Combinatorial engineering of 3-hydroxypropionate production from hemicellulose hydrolysate

Rongming Liu[1]* （Rongming.liu@colorado.edu）, Liya Liang[1], Andrew Garst[2], Alaksh Choudhurya[1], Violeta Sànchez i Noguéc[3], Gregg T. Beckhamc[3], and **Ryan T. Gill[1]**

[1]Renewable and Sustainable Energy Institute, University of Colorado Boulder, Boulder, Colorado; [2]Muse Biotechnology Inc., Boulder, Colorado; and [3]National Bioenergy Center, National Renewable Energy Laboratory, Golden, Colorado

Project Goals: Engineering of strains for industrial production requires the targeted improvement of multiple complex traits ranging from pathway flux to tolerance to mixed sugar utilization. Here, we report the use of an iterative CRISPR EnAbled Trackable genome Engineering (iCREATE) method for generating targeted genomic modifications at high efficiency along with high throughput phenotypic screening and growth strategies to rapidly engineer multiple traits in *Escherichia coli*.

Advances in DNA synthesis and sequencing have motivated increasingly complex efforts for programming cells on laboratory timescales. Realization of such efforts requires dramatically improved strategies for the precise and efficient editing of genomes with methods that can be performed at throughputs compatible with the latest sequencing technologies. CRISPR EnAbled Trackable genome Engineering (CREATE) couples the high efficiency CRISPR editing with the massively multiplexed rational design offered by parallel oligomer synthesis. This technology enables a single researcher to generate hundreds of thousands of designer variants in a few days and to map each of these variants to a selected phenotype using the designed barcode. To meet complex lab and industrial environments, an iterative CREATE (iCREATE) strategy was constructed and tested in *E. coli* for enhancement of its sugar mixture utilization rate and tolerance of typical hydrolysate inhibitors. Optimal combinations identified were also tested for 3-hydroxypropionate (3HP) production.

After pretreatment of lignocellulose, glucose and xylose are the main carbon sources in the hydrolysate. In *E. coli,* glucose completely inhibits the uptake of xylose, thus limiting the conversion of sugars to product molecules in fermentation of cellulosic biomass. To solve this problem, deletion of *ptsHI* genes was applied in *E. coli* BG. As a result, the strain BGgx (BG, Δ*ptsHI*) can utilize glucose and xylose simultaneously. However, the glucose consumption rate was significantly lower than that of xylose during the culture. To obtain an efficient sugar mixture utilization strain, RBS Library of *galP* and *glk* gene was constructed in BGgx by iCREATE. After plasmid and genomic sequencing of the top 5 variants, the results showed that $BGgxk_4P_1$ and $BGgxk_5P_1$ were the positive variants

during first-round screening, with cell growth rates 36% higher than BG in 50-ml bioreactor tubes. A second round of iCREATE was then used for $BGgxk_4P_1$ and $BGgxk_5P_1$ after gRNA plasmid curing. Due to 80% editing efficiency with 410 of CFU/μl, another 3-fold more colonies than the design size were tested in 96-well plates. The results showed that $BGgxk_4P_4$ was the best strain in the second round of iCREATE after sequencing of the plasmids and targeting genes of the top 5 variants.

During the pretreatment of corn stover or corn stalk, side-reaction products (furfural, 5-hydroxymethylfurfural, formate, acetate, and soluble lignin products) are formed. Here, we used the iCREATE method to construct a 27-gene library about 40,000 mutations for enhancement of hydrolysate tolerance in $BGgxk_4P_4$. These targeted genes encompass the global high level regulators that regulate the central pathway of metabolism, the transcription factors that play important roles in genome level transcription, and enzymes that function in NAD(P)H metabolism and the aldehyde reduction system. After another two round iCREATE, the best producing quadruple mutant strain $BGHP_{ht}$ was tested under high furfural and high acetate hydrolysate fermentation, demonstrating a 6.3-7 fold increase in productivity relative to the parent strain.

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