Final Technical Report

Systems Biology-Based Optimization of Extremely Thermophilic Lignocellulose Conversion to Bioproducts

DE-SC0019391

PI: Michael W. W. Adams (University of Georgia)

9/1/2018 - 8/31/2023

E-Link Report/Product Number: DOE-UGA-19391

Project Summary

This was a collaborative project involving researchers at the University of Georgia, North Carolina State University, Sanford-Burnham-Prebys Med. Discovery Institute and the University of Rhode Island. The over-arching goal was to demonstrate that non-model microorganisms, specifically extreme thermophiles, can be a strategic metabolic engineering platform for industrial biotechnology. We engineered the most thermophilic lignocellulose-degrading organism known, *Caldicellulosiruptor bescii* (*Cbes*), which grows optimally near 80°C, and the most thermophilic fermentative organism known, *Pyrococcus furiosus* (*Pfu*), which grows optimally at 100°C, to produce several key industrial chemicals. This work leveraged recent breakthrough advances in the development of molecular genetic tools for these organisms, complemented by a deep understanding of its metabolism and physiology gained over the past decade of study in the PIs' laboratories. We applied the latest metabolic reconstruction and modeling approaches to optimize biomass to product conversion. Bio-processing above 70°C can have important advantages over near-ambient operations. Highly genetically-modified microorganisms usually have a fitness disadvantage and can be easily overtaken in culture when contaminating microbes are present. The high growth temperature of extreme thermophiles precludes growth or survival of virtually any contaminating organism or phage. This reduces operating costs associated with reactor sterilization and maintaining a sterile facility. In addition, at industrial scales, heat production from microbial metabolic activity vastly outweighs heat loss through bioreactor walls such that cooling is required. Extreme thermophiles have the advantage that non-refrigerated cooling water can be used if needed, and heating requirements can be met with low-grade steam typically in excess capacity on plant sites. We assembled a highly interdisciplinary team that brought together all of the expertise for the project to have successful outcomes. This project also built upon and utilized extensive information already available in the PIs' labs for both *Cbes* and *Pfu* to develop models that provide a comprehensive description of these organisms' physiology and metabolism that were utilized to inform metabolic engineering strategies. The models were validated with experimental data and the results demonstrated that unpretreated lignocellulose has the potential to be converted into value-added industrial chemicals at high loading at bioreactor scale. The data generated from this research were published in twenty-one peer-reviewed papers in international journals with online access.

Technical Report

The research funded by DE-SC0019391 resulted in the following 21 publications with the indicated access information, along with one manuscript under revision. Abstracts of each publication are also provided in a following section.

Lee, L. L., Hart, W. S., Lunin, V. V., Alahuhta, M., Bomble, Y. J., Himmel, M. E., Blumer-Schuette, S. E., Adams, M. W. W. and Kelly, R. M. (2019) "Comparative biochemical and structural analysis of novel cellulose binding proteins (Tāpirins) from extremely thermophilic *Caldicellulosiruptor* species" *Appl. Environ. Microbiol.* **85** (doi: 10.1128/AEM.01983-18)

Crosby, J. R., Laemthong, T., Lewis, A. M., Straub, C. T., Adams, M. W. W. and Kelly, R. M. (2019) "Extreme thermophiles as emerging metabolic engineering platforms" *Curr. Opin. Biotechnol.* **59,** 55-64 (doi: 10.1016/j.copbio.2019.02.006)

Scott, I. M., Rubinstein, G. M., Poole, F. L, Lipscomb, G. L., Schut, G. J., Williams-Rhaesa, A. M., Stevenson, D. M., Amador-Noguez, D., Kelly, R. M. and Adams, M. W. W. (2019) "The thermophilic biomass-degrading bacterium *Caldicellulosiruptor bescii* utilizes two enzymes to oxidize glyceraldehyde-3-phosphate during glycolysis" *J. Biol. Chem.* **294,** 9995-10005 (doi: 10.1074/jbc.RA118.007120)

Straub, C. T., Khatibi, P. A., Wang, J. P., Conway, J. M., Williams-Rhaesa, A. M., Peszlen, I. M., Chiang, V. L., Adams, M. W. W. and Kelly, R. M. (2019) "Quantitative fermentation of unpretreated transgenic poplar by *Caldicellulosiruptor bescii*" *Nature Comm.* **10,** 3548 (doi: 10.1038/s41467-019-11376-6)

Straub, C. T., Khatibi, P. A., Otten, J. K., Adams, M. W. W. and Kelly, R. M. (2019) "Lignocellulose solubilization and conversion by extremely thermophilic *Caldicellulosiruptor bescii* improves by maintaining metabolic activity" *Biotech. Bioengin.* **116,** 1901-1908 (doi: 10.1002/bit.26993)

Lee, L.L., Crosby, J. R., Rubinstein, G. M., Laemthong, T., Bing, R. G., Straub, C. T., Adams, M. W. W. and Kelly, R. M. (2020) "The biology and biotechnology of the genus *Caldicellulosiruptor*: recent developments in Caldi world" *Extremophiles* **24,** 1-15 (doi: 10.1007/s00792-019-01116-5)

Straub, C. T., Bing, R. G,, Wang, J. P., Chiang, V. L., Adams, M. W. W. and Kelly, R. M. (2020) "Use of the lignocellulose-degrading bacterium *Caldicellulosiruptor bescii* to assess recalcitrance and conversion of wild-type and transgenic poplar" *Biotechnol. Biofuels* **11,** 43 (doi: 10.1186/s13068-020-01675-2)

Rubinstein, G. M., Lipscomb, G. L., Williams-Rhaesa, A. M., Kelly, R. M. and Adams, M. W. W. (2020) "Engineering the cellulolytic extreme thermophile *Caldicellulosiruptor bescii* to reduce carboxylic acids to alcohols using plant biomass as the energy source" *J. Indust. Micro. Biotech.* **47,** 585-597 (doi: 10.1007/s10295-020-02299-z)

Straub C. T., Schut, G. J., Otten J. K., Keller, L. M., Adams, M. W. W. and Kelly R. M. (2020) Modification of the glycolytic pathway in *Pyrococcus furiosus* and the implications for metabolic engineering. *Extremophiles* **24,** 511-518 (doi: 10.1007/s00792-020-01172-2)

Straub, C. T., Bing, R. G., Otten, J. K., Keller, L. M., Zeldes, B. M., Adams, M. W. W. and Kelly, R. M. (2020) "Metabolically engineered *Caldicellulosiruptor bescii* as a platform for producing acetone and hydrogen from lignocellulose" *Biotechnol. Bioeng*. **117,** 3799-3808 (doi: 10.1002/bit.27529)

Zhang, K., Zhao, W., Rodionov, D., Rubinstein, G. M., Nguyen, D. N., Tanwee, T. N. N., Crosby, J., Bing, R. G., Kelly, R. M., Adams, M. W. W. and Zhang, Y. (2021) "Genome-scale metabolic model of *Caldicellulosiruptor bescii* reveals optimal metabolic engineering strategies for ethanol production" *mSystems* 6, e0135120 (doi: 10.1128/mSystems.01351-20)

Rodionov, D. A., Rodionova, I. A., Rodionov, V. A., Zhang, K., Rubinstein, G. M., Tanwee, T. N. N., Crosby, J., Bing, R. G., Nookaew, I., Basen, M., Brown, S. D., Wilson, C., Klingeman, D. M., Poole, F. L., Zhang, Y., Kelly, R. M. and Adams, M. W. W. (2021) "Transcriptional regulation of plant biomass degradation and carbohydrate utilization genes in *Caldicellulosiruptor bescii*" *mSystems* 6, e0134520 (doi: 10.1128/mSystems.01345-20)

Bing, R. G., Sulis, D. B., Wang, J. P., Adams, M. W. W. and Kelly, R. M. (2021) "Thermophilic microbial deconstruction and conversion of natural and transgenic lignocellulose" *Environ. Microbiol. Rep.* **13**, 272-293 (doi: 10.1111/1758-2229.12943)

Bing, R. G., Straub, C. T., Sulis, D. B., Wang, J. P., Adamd, M. W. W. and Kelly, R. M. 2022. Plant biomass fermentation by the extreme thermophile *Caldicellulosiruptor bescii* for co-production of green hydrogen and acetone: Technoeconomic analysis. *Bioresource Technol*. 348:126780 (doi: 10.1016/j.biortech.2022.126780)

Crosby, J. R., Laemthong, T., Bing, R. G., Zhang, K., Tanwee, T. N. N., Lipscomb, G. L., Rodionov, D. A., Zhang, Y., Adams, M. W. W. and Kelly, R. M. 2022. Biochemical and regulatory analyses of xylanolytic regulons in *Caldicellulosiruptor bescii* reveal genus-wide features of hemicellulose utilization. *Appl. Environ. Microbiol*. 88, e0130222. (doi: 10.1128/aem.01302-22)

Laemthong, T., Bing, R. G., Crosby, J. R., Adams, M. W. W. and Kelly, R. M. 2022. Engineering *Caldicellulosiruptor bescii* with surface layer homology domain glycoside hydrolases to improve plant biomass solubilization. *Appl. Environ. Microbiol.* 88:e01274-22 (doi: 10.1128/aem.01274-22)

Bing, R. G., Carey, M. J., Laemthong, T., Willard, D. J., Crosby, J. R., Sulis, D. B., Wang, J. P., Adams, M. W. W. and Kelly, R. M. 2023a. Fermentative conversion of unpretreated plant biomass: A thermophilic threshold for indigenous microbial growth. *Bioresource Technol.* **367**, 128275 (doi: 10.1016/j.biortech.2022.128275)

Bing, R.G., Willard, D. J., Crosby, J. R., Adams, M. W. W. and Kelly, R. M. 2023d. Whither the genus *Caldicellulosiruptor* and the Order Thermoanaerobacterales: Phylogeny, taxonomy, ecology, and phenotype. *Front. Microbiol*. **14,** 1212538 (doi: 10.3389/fmicb.2023.1212538)

Laemthong, T., Bing, R. G., Crosby, J. R., Manesh, M. J. H., Adams, M. W. W. and Kelly, R. M. 2023. Role of cell-substrate association during plant biomass solubilization by the extreme thermophile *Caldicellulosiruptor bescii. Extremophiles* **27,** 6 (doi: 10.1007/s00792-023-01290-7)

Lipscomb, G. L., Crowley, A., Nguyen, D. M., Keller, M. W., O,Quinn, H. C., Tanwee, T. N. N., Vailionis, J. L., Zhang, K., Zhang, Y., Kelly, R. M. and Adams, M. W. W. 2023. Manipulating fermentation pathways in the hyperthermophilic archaeon *Pyrococcus furious* for ethanol production up to 95°C driven by carbon monoxide oxidation. *Appl. Environ. Microbiol.* May 10; e0001223. (doi: 10.1128/aem.00012-23)

Vailionis, J. L., Zhao, W., Zhang, K., Rodionov, D. A., Lipscomb, G. L., Tanwee, T. N. N., O'Quinn, H. C., Kelly R. M., Adams, M. W. W. and Zhang, Y. 2023. Optimizing strategies for bio-based ethanol production using genome-scale metabolic modeling of the hyperthermophilic archaeon, *Pyrococcus furiosus*. *Appl. Environ. Microbiol.* **89**, e0056323 (doi: 10.1128/aem.00563-23)

Crosby, J. R., Laemthong, T., Bing, R. G., Chen, S. L., Zhang, K., Tanwee, T. N. N., Lipscomb, G. L., Rodionov, D. A., Zhang, Y., Adams, M. W. W. and Kelly, R. M. 2023. Xylanolytic metabolism is regulated by coordination of transcription factors XynR and XylR in extremely thermophilic *Caldicellulosiruptor* species. *Appl. Environ. Microbiol*. **(**in revision).

Publications with Abstracts

Lee, L. L., Hart, W. S., Lunin, V. V., Alahuhta, M., Bomble, Y. J., Himmel, M. E., Blumer-Schuette, S. E., Adams, M. W. W. and Kelly, R. M. (2019) "Comparative biochemical and structural analysis of novel cellulose binding proteins (Tāpirins) from extremely thermophilic *Caldicellulosiruptor* species" *Appl. Environ. Microbiol.* **85** (doi: 10.1128/AEM.01983-18)

Genomes of extremely thermophilic *Caldicellulosiruptor* species encode novel cellulose binding proteins, called tāpirins, located proximate to the type IV pilus locus. The C-terminal domain of *Caldicellulosiruptor kronotskyensis* tāpirin 0844 (Calkro_0844) is structurally unique and has a cellulose binding affinity akin to that seen with family 3 carbohydrate binding modules (CBM3s). Here, full-length and C-terminal versions of tāpirins from *C. bescii* (Athe_1870), *C. hydrothermalis* (Calhy_0908), *C. kristjanssonii* (Calkr_0826), and *C. naganoensis* (NA10_0869) were produced recombinantly in *Escherichia coli* and compared to Calkro_0844. All five tāpirins bound to microcrystalline cellulose, switchgrass, poplar, and filter paper but not to xylan. Densitometry analysis of bound protein fractions visualized by SDS-PAGE revealed that Calhy_0908 and Calkr_0826 (from weakly cellulolytic species) associated with the cellulose substrates to a greater extent than Athe_1870, Calkro_0844, and NA10_0869 (from strongly cellulolytic species). Perhaps this relates to their specific needs to capture glucans released from lignocellulose by cellulases produced in *Caldicellulosiruptor* communities. Calkro_0844 and NA10_0869 share a higher degree of amino acid sequence identity (>80% identity) with each other than either does with Athe_1870 (~50%). The levels of amino acid sequence identity of Calhy_0908 and Calkr_0826 to Calkro_0844 were only 16% and 36%, respectively, although the three-dimensional structures of their C-terminal binding regions were closely related. Unlike the parent strain, *C. bescii* mutants lacking the tāpirin genes did not bind to cellulose following short-term incubation, suggesting a role in cell association with plant biomass. Given the scarcity of carbohydrates in neutral terrestrial hot springs, tāpirins likely help scavenge carbohydrates from lignocellulose to support growth and survival of *Caldicellulosiruptor* species.

Crosby, J. R., Laemthong, T., Lewis, A. M., Straub, C. T., Adams, M. W. W. and Kelly, R. M. (2019) "Extreme thermophiles as emerging metabolic engineering platforms" *Curr. Opin. Biotechnol.* **59,** 55-64 (doi: 10.1016/j.copbio.2019.02.006)

Going forward, industrial biotechnology must consider non-model metabolic engineering platforms if it is to have maximal impact. This will include microorganisms that natively possess strategic physiological and metabolic features but lack either molecular genetic tools or such tools are rudimentary, requiring further development. If non-model platforms are successfully deployed, new avenues for production of fuels and chemicals from renewable feedstocks or waste materials will emerge. Here, the challenges and opportunities for extreme thermophiles as metabolic engineering platforms are discussed.

Scott, I. M., Rubinstein, G. M., Poole, F. L, Lipscomb, G. L., Schut, G. J., Williams-Rhaesa, A. M., Stevenson, D. M., Amador-Noguez, D., Kelly, R. M. and Adams, M. W. W. (2019) "The thermophilic biomass-degrading bacterium *Caldicellulosiruptor bescii* utilizes two enzymes to oxidize glyceraldehyde-3-phosphate during glycolysis" *J. Biol. Chem.* **294,** 9995-10005 (doi: 10.1074/jbc.RA118.007120)

Caldicellulosiruptor bescii is an extremely thermophilic, cellulolytic bacterium with a growth optimum at 78 °C and is the most thermophilic cellulose degrader known. It is an attractive target for biotechnological applications, but metabolic engineering will require an in-depth understanding of its primary pathways. A previous analysis of its genome uncovered evidence that *C. bescii* may have a completely uncharacterized aspect to its redox metabolism, involving a tungsten-containing oxidoreductase of unknown function. Herein, we purified and characterized this new member of the aldehyde ferredoxin oxidoreductase family of tungstoenzymes. We show that it is a heterodimeric glyceraldehyde-3-phosphate (GAP) ferredoxin oxidoreductase (GOR) present not only in all known *Caldicellulosiruptor* species, but also in 44 mostly anaerobic bacterial genera. GOR is phylogenetically distinct from the monomeric GAP-oxidizing enzyme found previously in several Archaea. We found that its large subunit (GOR-L) contains a single tungstopterin site and one iron-sulfur [4Fe-4S] cluster, that the small subunit (GOR-S) contains four [4Fe-4S] clusters, and that GOR uses ferredoxin as an electron acceptor. Deletion of either subunit resulted in a distinct growth phenotype on both C_5 and C_6 sugars, with an increased lag phase, but higher cell densities. Using metabolomics and kinetic analyses, we show that GOR functions in parallel with the conventional GAP dehydrogenase, providing an alternative ferredoxin-dependent glycolytic pathway. These two pathways likely facilitate the recycling of reduced redox carriers (NADH and ferredoxin) in response to environmental H_2 concentrations. This metabolic flexibility has important implications for the future engineering of this and related species.

Straub, C. T., Khatibi, P. A., Wang, J. P., Conway, J. M., Williams-Rhaesa, A. M., Peszlen, I. M., Chiang, V. L., Adams, M. W. W. and Kelly, R. M. (2019) "Quantitative fermentation of unpretreated transgenic poplar by *Caldicellulosiruptor bescii*" *Nature Comm.* **10,** 3548 (doi: 10.1038/s41467-019-11376-6)

Microbial fermentation of lignocellulosic biomass to produce industrial chemicals is exacerbated by the recalcitrant network of lignin, cellulose and hemicelluloses comprising the plant secondary cell wall. In this study, we show that transgenic poplar (Populus trichocarpa) lines can be solubilized without any pretreatment by the extreme thermophile Caldicellulosiruptor bescii that has been metabolically engineered to shift its fermentation products away from inhibitory organic acids to ethanol. Carbohydrate solubilization and conversion of unpretreated milled biomass is nearly 90% for two transgenic lines, compared to only 25% for wild-type poplar. Unexpectedly, unpretreated intact poplar stems achieved nearly 70% of the fermentation production observed with milled poplar as the substrate. The nearly quantitative microbial conversion of the carbohydrate content of unpretreated transgenic lignocellulosic biomass bodes well for full utilization of renewable biomass feedstocks.

Straub, C. T., Khatibi, P. A., Otten, J. K., Adams, M. W. W. and Kelly, R. M. (2019) "Lignocellulose solubilization and conversion by extremely thermophilic *Caldicellulosiruptor bescii* improves by maintaining metabolic activity" *Biotech. Bioengin.* **116,** 1901-1908 (doi: 10.1002/bit.26993)

The extreme thermophile Caldicellulosiruptor bescii solubilizes and metabolizes the carbohydrate content of lignocellulose, a process that ultimately ceases because of biomass recalcitrance, accumulation of fermentation products, inhibition by lignin moieties, and reduction of metabolic activity. Deconstruction of low loadings of lignocellulose (5 g/L), either natural or transgenic, whether unpretreated or subjected to hydrothermal processing, by C. bescii typically results in less than 40% carbohydrate solubilization. Mild alkali pretreatment (up to 0.09 g NaOH/g biomass) improved switchgrass carbohydrate solubilization by C. bescii to over 70% compared to less than 30% for no pretreatment, with two-thirds of the carbohydrate content in the treated switchgrass converted to acetate and lactate. C. bescii grown on high loadings of unpretreated switchgrass (50 g/L) retained in a pH-controlled bioreactor slowly purged (τ = 80 hr) with growth media without a carbon source improved carbohydrate solubilization to over 40% compared to

batch culture at 29%. But more significant was the doubling of solubilized carbohydrate conversion to fermentation products, which increased from 40% in batch to over 80% in the purged system, an improvement attributed to maintaining the bioreactor culture in a metabolically active state. This strategy should be considered for optimizing solubilization and conversion of lignocellulose by C. bescii and other lignocellulolytic microorganisms.

Lee, L.L., Crosby, J. R., Rubinstein, G. M., Laemthong, T., Bing, R. G., Straub, C. T., Adams, M. W. W. and Kelly, R. M. (2020) "The biology and biotechnology of the genus *Caldicellulosiruptor*: recent developments in Caldi world" *Extremophiles* **24,** 1-15 (doi: 10.1007/s00792-019-01116-5)

Terrestrial hot springs near neutral pH harbor extremely thermophilic bacteria from the genus *Caldicellulosiruptor,* which utilize the carbohydrates of lignocellulose for growth. These bacteria are technologically important because they produce novel, multi-domain glycoside hydrolases that are prolific at deconstructing microcrystalline cellulose and hemicelluloses found in plant biomass. Among other interesting features, *Caldicellulosiruptor* species have successfully adapted to bind specifically to lignocellulosic substrates via surface layer homology (SLH) domains associated with glycoside hydrolases and unique binding proteins (tāpirins) present only in these bacteria. They also utilize a parallel pathway for conversion of glyceraldehyde-3-phosphate into 3-phosphoglycerate via a ferredoxin-dependent oxidoreductase that is conserved across the genus. Advances in the genetic tools for *Caldicellulosiruptor bescii*, including the development of a high-temperature kanamycin-resistance marker and xylose-inducible promoter, have opened the door for metabolic engineering applications and some progress along these lines has been reported. While several species of *Caldicellulosiruptor* can readily deconstruct lignocellulose, improvements in the amount of carbohydrate released and in the production of bio-based chemicals are required to successfully realize the biotechnological potential of these organisms.

Straub, C. T., Bing, R. G,, Wang, J. P., Chiang, V. L., Adams, M. W. W. and Kelly, R. M. (2020) "Use of the lignocellulose-degrading bacterium *Caldicellulosiruptor bescii* to assess recalcitrance and conversion of wild-type and transgenic poplar" *Biotechnol. Biofuels* **11,** 43 (doi: 10.1186/s13068-020-01675-2)

Biological conversion of lignocellulosic biomass is significantly hindered by feedstock recalcitrance, which is typically assessed through an enzymatic digestion assay, often preceded by a thermal and/or chemical pretreatment. Here, we assay 17 lines of unpretreated transgenic black cottonwood (*Populus trichocarpa*) utilizing a lignocellulose-degrading, metabolically engineered bacterium, *Caldicellulosiruptor bescii*. The poplar lines were assessed by incubation with an engineered *C. bescii* strain that solubilized and converted the hexose and pentose carbohydrates to ethanol and acetate. The resulting fermentation titer and biomass solubilization were then utilized as a measure of biomass recalcitrance and compared to data previously reported on the transgenic poplar samples. Of the 17 transgenic poplar lines examined with *C. bescii*, a wide variation in solubilization and fermentation titer was observed. While the wild type poplar control demonstrated relatively high recalcitrance with a total solubilization of only 20% and a fermentation titer of 7.3 mM, the transgenic lines resulted in solubilization ranging from 15 to 79% and fermentation titers from 6.8 to 29.6 mM. Additionally, a strong inverse correlation (R^2 = 0.8) between conversion efficiency and lignin content was observed with lower lignin samples more easily converted and solubilized by *C. bescii*. Feedstock recalcitrance can be significantly reduced with transgenic plants, but finding the correct modification may require a large sample set to identify the most advantageous genetic modifications for the feedstock. Utilizing *C. bescii* as a screening assay for recalcitrance, poplar lines with down-regulation of coumarate 3-hydroxylase 3 (C3H3) resulted in the highest degrees of solubilization and conversion by *C. bescii*. One such line, with a growth phenotype similar to the wild-type,

generated more than three times the fermentation products of the wild-type poplar control, suggesting that excellent digestibility can be achieved without compromising fitness of the tree.

Rubinstein, G. M., Lipscomb, G. L., Williams-Rhaesa, A. M., Kelly, R. M. and Adams, M. W. W. (2020) “Engineering the cellulolytic extreme thermophile *Caldicellulosiruptor bescii* to reduce carboxylic acids to alcohols using plant biomass as the energy source” *J. Indust. Micro. Biotech.* **47,** 585-597 (doi: 10.1007/s10295-020-02299-z)

Caldicellulosiruptor bescii is the most thermophilic cellulolytic organism yet identified (T_{opt} 78 °C). It grows on untreated plant biomass and has an established genetic system thereby making it a promising microbial platform for lignocellulose conversion to bio-products. Here, we investigated the ability of engineered C. bescii to generate alcohols from carboxylic acids. Expression of aldehyde ferredoxin oxidoreductase (aor from Pyrococcus furiosus) and alcohol dehydrogenase (adhA from Thermoanaerobacter sp. X514) enabled C. bescii to generate ethanol from crystalline cellulose and from biomass by reducing the acetate produced by fermentation. Deletion of lactate dehydrogenase in a strain expressing the AOR-Adh pathway increased ethanol production. Engineered strains also converted exogenously supplied organic acids (isobutyrate and n-caproate) to the corresponding alcohol (isobutanol and hexanol) using both crystalline cellulose and switchgrass as sources of reductant for alcohol production. This is the first instance of an acid to alcohol conversion pathway in a cellulolytic microbe.

Straub C. T., Schut, G. J., Otten J. K., Keller, L. M., Adams, M. W. W. and Kelly R. M. (2020) Modification of the glycolytic pathway in *Pyrococcus furiosus* and the implications for metabolic engineering. *Extremophiles* **24,** 511-518 (doi: 10.1007/s00792-020-01172-2)

The key difference in the modified Embden-Meyerhof glycolytic pathway in hyperthermophilic Archaea, such as *Pyrococcus furiosus*, occurs at the conversion from glyceraldehyde-3-phosphate (GAP) to 3-phosphoglycerate (3-PG) where the typical intermediate 1,3-bisphosphoglycerate (1,3-BPG) is not present. The absence of the ATP-yielding step catalyzed by phosphoglycerate kinase (PGK) alters energy yield, redox energetics, and kinetics of carbohydrate metabolism. Either of the two enzymes, ferredoxin-dependent glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR) or $NADP^+$-dependent non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPN), responsible for this "bypass" reaction, could be deleted individually without impacting viability, albeit with differences in native fermentation product profiles. Furthermore, *P. furiosus* was viable in the gluconeogenic direction (growth on pyruvate or peptides plus elemental sulfur) in a ΔgapnΔgapor strain. Ethanol was utilized as a proxy for potential heterologous products (e.g., isopropanol, butanol, fatty acids) that require reducing equivalents (e.g., NAD(P)H, reduced ferredoxin) generated from glycolysis. Insertion of a single gene encoding the thermostable NADPH-dependent primary alcohol dehydrogenase (adhA) (Tte_0696) from *Caldanaerobacter subterraneus*, resulted in a strain producing ethanol via the previously established aldehyde oxidoreductase (AOR) pathway. This strain demonstrated a high ratio of ethanol over acetate (> 8:1) at 80 °C and enabled ethanol production up to 85 °C, the highest temperature for bio-ethanol production reported to date.

Straub, C. T., Bing, R. G., Otten, J. K., Keller, L. M., Zeldes, B. M., Adams, M. W. W. and Kelly, R. M. (2020) “Metabolically engineered *Caldicellulosiruptor bescii* as a platform for producing acetone and hydrogen from lignocellulose” *Biotechnol. Bioeng*. **117,** 3799-3808 (doi: 10.1002/bit.27529)

The production of volatile industrial chemicals utilizing metabolically engineered extreme thermophiles offers the potential for processes with simultaneous fermentation and product separation. An excellent target chemical for such a process is acetone (T_b = 56°C), ideally produced from lignocellulosic biomass. *Caldicellulosiruptor bescii* (T_{opt} 78°C), an extremely thermophilic fermentative bacterium naturally capable of deconstructing and fermenting

lignocellulose, was metabolically engineered to produce acetone. When the acetone pathway construct was integrated into a parent strain containing the bifunctional alcohol dehydrogenase from *Clostridium thermocellum*, acetone was produced at 9.1 mM (0.53 g/L), in addition to minimal ethanol 3.3 mM (0.15 g/L), along with net acetate consumption. This demonstrates that C. bescii can be engineered with balanced pathways in which renewable carbohydrate sources are converted to useful metabolites, primarily acetone and H_2 , without net production of its native fermentation products, acetate and lactate.

Zhang, K., Zhao, W., Rodionov, D., Rubinstein, G. M., Nguyen, D. N., Tanwee, T. N. N., Crosby, J., Bing, R. G., Kelly, R. M., Adams, M. W. W. and Zhang, Y. (2021) "Genome-scale metabolic model of *Caldicellulosiruptor bescii* reveals optimal metabolic engineering strategies for ethanol production" *mSystems* 6, e0135120 (doi: 10.1128/mSystems.01351-20)

Rodionov, D. A., Rodionova, I. A., Rodionov, V. A., Zhang, K., Rubinstein, G. M., Tanwee, T. N. N., Crosby, J., Bing, R. G., Nookaew, I., Basen, M., Brown, S. D., Wilson, C., Klingeman, D. M., Poole, F. L., Zhang, Y., Kelly, R. M. and Adams, M. W. W. (2021) "Transcriptional regulation of plant biomass degradation and carbohydrate utilization genes in *Caldicellulosiruptor bescii*" *mSystems* 6, e0134520 (doi: 10.1128/mSystems.01345-20)

Metabolic modeling was used to examine potential bottlenecks that could be encountered for metabolic engineering of the cellulolytic extreme thermophile *Caldicellulosiruptor bescii* to produce bio-based chemicals from plant biomass. The model utilizes subsystems-based genome annotation, targeted reconstruction of carbohydrate utilization pathways, and biochemical and physiological experimental validations. Specifically, carbohydrate transport and utilization pathways involving 160 genes and their corresponding functions were incorporated, representing the utilization of C5/C6 monosaccharides, disaccharides, and polysaccharides such as cellulose and xylan. To illustrate its utility, the model predicted that optimal production from biomass-based sugars of the model product, ethanol, was driven by ATP production, redox balancing, and proton translocation, mediated through the interplay of an ATP synthase, a membrane-bound hydrogenase, a bifurcating hydrogenase, and a bifurcating NAD- and NADP-dependent oxidoreductase. These mechanistic insights guided the design and optimization of new engineering strategies for product optimization, which were subsequently tested in the *C. bescii* model, showing a nearly 2-fold increase in ethanol yields. The *C. bescii* model provides a useful platform for investigating the potential redox controls that mediate the carbon and energy flows in metabolism and sets the stage for future design of engineering strategies aiming at optimizing the production of ethanol and other bio-based chemicals.

Bing, R. G., Sulis, D. B., Wang, J. P., Adams, M. W. W. and Kelly, R. M. (2021) "Thermophilic microbial deconstruction and conversion of natural and transgenic lignocellulose" *Environ. Microbiol. Rep.* **13**, 272-293 (doi: 10.1111/1758-2229.12943)

The potential to convert renewable plant biomasses into fuels and chemicals by microbial processes presents an attractive, less environmentally intense alternative to conventional routes based on fossil fuels. This would best be done with microbes that natively deconstruct lignocellulose and concomitantly form industrially relevant products, but these two physiological and metabolic features are rarely and simultaneously observed in nature. Genetic modification of both plant feedstocks and microbes can be used to increase lignocellulose deconstruction capability and generate industrially relevant products. Separate efforts on plants and microbes are ongoing, but these studies lack a focus on optimal, complementary combinations of these disparate biological systems to obtain a convergent technology. Improving genetic tools for plants have given rise to the generation of low-lignin lines that are more readily solubilized by microorganisms. Most focus on the microbiological front has involved thermophilic bacteria from the genera *Caldicellulosiruptor* and *Clostridium*, given their capacity to degrade lignocellulose and

to form bio-products through metabolic engineering strategies enabled by ever-improving molecular genetics tools. Bioengineering plant properties to better fit the deconstruction capabilities of candidate consolidated bioprocessing microorganisms has potential to achieve the efficient lignocellulose deconstruction needed for industrial relevance.

Bing, R. G., Straub, C. T., Sulis, D. B., Wang, J. P., Adamd, M. W. W. and Kelly, R. M. 2022. Plant biomass fermentation by the extreme thermophile *Caldicellulosiruptor bescii* for co-production of green hydrogen and acetone: Technoeconomic analysis. *Bioresource Technol*. 348:126780 (doi: 10.1016/j.biortech.2022.126780)

A variety of chemical and biological processes have been proposed for conversion of sustainable low-cost feedstocks into industrial products. Here, a biorefinery concept is formulated, modeled, and analyzed in which a naturally (hemi)cellulolytic and extremely thermophilic bacterium, *Caldicellulosiruptor bescii*, is metabolically engineered to convert the carbohydrate content of lignocellulosic biomasses (i.e., soybean hulls, transgenic poplar) into green hydrogen and acetone. Experimental validation of *C. bescii* fermentative performance demonstrated 82% carbohydrate solubilization of soybean hulls and 55% for transgenic poplar. A detailed technical design, including equipment specifications, provides the basis for an economic analysis that establishes metabolic engineering targets. This robust industrial process leveraging metabolically engineered *C. bescii* yields 206 kg acetone and 25 kg H_2 per metric ton of soybean hull, or 174 kg acetone and 21 kg H_2 per metric ton transgenic poplar. Beyond this specific case, the model demonstrates industrial feasibility and economic advantages of thermophilic fermentation.

Crosby, J. R., Laemthong, T., Bing, R. G., Zhang, K., Tanwee, T. N. N., Lipscomb, G. L., Rodionov, D. A., Zhang, Y., Adams, M. W. W. and Kelly, R. M. 2022. Biochemical and regulatory analyses of xylanolytic regulons in *Caldicellulosiruptor bescii* reveal genus-wide features of hemicellulose utilization. *Appl. Environ. Microbiol*. 88, e0130222. (doi: 10.1128/aem.01302-22)

Caldicellulosiruptor species scavenge carbohydrates from runoff containing plant biomass that enters hot springs and from grasses that grow in more moderate parts of thermal features. While only a few *Caldicellulosiruptor* species can degrade cellulose, all known species are hemicellulolytic. The most well-characterized species, Caldicellulosiruptor bescii, decentralizes its hemicellulase inventory across five different genomic loci and two isolated genes. Transcriptomic analyses, comparative genomics, and enzymatic characterization were utilized to assign functional roles and determine the relative importance of its six putative endoxylanases (five glycoside hydrolase family 10 [GH10] enzymes and one GH11 enzyme) and two putative exoxylanases (one GH39 and one GH3) in C. bescii. Two genus-wide conserved xylanases, *C. bescii* XynA (GH10) and *C. bescii* Xyl3A (GH3), had the highest levels of sugar release on oat spelt xylan, were in the top 10% of all genes transcribed by *C. bescii*, and were highly induced on xylan compared to cellulose. This indicates that a minimal set of enzymes are used to drive xylan degradation in the genus *Caldicellulosiruptor*, complemented by hemicellulolytic inventories that are tuned to specific forms of hemicellulose in available plant biomasses. To this point, synergism studies revealed that the pairing of specific GH family proteins (GH3, -11, and -39) with *C. bescii* GH10 proteins released more sugar *in vitro* than mixtures containing five different GH10 proteins. Overall, this work demonstrates the essential requirements for *Caldicellulosiruptor* to degrade various forms of xylan and the differences in species genomic inventories that are tuned for survival in unique biotopes with variable lignocellulosic substrates.

Laemthong, T., Bing, R. G., Crosby, J. R., Adams, M. W. W. and Kelly, R. M. 2022. Engineering *Caldicellulosiruptor bescii* with surface layer homology domain glycoside hydrolases to improve plant biomass solubilization. *Appl. Environ. Microbiol.* 88:e01274-22 (doi: 10.1128/aem.01274-22)

Extremely thermophilic *Caldicellulosiruptor* species solubilize carbohydrates from lignocellulose through glycoside hydrolases (GHs) that can be extracellular, intracellular, or cell surface layer (S-layer) associated. *Caldicellulosiruptor* genomes sequenced so far encode at least one surface layer homology domain glycoside hydrolase (SLH-GH), representing six different classes of these enzymes; these can have multiple binding and catalytic domains. Biochemical characterization of a representative from each class was done to determine their biocatalytic features: four SLH-GHs from Caldicellulosiruptor kronotskyensis (Calkro_0111, Calkro_0402, Calkro_0072, and Calkro_2036) and two from Caldicellulosiruptor hydrothermalis (Calhy_1629 and Calhy_2383). Calkro_0111, Calkro_0072, and Calhy_2383 exhibited β-1,3-glucanase activity, Calkro_0402 was active on both β-1,3/1,4-glucan and β-1,4-xylan, Calkro_2036 exhibited activity on both β-1,3/1,4-glucan and β-1,4-glucan, and Calhy_1629 was active only on arabinan. Caldicellulosiruptor bescii, the only species with molecular genetic tools as well as already a strong cellulose degrader, contains only one SLH-GH, Athe_0594, a glucanase that is a homolog of Calkro_2036; the other 5 classes of SLH-GHs are absent in *C. bescii*. The *C. bescii* secretome, supplemented with individual enzymes or cocktails of SLH-GHs, increased *in vitro* sugar release from sugar cane bagasse and poplar. Expression of non-native SLH-GHs *in vivo*, either associated with the S-layer or as freely secreted enzymes, improved total carbohydrate solubilization of sugar cane bagasse and poplar by up to 45% and 23%, respectively. Most notably, expression of Calkro_0402, a xylanase/glucanase, improved xylose solubilization from poplar and bagasse by over 70% by *C. bescii*. While these *Caldicellulosiruptor* species are already prolific lignocellulose degraders, they can be further improved by the strategy described here. Hence, *Caldicellulosiruptor* species hold promise as microorganisms that can solubilize the carbohydrate portion of lignocellulose and subsequently convert fermentable sugars into bio-based chemicals and fuels. Members of the genus have surface layer (S-layer) homology domain-associated glycoside hydrolases (SLH-GHs) that mediate attachment to biomass as well as hydrolysis of carbohydrates. *Caldicellulosiruptor bescii,* the most studied member of the genus, has only one SLH-GH. Expression of SLH-GHs from other *Caldicellulosiruptor* species in *C. bescii* significantly improved degradation of sugar cane bagasse and poplar. This suggests that this extremely thermophilic bacterium can be engineered to further improve its ability to degrade specific plant biomasses by inserting genes encoding SLH-GHs recruited from other *Caldicellulosiruptor* species.

Bing, R. G., Carey, M. J., Laemthong, T., Willard, D. J., Crosby, J. R., Sulis, D. B., Wang, J. P., Adams, M. W. W. and Kelly, R. M. 2023a. Fermentative conversion of unpretreated plant biomass: A thermophilic threshold for indigenous microbial growth. *Bioresource Technol.* **367**, 128275 (doi: 10.1016/j.biortech.2022.128275)

Naturally occurring, microbial contaminants were found in plant biomasses from common bioenergy crops and agricultural wastes. Unexpectedly, indigenous thermophilic microbes were abundant, raising the question of whether they impact thermophilic consolidated bioprocessing fermentations that convert biomass directly into useful bioproducts. Candidate microbial platforms for biomass conversion, *Acetivibrio thermocellus* (basionym *Clostridium thermocellum*; T_{opt} 60 °C) and *Caldicellulosiruptor bescii* (T_{opt} 78 °C), each degraded a wide variety of plant biomasses, but only *A. thermocellus* was significantly affected by the presence of indigenous microbial populations harbored by the biomass. Indigenous microbial growth was eliminated at ≥75 °C, conditions where *C. bescii* thrives, but where *A. thermocellus* cannot survive. Therefore, 75 °C is the thermophilic threshold to avoid sterilizing pre-treatments on the biomass that prevents native microbes from competing with engineered microbes and forming undesirable by-products. Thermophiles that naturally grow at and above 75 °C offer specific advantages as platform microorganisms for biomass conversion into fuels and chemicals.

Bing, R.G., Willard, D. J., Crosby, J. R., Adams, M. W. W. and Kelly, R. M. 2023d. Whither the genus *Caldicellulosiruptor* and the Order Thermoanaerobacterales: Phylogeny, taxonomy, ecology, and phenotype. *Front. Microbiol*. **14,** 1212538 (doi: 10.3389/fmicb.2023.1212538)

The order Thermoanaerobacterales currently consists of fermentative anaerobic bacteria, including the genus *Caldicellulosiruptor*. *Caldicellulosiruptor* are represented by thirteen species; all, but one, have closed genome sequences. Interest in these extreme thermophiles has been motivated not only by their high optimal growth temperatures (≥70°C), but also by their ability to hydrolyze polysaccharides including, for some species, both xylan and microcrystalline cellulose. *Caldicellulosiruptor* species have been isolated from geographically diverse thermal terrestrial environments located in New Zealand, China, Russia, Iceland and North America. Evidence of their presence in other terrestrial locations is apparent from metagenomic signatures, including volcanic ash in permafrost. Here, phylogeny and taxonomy of the genus *Caldicellulosiruptor* was re-examined in light of new genome sequences. Based on genome analysis of 15 strains, a new order, Caldicellulosiruptorales, is proposed containing the family *Caldicellulosiruptoraceae*, consisting of two genera, *Caldicellulosiruptor* and *Anaerocellum*. Furthermore, the order Thermoanaerobacterales also was re-assessed, using 91 genome-sequenced strains, and should now include the family Thermoanaerobacteraceae containing the genera *Thermoanaerobacter, Thermoanaerobacterium, Caldanaerobacter,* the family Caldanaerobiaceae containing the genus *Caldanaerobius*, and the family Calorimonaceae containing the genus *Calorimonas*. A main outcome of ANI/AAI analysis indicates the need to reclassify several previously designated species in the Thermoanaerobacterales and Caldicellulosiruptorales by condensing them into strains of single species. Comparative genomics of carbohydrate-active enzyme inventories suggested differentiating phenotypic features, even among strains of the same species, reflecting available nutrients and ecological roles in their native biotopes.

Laemthong, T., Bing, R. G., Crosby, J. R., Manesh, M. J. H., Adams, M. W. W. and Kelly, R. M. 2023. Role of cell-substrate association during plant biomass solubilization by the extreme thermophile *Caldicellulosiruptor bescii. Extremophiles* **27,** 6 (doi: 10.1007/s00792-023-01290-7)

Caldicellulosiruptor species are proficient at solubilizing carbohydrates in lignocellulosic biomass through surface (S)-layer bound and secretomic glycoside hydrolases. Tāpirins, surface-associated, non-catalytic binding proteins in *Caldicellulosiruptor* species, bind tightly to microcrystalline cellulose, and likely play a key role in natural environments for scavenging scarce carbohydrates in hot springs. However, the question arises: If tāpirin concentration on *Caldicellulosiruptor* cell walls increased above native levels, would this offer any benefit to lignocellulose carbohydrate hydrolysis and, hence, biomass solubilization? This question was addressed by engineering the genes for tight-binding, non-native tāpirins into *C. bescii*. The engineered *C. bescii* strains bound more tightly to microcrystalline cellulose (Avicel) and biomass compared to the parent. However, tāpirin overexpression did not significantly improve solubilization or conversion for wheat straw or sugarcane bagasse. When incubated with poplar, the tāpirin-engineered strains increased solubilization by 10% compared to the parent, and corresponding acetate production, a measure of carbohydrate fermentation intensity, was 28% higher for the Calkr_0826 expression strain and 18.5% higher for the Calhy_0908 expression strain. These results show that enhanced binding to the substrate, beyond the native capability, did not improve *C. bescii* solubilization of plant biomass, but in some cases may improve conversion of released lignocellulose carbohydrates to fermentation products.

Lipscomb, G. L., Crowley, A., Nguyen, D. M., Keller, M. W., O,Quinn, H. C., Tanwee, T. N. N., Vailionis, J. L., Zhang, K., Zhang, Y., Kelly, R. M. and Adams, M. W. W. 2023. Manipulating fermentation pathways in the hyperthermophilic archaeon *Pyrococcus furious* for ethanol

production up to 95°C driven by carbon monoxide oxidation. *Appl. Environ. Microbiol.* May 10; e0001223. (doi: 10.1128/aem.00012-23)

Genetic engineering of hyperthermophilic organisms for the production of fuels and other useful chemicals is an emerging biotechnological opportunity. In particular, for volatile organic compounds such as ethanol, fermentation at high temperatures could allow for straightforward separation by direct distillation. Currently, the upper growth temperature limit for native ethanol producers is 72°C in the bacterium *Thermoanaerobacter ethanolicus* JW200, and the highest temperature for heterologously-engineered bioethanol production was recently demonstrated at 85°C in the archaeon *Pyrococcus furiosus*. Here, we describe an engineered strain of *P. furiosus* that synthesizes ethanol at 95°C, utilizing a homologously-expressed native alcohol dehydrogenase, termed AdhF. Ethanol biosynthesis was compared at 75°C and 95°C with various engineered strains. At lower temperatures, the acetaldehyde substrate for AdhF is most likely produced from acetate by aldehyde ferredoxin oxidoreductase (AOR). At higher temperatures, the effect of AOR on ethanol production is negligible, suggesting that acetaldehyde is produced by pyruvate ferredoxin oxidoreductase (POR) via oxidative decarboxylation of pyruvate, a reaction known to occur only at higher temperatures. Heterologous expression of a carbon monoxide dehydrogenase complex in the AdhF overexpression strain enabled it to use CO as a source of energy, leading to increased ethanol production. A genome reconstruction model for P. furiosus was developed to guide metabolic engineering strategies and understand outcomes. This work opens the door to the potential for 'bioreactive distillation' since fermentation can be performed well above the normal boiling point of ethanol. Previously, the highest temperature for biological ethanol production was 85°C. Here, we have engineered ethanol production at 95°C by the hyperthermophilic archaeon *Pyrococcus furiosus*. Using mutant strains, we showed that ethanol production occurs by different pathways at 75°C and 95°C. In addition, by heterologous expression of a carbon monoxide dehydrogenase complex, ethanol production by this organism was driven by the oxidation of carbon monoxide. A genome reconstruction model for *P. furiosus* was developed to guide metabolic engineering strategies and understand outcomes.

Vailionis, J. L., Zhao, W., Zhang, K., Rodionov, D. A., Lipscomb, G. L., Tanwee, T. N. N., O'Quinn, H. C., Kelly R. M., Adams, M. W. W. and Zhang, Y. 2023. Optimizing strategies for bio-based ethanol production using genome-scale metabolic modeling of the hyperthermophilic archaeon, *Pyrococcus furiosus*. *Appl. Environ. Microbiol.* **89**, e0056323 (doi: 10.1128/aem.00563-23)

A genome-scale metabolic model, encompassing a total of 623 genes, 727 reactions, and 865 metabolites, was developed for *Pyrococcus furiosus*, an archaeon that grows optimally at 100°C by carbohydrate and peptide fermentation. The model uses subsystem-based genome annotation, along with extensive manual curation of 237 gene-reaction associations including those involved in central carbon metabolism, amino acid metabolism, and energy metabolism. The redox and energy balance of *P. furiosus* was investigated through random sampling of flux distributions in the model during growth on disaccharides. The core energy balance of the model was shown to depend on high acetate production and the coupling of a sodium-dependent ATP synthase and membrane-bound hydrogenase, which generates a sodium gradient in a ferredoxin-dependent manner, aligning with existing understanding of P. furiosus metabolism. The model was utilized to inform genetic engineering designs that favor the production of ethanol over acetate by implementing an NADPH and CO-dependent energy economy. The P. furiosus model is a powerful tool for understanding the relationship between generation of end products and redox/energy balance at a systems-level that will aid in the design of optimal engineering strategies for production of bio-based chemicals and fuels. This work is important because the bio-based production of organic chemicals provides a sustainable alternative to fossil-based production in the face of today's climate challenges. In this work, we present a genome-scale metabolic reconstruction of *Pyrococcus furiosus*, a well-established platform organism that has

been engineered to produce a variety of chemicals and fuels. The metabolic model was used to design optimal engineering strategies to produce ethanol. The redox and energy balance of P. furiosus was examined in detail, which provided useful insights that will guide future engineering designs.

Crosby, J. R., Laemthong, T., Bing, R. G., Chen, S. L., Zhang, K., Tanwee, T. N. N., Lipscomb, G. L., Rodionov, D. A., Zhang, Y., Adams, M. W. W. and Kelly, R. M. 2023. Xylanolytic metabolism is regulated by coordination of transcription factors XynR and XylR in extremely thermophilic *Caldicellulosiruptor* species. *Appl. Environ. Microbiol*. (in revision)

Global transcription factors control metabolic processes in bacteria to efficiently utilize available carbon. The genus *Caldicellulosiruptor* has drawn interest due to the ability its members to degrade lignocellulosic biomass. Regulatory reconstruction of *C. bescii* identified two major global transcription factors for xylan utilization, XynR and XylR, and the corresponding putative transcription factor binding sites. Recombinant versions of XynR (LacI family) and XylR (ROK family) were produced and subjected to biolayer interferometry analysis to confirm binding sites. Four XynR sites and two XylR sites were validated, accounting for 20 of 26 genes regulated by XynR and 6 of 7 genes regulated by XylR. Kinetic titration series showed that XynR can bind as either a dimer or tetramer, with affinities (K_D) at 25°C in the 50-70 nM range. Bioinformatic analysis of the individual genes controlled by the two regulators showed an inter-dependent scheme for xylan conversion; the transport of xylooligosaccharides is dependent on XylR, while enzymes responsible for hydrolysis are controlled by both regulators. For xylose catabolism by the xylose isomerase-xylulose kinase, regulation is also split with XylR controlling XI and XynR controlling XK. The XynR/XylR regulator pair within *C. bescii* is conserved across 12 of the 13 sequenced species of *Caldicellulosiruptor*, suggesting similarities in regulating linear xylan conversion. In other xylanolytic thermophiles, XylR homologs control xylan degradation, compared to just seven out of 26 genes for *C. bescii*. These results show that two separate regulatory schemes (dual repression) are coordinated by *C. bescii* to effectively regulate hemicellulase inventory and xylan catabolism.