Glycosylation is Vital for Industrial Performance of Hyper-Active Cellulases

Brandon C. Knott[1*] (brandon.knott@nrel.gov), Daehwan Chung[1], Nicholas S. Sarai[1], Neal Hengge[1], Jordan F. Russell[3], Roman Brunecky[1], Nitin Supekar,[3] Todd VanderWall[1], Deanne W. Sammond[1], Michael F. Crowley[1], Parastoo Azadi[3], Janet Westpheling[3], Michael E. Himmel[1], Yannick J. Bomble[1], and **Gerald A. Tuskan**[2]

[1]National Renewable Energy Laboratory Golden, CO; [2]Center for Bioenergy Innovation, Oak Ridge National Laboratory, Oak Ridge, TN; [3]University of Georgia, Athens

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Project Goals: The Center for Bioenergy Innovation (CBI) vision is *to accelerate domestication of bioenergy-relevant, non-model plants and microbes to enable high-impact innovations at multiple points in the bioenergy supply chain.* CBI will address strategic barriers to the current bioeconomy in the areas of: 1) high-yielding, robust feedstocks, 2) lower capital and processing costs via consolidated bioprocessing (CBP) to specialty biofuels, and 3) methods to create valuable byproducts from the lignin. CBI will identify and utilize key plant genes for growth, composition and sustainability phenotypes as a means of achieving lower feedstock costs, focusing on poplar and switchgrass. We will convert these feedstocks to specialty biofuels (C4 alcohols and C6 esters) using CBP at high rates, titers and yield in combination with cotreatment or pretreatment. CBI will maximize product value by *in planta* modifications and biological funneling of lignin to value-added chemicals.

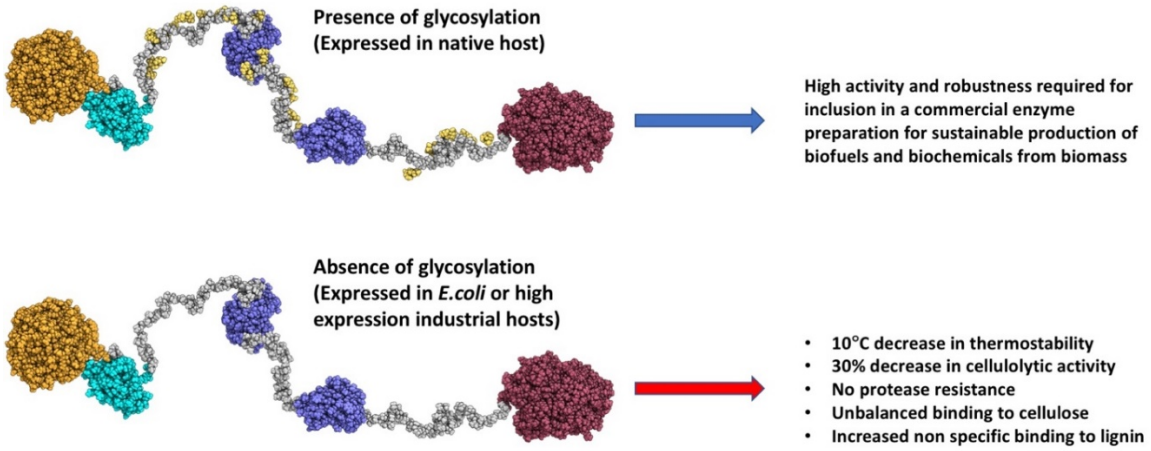
In the terrestrial biosphere, biomass deconstruction is conducted by microbes employing a variety of complementary strategies, many of which remain to be discovered. These strategies are also employed industrially, where the development of cost-effective biofuels requires increasingly more efficient (and less costly) cellulase formulations. The glycan decoration of fungal cellulases has been shown to protect these enzymes from protease action and to enhance binding to cellulose. Protein glycosylation is one of the most common protein post-translational modifications (PTM) and is thought to permit microorganisms to expand the combinatorial complexity of their gene products at a level beyond sequence space alone, opening new routes to structural, catalytic, and thermodynamic diversity. Glycosylation as a PTM in Nature has a variety of proposed roles that include enhancing protein solubility, biasing protein folding pathways, providing stability against proteolysis, and modulating signaling and molecular recognition pathways. Eukaryotic cellulolytic enzymes are often glycosylated, and this PTM has been shown to be important for both function and stability. Here we examine glycosylation's importance in cellulolytic procaryotes.

The hyperthermophilic anaerobic bacterium, *Caldicellulosiruptor bescii,* isolated from hot springs in the geothermally active Valley of Geysers in Siberia, can efficiently solubilize biomass without pretreatment. *C. bescii* relies primarily on a suite of complex multi-catalytic domain and multifunctional gene products to deconstruct biomass. The secretome of *C. bescii* displays high cellulolytic activity and the combination of the four most highly expressed enzymes in the secretome are enough to reproduce the activity of the entire secretome, making these enzymes appealing for the biofuels industry. One of these enzymes, CelA, has been shown to be the most efficient single gene product on several biomass substrates. CelA is a complex, thermally stable cellulase, containing an *N*-terminal GH9A-$CBM3_c$ processive endoglucanase, two family 3 carbohydrate-binding modules ($CBM3_b$), and a *C*-terminal GH48 exo-β-1,4-glucanase domain linked by Pro/Thr rich linkers. Recent

characterization of *C. bescii* carbohydrate-active enzymes (CAZymes), and especially native CelA has shown that the enzyme is glycosylated upon secretion from the cell. Despite the recognition of its important role in cellulolytic eukaryotes, detailed studies of bacterial cellulase glycosylation have not been reported.

We demonstrate that glycosylation of multimodular bacterial cellulase CelA is uniform across its three linker peptides and composed primarily of galactose disaccharides. This pattern of glycosylation is unique among previous studies of eukaryotic and bacterial cellulases. Furthermore, by combining experiment and computation, we find that glycosylation on the three intrinsically disordered regions of CelA plays key roles in modulating its proteolytic stability, thermodynamic stability, substrate binding, hydrolytic activity, and overall tertiary structure. The collective effects of glycosylation provide this multifunctional cellulase with the ability to function optimally in harsh environments, including geothermal hot springs and industrial biorefineries.

The understanding of bacterial cellulase glycosylation developed by this study opens the door to further studies of glycosylation in these systems but also to applications in the biofuels and biomaterials industries where better biomass-degrading enzymes are needed. For example, nearly all industrial applications of secreted enzymes require access to hosts capable of large-scale production yielding high titers of proteins. Challenges with the expression of bacterial glycosyl hydrolases, many of which are highly active or display diverse and important specificities, continue to limit the introduction of these enzymes into commercial markets. Enhancing our understanding of the roles played by glycans decorating bacterial enzymes will greatly enable use of these diverse catalysts at large scale to help enable the sustainable production of biofuels and biochemicals.



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