despite much effort, a similar bacterial set has not been developed. Furthermore, many of the bacterial cellulolytic enzymes cloned and expressed to date have significantly different pH and temperature optima, preventing their effective substitution for individual fungal enzymes in the set. Only a handful of thermophilic cellulolytic bacterial species have been described. For the past five years we have collaborated with the DOE Joint Genome Institute, Great Lakes Bioenergy Research Center and researchers at TBI and other universities to study the molecular diversity of microbes in thermal environments and to implement a genomics- and metagenomics-based enzyme discovery program focused on thermophilic microbes. In an attempt to generate a bacterial minimum cellulase set, we have cloned, expressed, and characterized full-length glycosyl hydrolase genes from *Acidothermus cellulolyticus*, *Dictyoglomus turgidum*, and a number of thermophilic bacillus. Results from experiments with individual enzymes and mixtures of the enzymes suggest that cellulose hydrolysis by bacterial cellulases proceeds via a different path than used by the *T. reesei* enzymes.

Table 1. Thermophilic microbial genomes sequenced via DOE Joint Genome Institute.

<i>Alicyclobacillus</i> sp.	<i>Hydrogenobaculum</i> strain Y04AAS1
<i>Dictyoglomus turgidum</i>	<i>Sulfolobus solfataricus</i> 98/2
<i>Geobacillus</i> sp. Y41MC1	<i>Sulfurihydrogenibium</i> sp. Y03AOP1
<i>Geobacillus</i> sp. Y41MC4	<i>Thermareobacter</i> sp. Y412MC57
<i>Geobacillus</i> sp. Y412MC10	<i>Thermus aquaticus</i> Y51MC23
<i>Geobacillus</i> sp. G11MC16	<i>Thermus brockianus</i>
<i>Geobacillus</i> sp. WCH70	<i>Obsidian Mixed Community</i>
<i>Geobacillus</i> sp. Y412MC61	<i>Bath Mixed Community</i>
<i>Geobacillus</i> sp. Y412MC52	

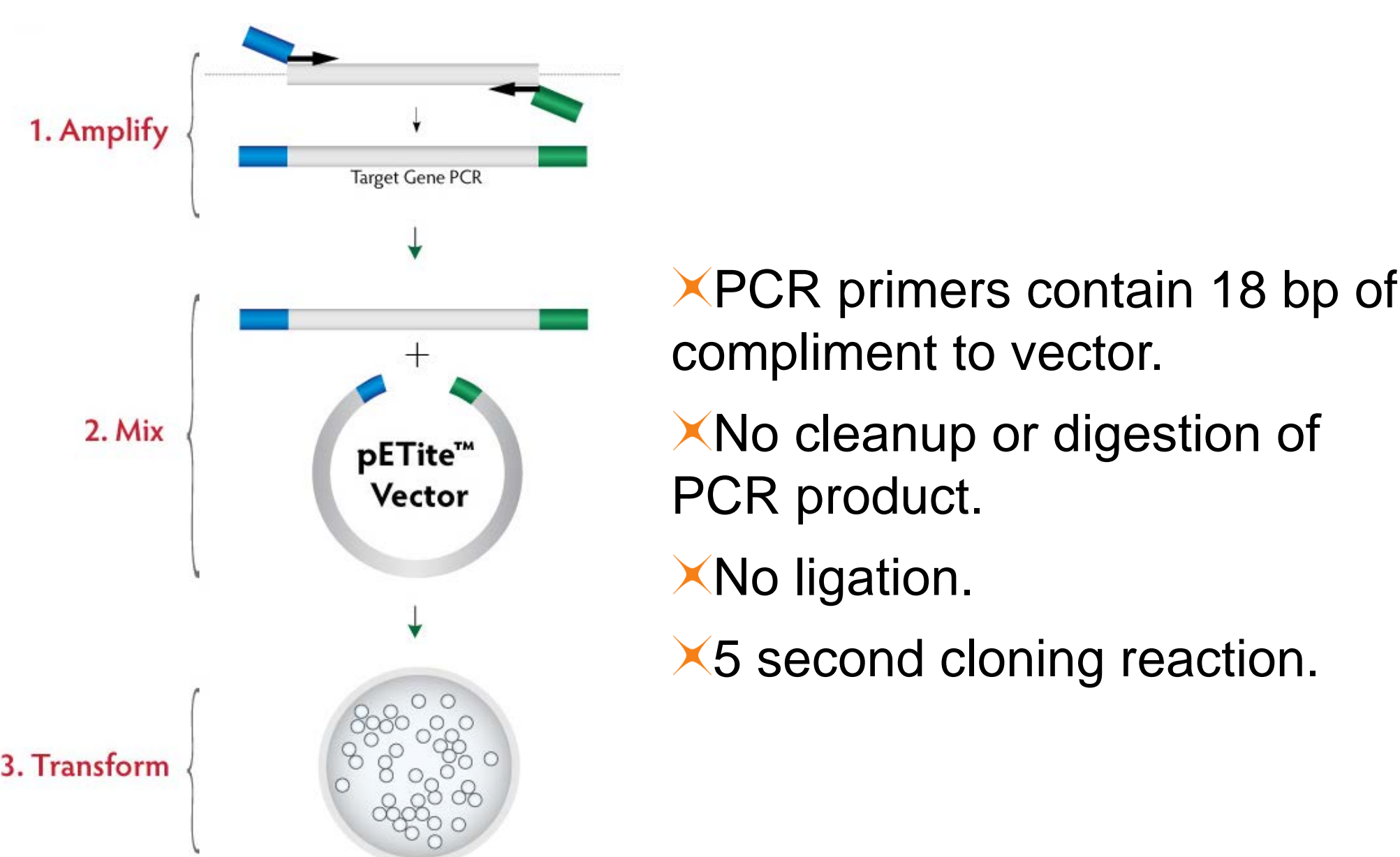
Figure 1. Random shotgun or PCR directed expression screening of thermostable cellulolytic enzymes.

Random Shotgun Expression Screen



PCR Directed Expression Screen

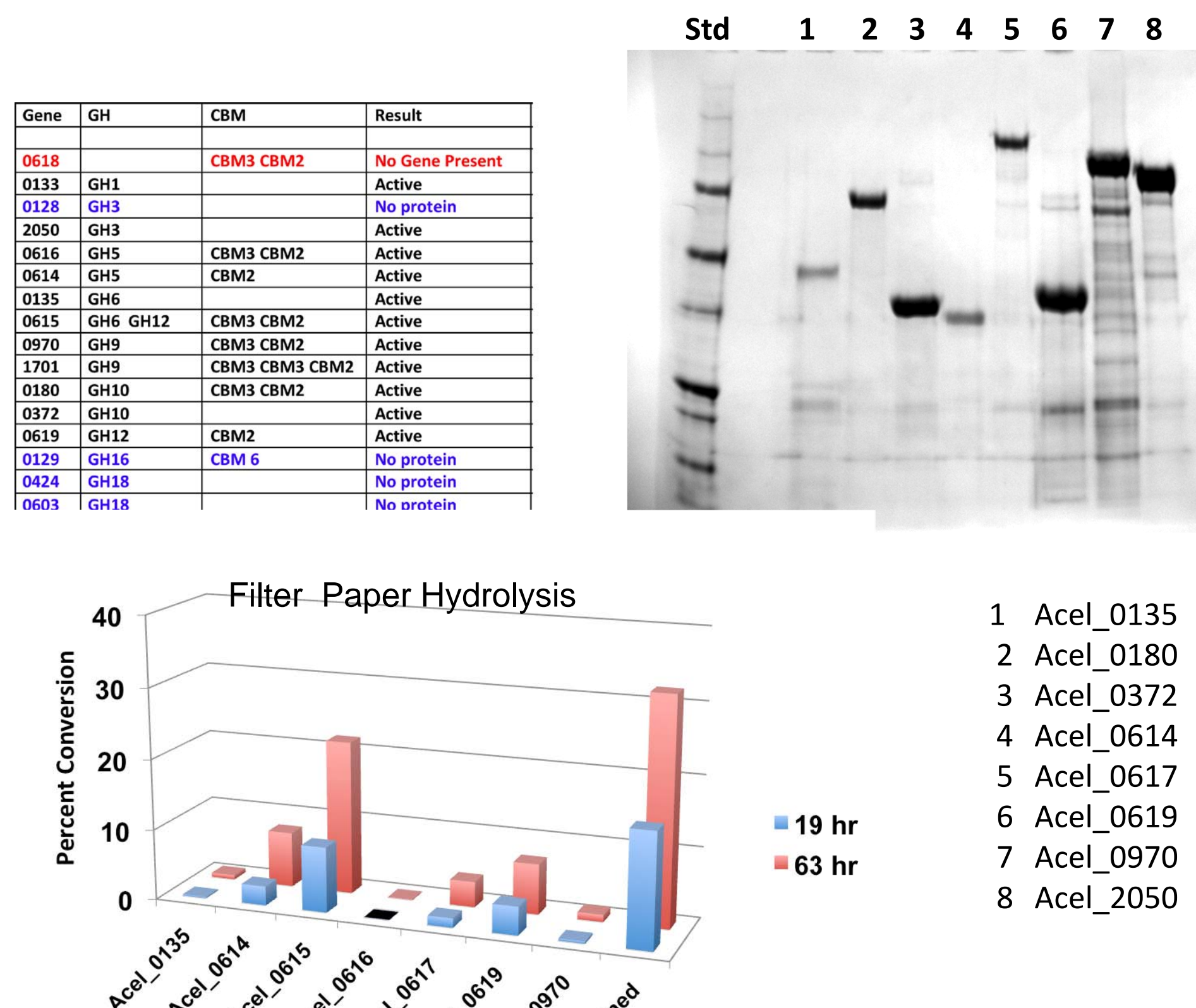
Steinmetz (2011) Expresso® Cloning and Expression Systems: Expressionengineering™ Technology streamlines recombinant protein expression. Nature Methods 8(6) iii-iv



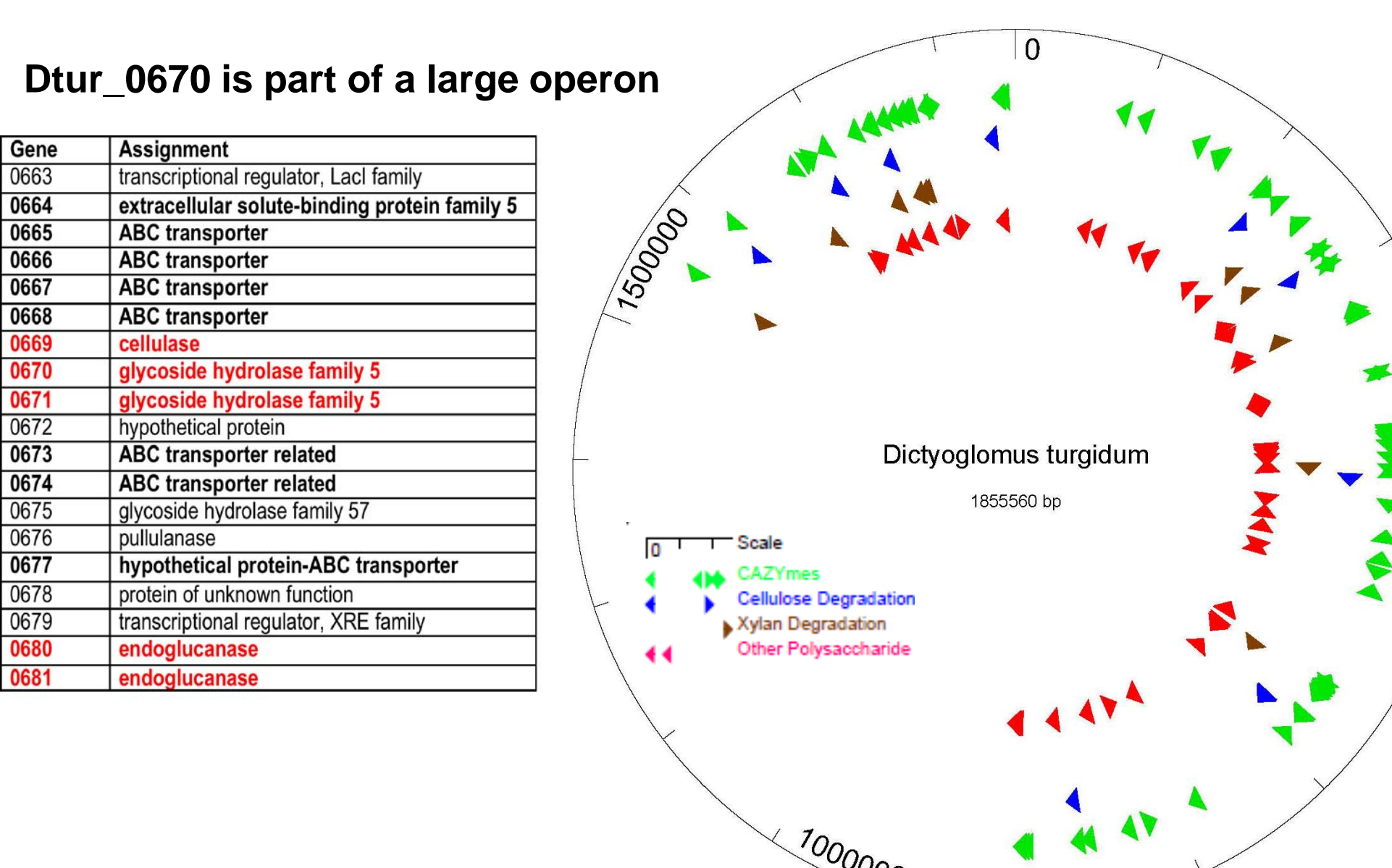
Multiplex Carbohydrate Active Enzyme Screening Assay

- ✦ Cultures are pelleted and lysed
- ✦ Multiplex substrate is added
 - 0.2% AZCL-HE-Cellulose
 - 0.2% AZCL-Arabinoxylan
 - 0.02% Methlumbelliferyl-β-D-Xylopyranoside
 - 0.02% Bromo-6-Chloro-3-Indoyle-β-D-Glucopyranoside
- ✦ Lysate and substrate is incubated overnight @ temperature optimal for organism enzymes

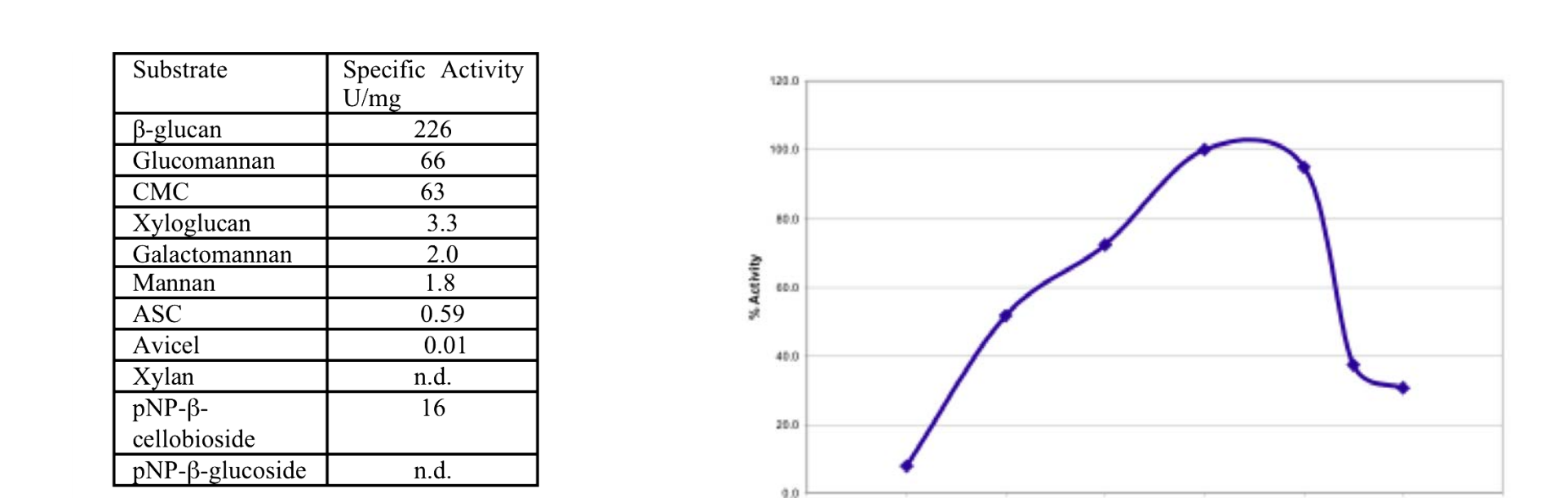
Acidothermus cellulolyticus Cellulase Cloning and Expression



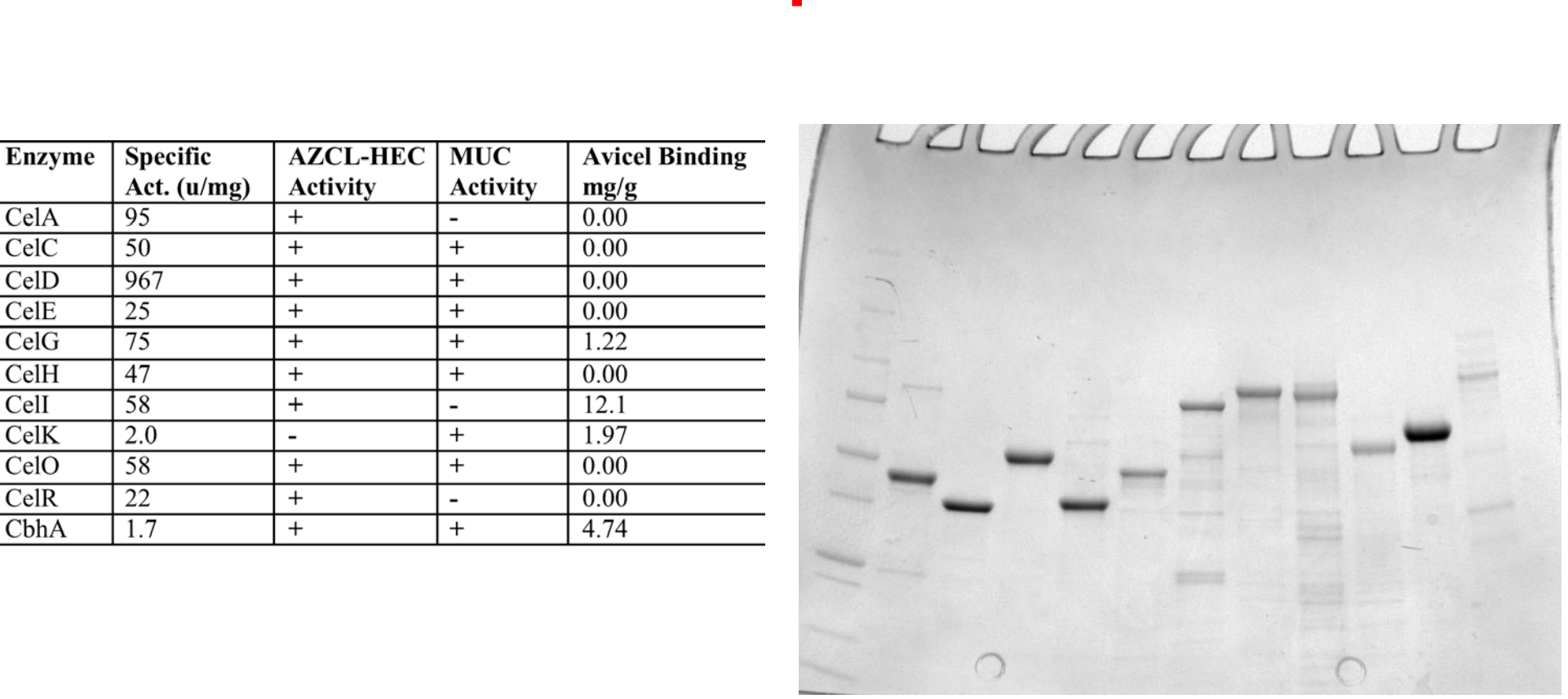
Dictyoglomus turgidum Cellulase Cloning and Expression



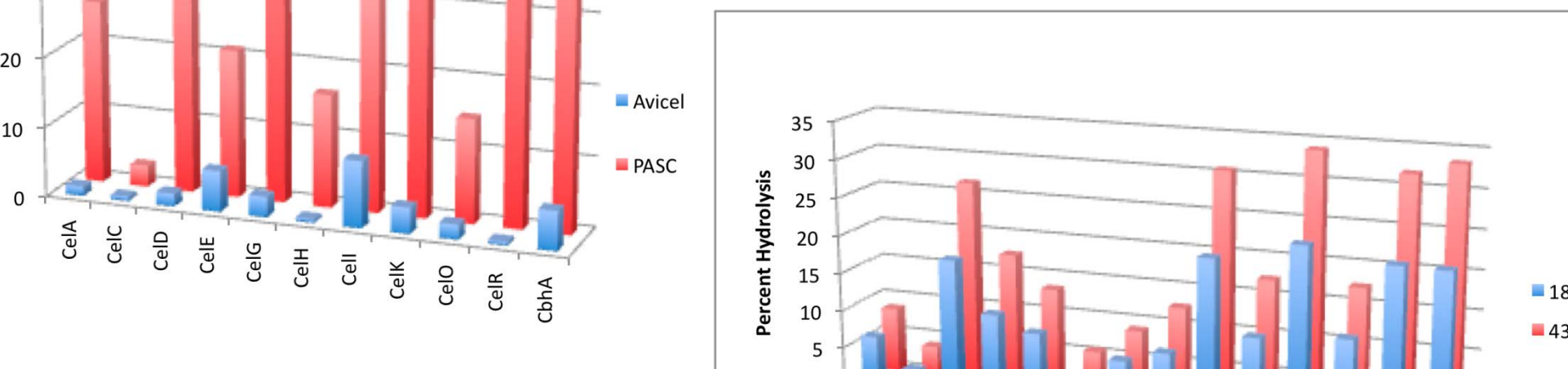
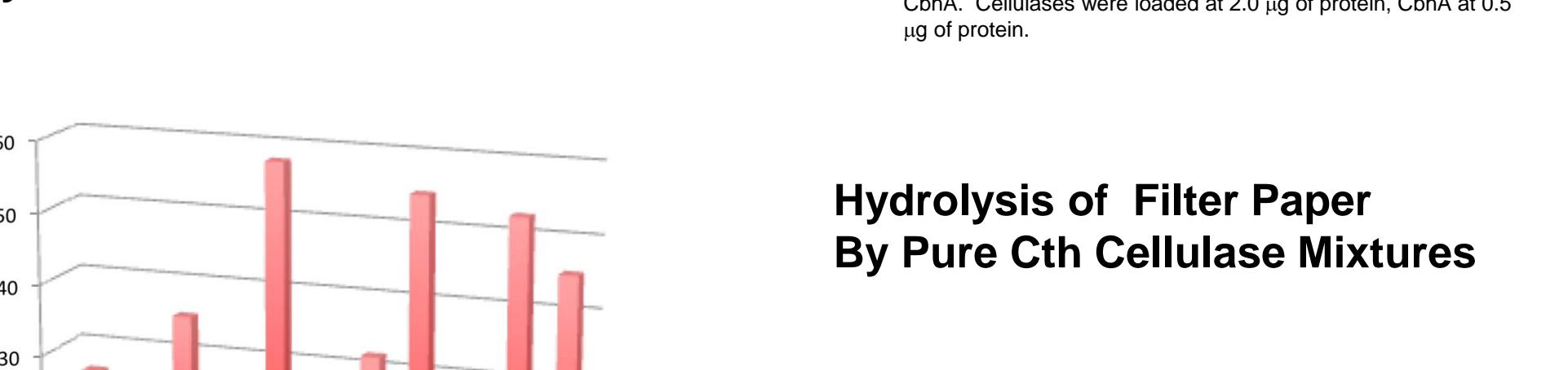
Characterization Of Dtur_0670 CelA



Clostridium thermocellum Cellulase Cloning and Expression



Hydrolysis of PASC and Avicel By Pure Cth Cellulases

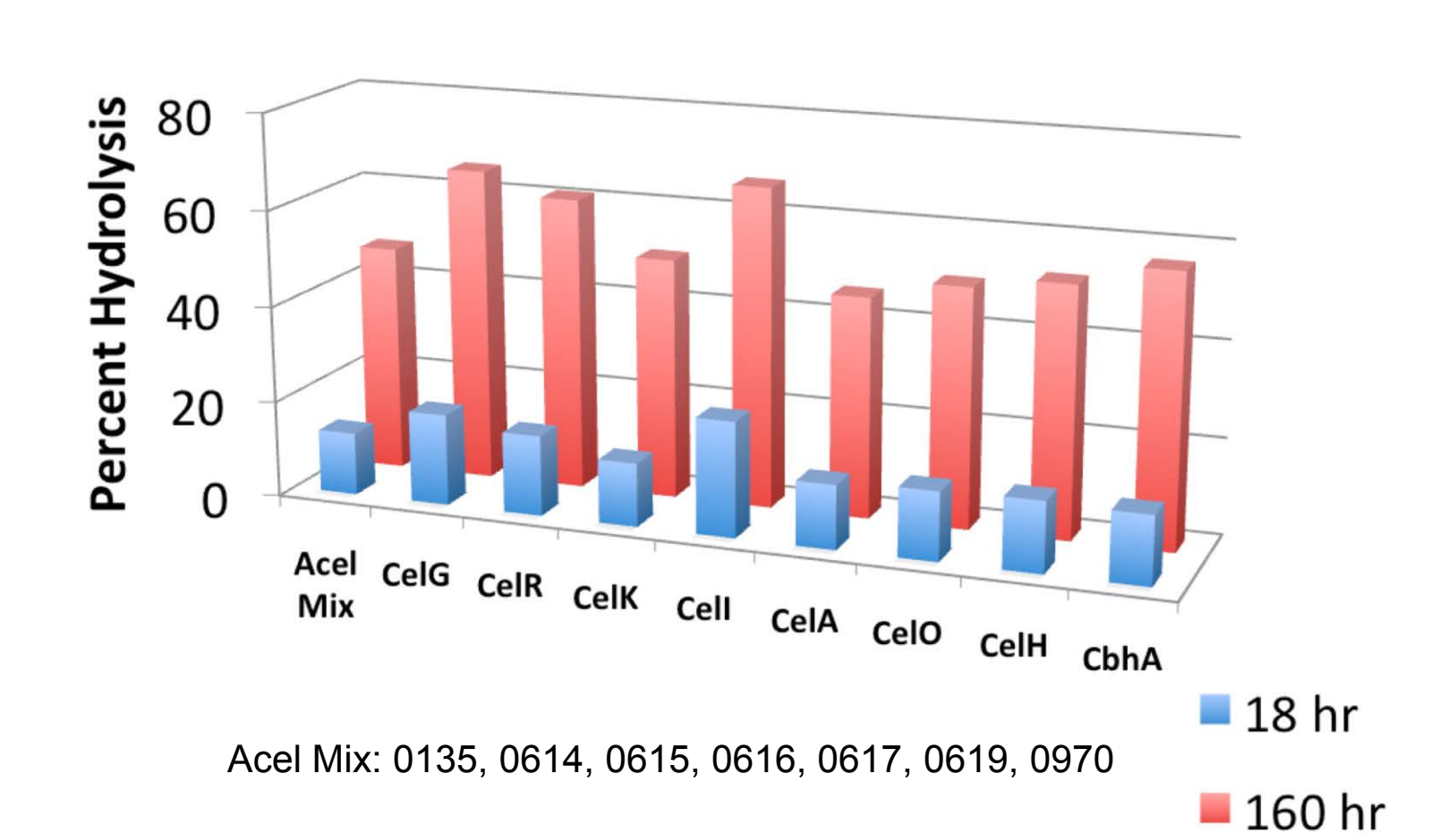


Cellulose hydrolysis was carried out using 3.4 mg Whatman 1 filter paper and either 0.1 mg or 0.5 mg of pure enzyme for 18 hr at 60 °C, pH 5.8

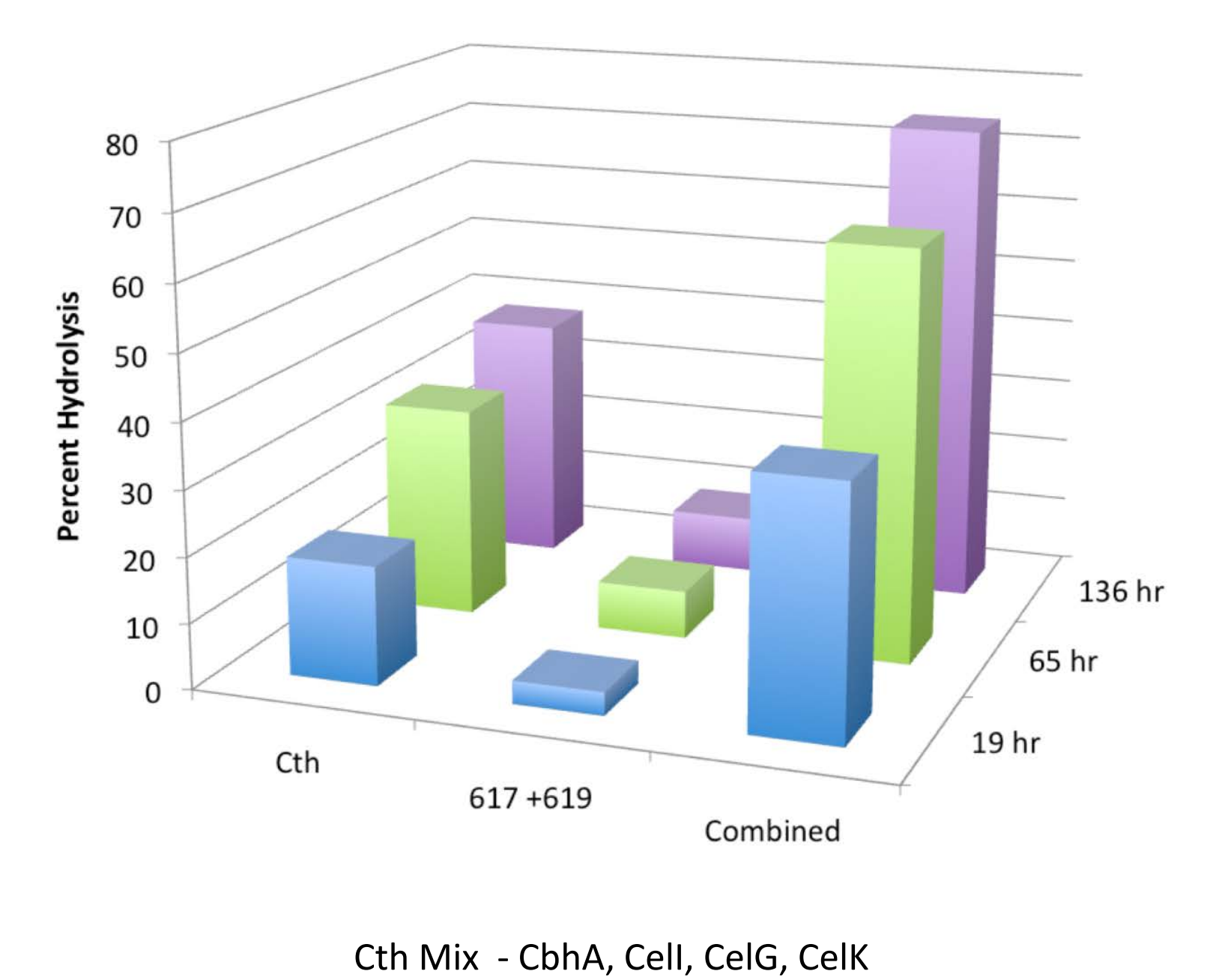
Cellulase	µg CbA	µg CeI	µg CeR	µg CeG	µg CeK
S1	100	0	0	0	100
S2	100	0	0	100	100
S3	0	100	0	0	100
S4	0	100	0	100	100
S5	0	0	100	0	100
S6	0	0	100	100	100
S7	100	0	0	100	100
S8	0	100	0	100	100
S9	0	0	100	100	100
S10	0	0	100	100	100
S11	0	100	100	100	100
S12	100	0	100	100	100
S13	100	100	0	100	100
S14	100	100	100	100	100

Mixtures of Cellulases Show Unique Synergies

Filter Paper Hydrolysis by Acel Mixture Plus Cth Cellulases



Filter Paper Hydrolysis by Cth Mixture Plus Acel Cellulases



RESULTS AND DISCUSSION

At Lucigen and C5-6 Technologies, we have utilized a combination of screening of shotgun libraries and mining of completed genomes to identify, clone, express and characterize over 200 carbohydrases. Of these 200 carbohydrases, over 30 have been thermophilic cellulases, from a variety of organisms including *Clostridium thermocellum*, *Dictyoglomus thermophilum*, and *Acidothermus cellulolyticus* as seen in the accompanying figures. The thermophilic cellulases hydrolyze a wide range of substrates including beta-glucans, amorphous cellulose, and crystalline celluloses. A number of these thermophilic cellulases also possess strong activity against xyloglucan, mannan, and substituted glucosammanans and galactosammanans. Combinations of these enzymes yield significant hydrolysis of both amorphous and crystalline celluloses; remarkable synergies exist when cellulases are combined from anaerobic and aerobic species.

CONCLUSIONS

Thermophiles are and will continue to be, an important source of new and novel cellulolytic enzymes. These enzymes show unique activities both alone and in mixtures. Results from studies on the pure, cloned enzymes and their mixtures suggest that these thermophilic enzyme systems may degrade cellulose via a different mechanism than used by fungal cellulases.

Selected References

- Brumm P.J., Hermanson S., Luedtke J., and D. Mead, "Identification, Cloning and Characterization of *Dictyoglomus turgidum* CelA, a Thermostable Endoglucanase with both Cellulase and Mannanase Activity" (2011) *J. Life Sciences* 5:488-496.
- Brumm P.J., Hermanson S., Hochstein R., Boyum J., Hermersmann N., Gowda K., and D. Mead, "Mining *Dictyoglomus turgidum* for Enzymatically Active Carbohydrases" *Applied Biochemistry and Biotechnology* (2011) 163:205-214.
- Gao D., Chundawat S., Tongjun Liu T., Spencer Hermanson S., Gowda K., Brumm P., Dale B.E., and V. Balan, (2010) "Strategy for Identification of Novel Fungal and Bacterial Glycosyl Hydrolase Hybrid Mixtures that can Efficiently Saccharify Pretreated Lignocellulosic Biomass", *Bioenergy Res.* DOI 10.1007/s12155-009-9066-6.