Synthetic Biology of Novel Thermophilic Bacteria for Enhanced Production of Ethanol from 5-Carbon Sugars

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INTRODUCTION

The Problem: Energy Independence. Part of The Energy Solution … Plant Biomass

Bioethanol And Bottlenecks with State of the Art

- Optimal temperature for enzymatic saccharification is 50-60°C, optimal temperature for most ethanol fermentations is 30°C

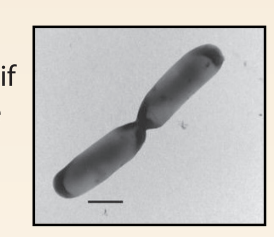
  Solution: Simultaneous Saccharification and Fermentation (SSF)

- Conventional fermentation organisms (Yeast) cannot process five-carbon sugars, reducing efficiency and increasing chance of process contamination.

  Solution: Organisms that utilize wide range of sugars for ethanol production

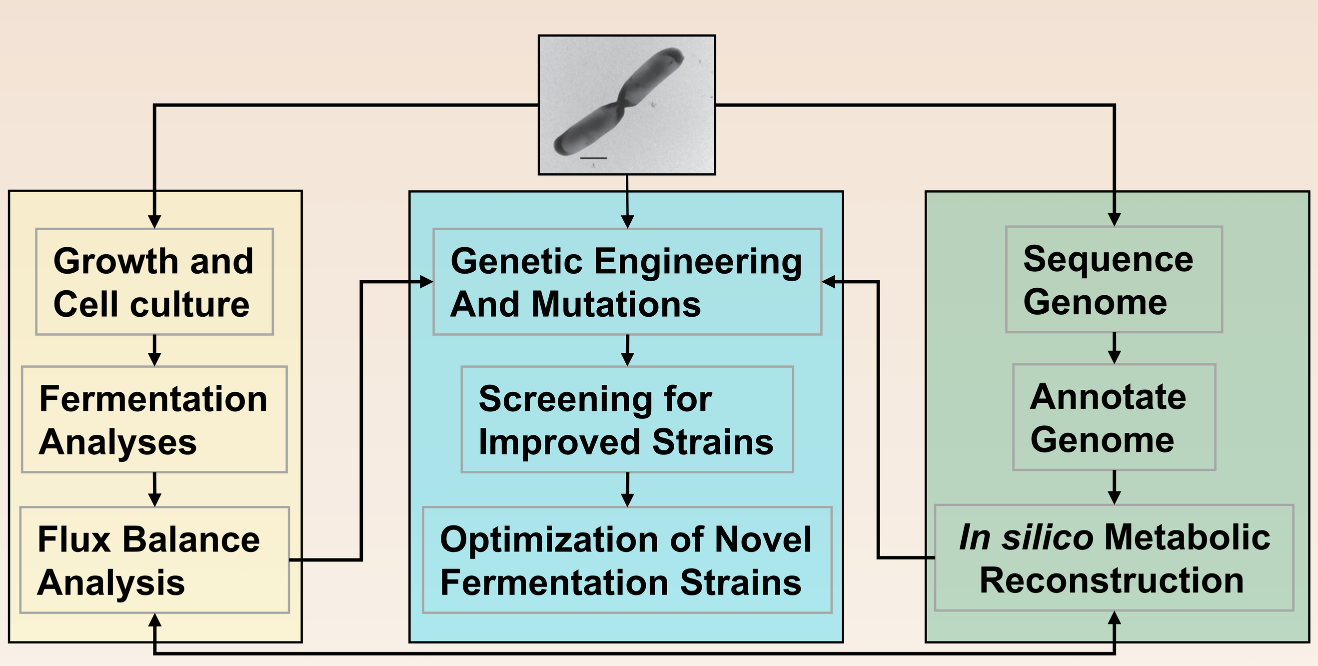
Our Proposed Solution: Engineer Thermophilic Bacteria for Ethanol Production

- Geobacillus thermoglucosidasius M10EXG grows optimally at ~60°C, is tolerant to 10% ethanol (v/v) and can grow in aerobic and anaerobic conditions
  - All other known thermophilic bacteria are tolerant to <4% (v/v) ethanol
- Produces ethanol, acetate, lactate, from five and six-carbon sugars
- Excellent candidate for SSF if engineered to overproduce ethanol



APPROACH

Synthetic Biology: Interdisciplinary Approach to Engineering Biological Systems



- G. *thermoglucosidasius* (Gth) M10EXG ferments wide range of sugars C5 sugars (xylose)
  - Fermentation enzyme already present in Gth M10EXG
- Metabolic engineering of bacterial pathways is currently employed for production of preferred molecules (Keasling, Nature 2006)
  - *Gth* M10EXG has the necessary prerequisites for metabolic engineering
- Engineering thermotolerance in a whole organism is difficult (~impossible)
  - *Gth* M10EXG grown optimally at 60°C
- Ethanol tolerance is dependent on multiple parameters — nature of membranes, membrane pumps for ethanol, enzymes and membrane proteins.
  - *Gth* M10EXG has the highest tolerance for ethanol among all known thermophilic bacteria

RESULTS

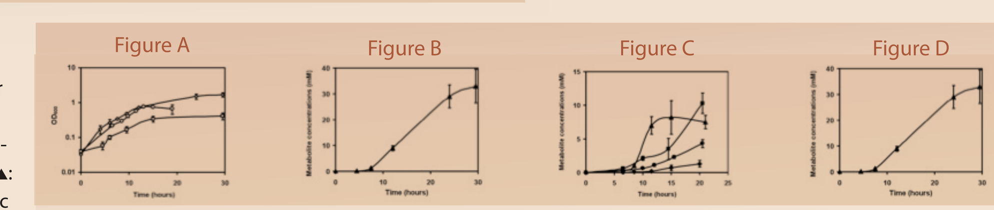
Growth kinetics and yield of Geobacillus thermoglucosidasius M10EXG

under three oxygen conditions (+O_2: aerobic growth, + μO_2: micro-aerobic growth, -O_2: anaerobic growth).

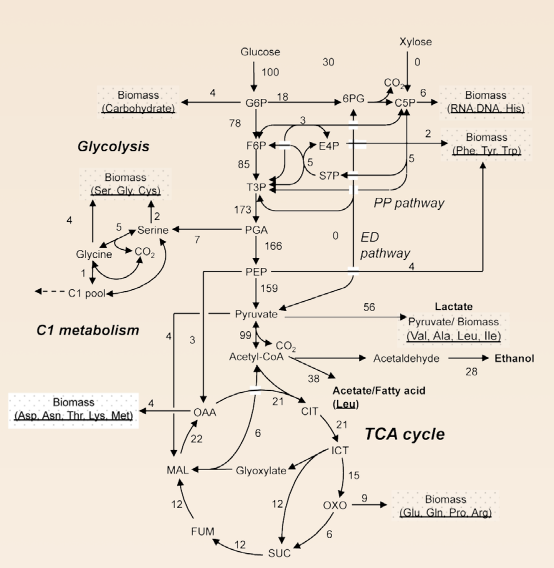
Yield[1]	G + O_2	G + μO_2	G-O_2[2]	Max[3]
Y_{acet}	0.64±0.12	0.40±0.05	0.62±0.11	2.6
Y_{lact}	0.02±0.01	0.67±0.07	0.81±0.07	2
Y_{etha}	0.01±0.01	0.28±0.04	0.32±0.05	2
Y_{form}	0	0.13±0.05	1.03±0.15	5.6
$Y_{biomass}$	0.27±0.05	0.19±0.04	0.08±0.03	0.34
Growth rate, hr^{-1}	0.31±0.04	0.20±0.04	0.13±0.03	0.44

1. Metabolite yield unit, mol metabolites mol^{-1} glucose. Biomass yield unit, g biomass g^{-1} glucose.
2. Small amount of succinate was also detected.
3. Max yield for each metabolite was predicted by single optimization using Simpheny. The model assumed the glucose uptake rate equal to 5mM $hr^{-1}g^{-1}$ biomss.

Biomass growth. Fig A (○: aerobic; ◇: micro-aerobic; □: anaerobic); Extra-cellular metabolite production. Fig. B: aerobic; Fig. C: micro-aerobic; Fig. D: anaerobic (▲: acetate; ■: Lactate; ♦: formic acid; ●: ethanol).



Flux Balance Analysis of Glucose Metabolism Under Micro-aerobic Growth by G. *thermoglucosidasius* M10EXG.

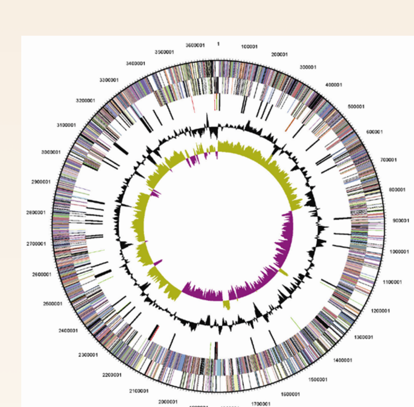


The amino acids used for isotopomer models are in parenthesis. Numbers denote the arbitrary flux indices used in modeling the pathways.
Abbreviations:

ACoA, acetyl-coenzyme A;
CIT, citrate;
E4P, erythrose-4-phosphate;
C1, 5,10-Me-THF;
F6P, fructose-6-phosphate;
G6P, glucose-6-phosphate;
6PG, 6-phosphogluconate;
ICT, isocitrate;
MAL, malate;
OAA, oxaloacetate; OXO, 2-oxoglutarate;
PEP, phosphoenolpyruvate;
PGA, 3-phosphoglycerate;
C5P, ribose-5-phosphate (or ribulose-5-phosphate or xylose-5-phosphate);
S7P, sedoheptulose-7-phosphate; SUC, succinate;
T3P, triose-3-phosphate;
PYR, pyruvate.

The pathway information gleaned will help us better design synthetic pathways

G. *thermoglucosidasius* M10EXG Chromosomal Map



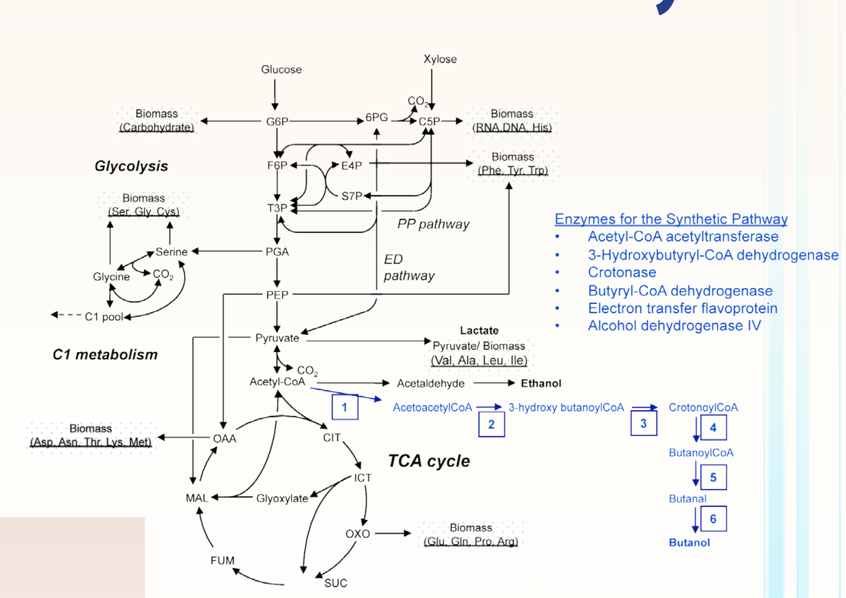
Genome features	*G. thermoglucosidasius* M10EXG	*G. kaustophilus* HTA426	*G. thermodenitrificans* NG80-2
Chromosome size	3773252	3592666	3608012
Protein coding regions	98.51%	95.52%	96.74%
G+C content	43.72%	52%	48.86%
tRNAs	66	87	87
rRNAs	8	9	11
Average ORF length	750 bp	862 bp	871 bp
Open reading frames	4354	3540	3471
Genes belonging to families in paralogs	660	1692	1623
Putative function assigned	72.69%	52.34	65.16%
Unknown function	25.81%	42.97%	30.15%
CRISPR count	7	3	2

Chromosomal map of genes (From outside of the circular chromosome to the center) 1.Genes on forward strand (color by COG categories), 2. Genes on reverse strand (color by COG categories), 3. RNA genes (tRNAs green, sRNAs red, other RNAs black)4. GC content5. GC skew

COG Code	COG Function Definition
[A]	RNA processing and modification
[B]	Chromatin structure and dynamics
[C]	Energy production and conversion
[D]	Cell cycle control, cell division, chromosome partitioning
[E]	Amino acid transport and metabolism
[F]	Nucleotide transport and metabolism
[G]	Carbohydrate transport and metabolism
[H]	Coenzyme transport and metabolism
[I]	Lipid transport and metabolism
[J]	Translation, ribosomal structure and biogenesis
[K]	Transcription
[L]	Replication, recombination and repair

[M]	Cell wall/membrane/envelope biogenesis
[N]	Cell motility
[O]	Posttranslational modification, protein turnover, chaperones
[P]	Inorganic ion transport and metabolism
[Q]	Secondary metabolites biosynthesis, transport and catabolism
[R]	General function prediction only
[S]	Function unknown
[T]	Signal transduction mechanisms
[U]	Intracellular trafficking, secretion, and vesicular transport
[V]	Defense mechanisms
[W]	Extracellular structures
[Y]	Nuclear structure
[Z]	Cytoskeleton

Fuel Map: Engineering Butanol Production Pathway



SIGNIFICANCE

- Development of bacterial fermentation at high temperatures. Yeast cannot ferment C-5 sugars while bacteria can utilize C5 and C6 sugars.
- High temperature fermentations are compatible with and ideal for Simultaneous Saccharification and Fermentation (SSF) and Consolidated Bioprocessing (CBP) for fuel production & recovery from biomass.
- Improvement in the conversion and recovery of lignocellulose biomass to ethanol.
- Engineered thermophilic platform can be used for development of second-generation hydrocarbon biofuels
- Project goals and approach fits well with the DOE mission and program for 5/10/15 year goals:
  - '…SSF in thermophilic bacteria for C5 and C6 sugars.'
- Development of cutting edge methods and protocols
  - ^{13}C isotopomer analysis for FBA is a state-of-the-art HTP method of flux pathways.
  - Pathway engineering in thermophilic microorganisms- new genetic tools.
  - Metabolic and Kinetic Modeling to rationally engineer flux towards ethanol production

Strengthening America's Infrastructure Security

NNSA National Nuclear Security Administration

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