Linking Overflow Metabolism and Growth Cessation in *Clostridium thermocellum* DSM1313 during High Cellulose Loading Fermentations

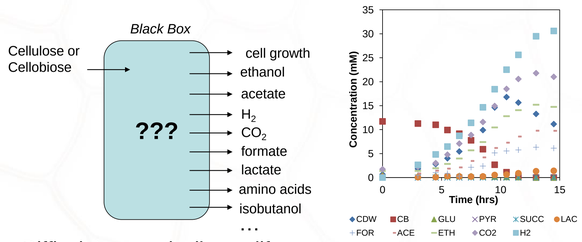
R. Adam Thompson[2,3], Sergio Garcia[1,2], and Cong T. Trinh[1,2,3]

[1] Department of Chemical and Biomolecular Engineering, University of Tennessee; Knoxville, Tennessee; [2]BioEnergy Science Center, Oak Ridge, Tennessee; [3]Bredesen Center for Interdisciplinary Research and Education

Abstract: As a model thermophilic bacterium for the production of second-generation biofuels, the metabolism of *Clostridium thermocellum* has been widely studied. However, most studies have characterized C. thermocellum metabolism for growth on soluble model substrates (i.e. cellobiose) and at relatively low substrate concentrations. This narrow outlook is not industrially relevant, however, as commercial viability requires substrate loadings of at least 100 g/L cellulosic materials. Recently, wild-type C. thermocellum was cultured on high cellulose loading batch fermentations and reported to produce a wide range of fermentative products not seen at lower substrate concentrations, opening the door for a more in-depth analysis of how this organism will behave in industrially relevant conditions. In this work, we elucidated the interconnectedness of overflow metabolism and growth cessation in C. thermocellum during high cellulose loading batch fermentations. Metabolic flux and thermodynamic analyses suggested that hydrogen and formate accumulation perturbed the complex redox metabolism and limited conversion of pyruvate to acetyl-CoA conversion, likely leading to overflow metabolism and growth cessation in C. thermocellum. Pyruvate formate lyase (PFL) acts as an important redox valve and is inhibited by formate accumulation. Finally, we demonstrated that manipulation of fermentation conditions to alleviate hydrogen accumulation could dramatically alter the fate of pyruvate, providing valuable insight into process design for enhanced *C. thermocellum* production of chemicals and biofuels.

1. Experimental Background
High Substrate Loading Fermentations

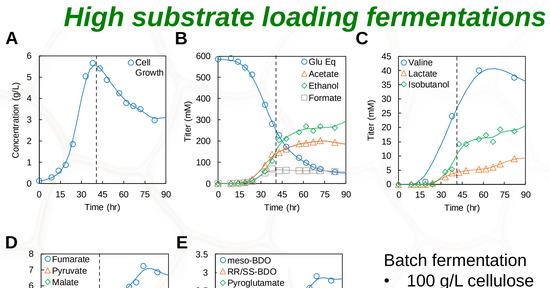
Clostridium thermocellum



- Difficult to genetically modify
- Branched metabolism gives a distribution of products
- Plenty of questions remain about its metabolism

Question: How can we elucidate, predict, and control cellular metabolism?
Approach: Employ metabolic network modeling and analysis

High substrate loading fermentations



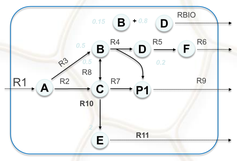
Batch fermentation
- 100 g/L cellulose
- pH7 control
- No sparging

Data reproduced from Holwerda *et al*, Biotechnol Biofuels, 2014

Key questions are:
1) Why did cell growth stop around 30-40 h?
2) What caused the formation of reduced metabolites?
3) What is the link between overflow metabolism and growth cessation?
4) How can overflow metabolism be controlled to enhance production of target chemicals?

2. Modeling Background
iAT601 Genome Scale Metabolic Model

Constraint Based Modeling of Metabolism



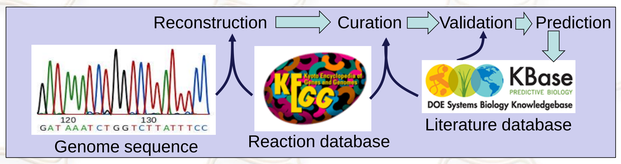
S_{ij} ≔ Stoichiometric coefficient of metabolite i in reaction j
r_j ≔ Flux of reaction j (mmol/gCDW/hr)
C_i ≔ Concentration of metabolite i
l_j ≔ Lower flux bound of reaction j
u_j ≔ Upper flux bound of reaction j

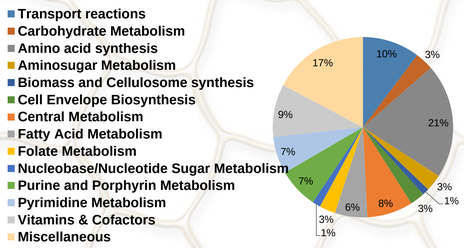
$$\sum_{j\in} S_{ij} r_j = \frac{dC_i}{dt} = 0 \text{ for all } i \in Metabolites \quad \text{Mass balance}$$

$$l_j \le r_j \le u_j \text{ for all } j \in Reactions \quad \text{Reaction rate bounds}$$

The model can predict reaction fluxes by:
- Optimizing an objective function (e.g. growth rate)
- Homogeneously sampling the solution space to create an ensemble of feasible flux distributions. In this study, to circumvent the single solution limitation of FBA and bias of using an objective growth function, we used the flux sampling approach to predict flux distribution throughout batch fermentation.

*i*AT601



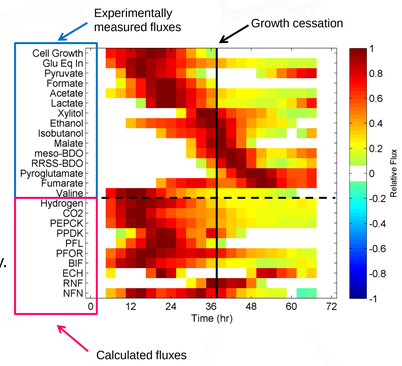


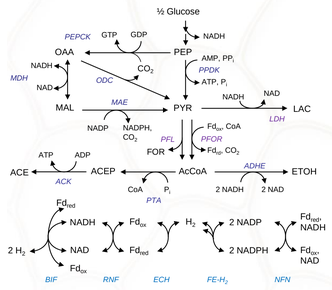
The refined genome scale model consists of 871 reactions encoded by 601 genes, spanning multiple KEGG ontology categories

3. Quantitative Phenotype Analysis
Metabolic Modeling and Thermodynamic Calculations

Dynamic flux analysis during high substrate loading fermentations

- Experimentally measured fluxes, that were normalized with their respective maximum values, varied throughout the fermentations
- Intracellular fluxes at key nodes such as pyruvate and acetyl CoA varied accordingly.
- Restructuring of redox metabolism were also observed.
- Notably, at growth cessation, hydrogen and PFL fluxes were stalled.



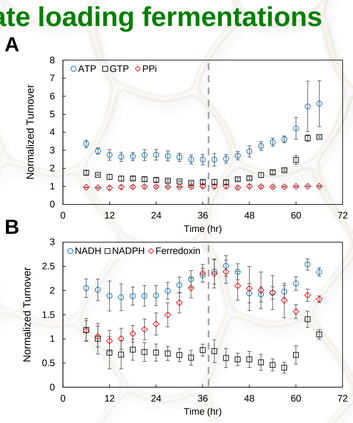


Distribution of global cofactors and ATP during high substrate loading fermentations

- Turnover rate Φ_i of a metabolite i determines how frequent that metabolite is biologically transformed and recycled at a given steady state.

$$\Phi_i = 0.5 \sum_j |s_{i,j} \cdot r_j|$$

- Cofactors (ATP, GTP, PPi) were fairly consistent throughout the batch until late stationary phase where ATP and GTP increased.
- NADH and ferredoxin turnover rates increased as cell growth halted, with the ferredoxin turnover increasing roughly two-folds. This phenomenon suggests rewiring of redox metabolism.



Thermodynamic driving force of key metabolic reactions

Thermodynamic analysis

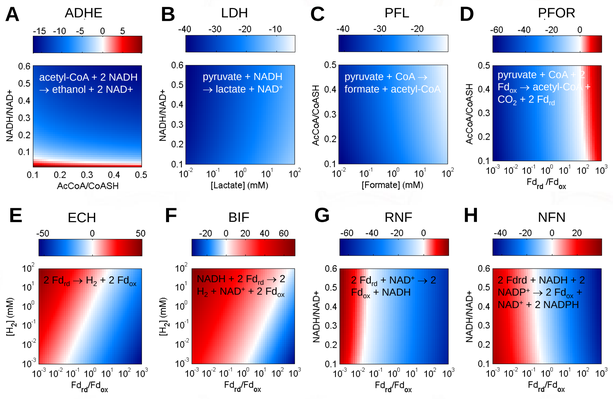
Given ϑ_A A + ϑ_B B $\underset{J^-}{\overset{J^+}{\rightleftarrows}}$ ϑ_C C + ϑ_D D

Gibbs free energy $\Delta_r G = \Delta_r G'^m + RTln\left(\prod_i C_i^{\vartheta_i}\right)$

Driving Force = $\frac{J^+ - J^-}{J^+} = 1 - exp\left(\frac{\Delta_r G}{RT}\right)$

If $\Delta_r G = -7.5 \frac{KJ}{mol}$, driving Force = 99%

- Key reactions around acetyl CoA and pyruvate nodes as well as redox reactions. Reactions ADHE, LDH, PFL and PFOR derived from acetyl-CoA and pyruvate are in general thermodynamically favorable while redox reactions ECH, BIF, RNF, NFN vary depending the redox states of the cell.
- From this analysis, thermodynamic constraint is not responsible for stall of PFL flux. So what truly caused the stall of PFL flux?

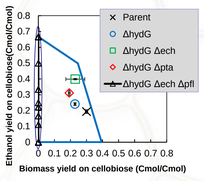


What caused growth cessation?

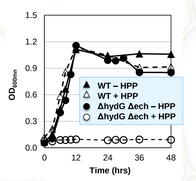
Experimental data shows large increase in formate production when Hydrogen is removed

Elementary mode analysis predicts elimination of hydrogen gas and formate production (ΔhydG Δech Δpfl) can't grow

Model Predictions



Experimental Confirmation
(HPP a Pfl inhibitor)



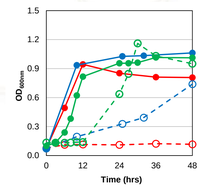
Probing Redox Bottlenecks using ΔhydG Δech strains and HPP[3]

***Exogenous electron acceptors*:**

SO4: SO4 + 2 Fdred = H2S + 2 Fdox

KIV: KIV + Fdox + 2 NADPH = iBuOH + CO2 + Fdred + 2 NADP

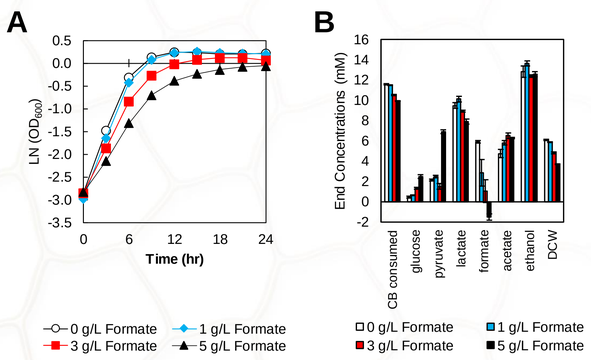
FUM: FUM + NADH = SUCC + NAD



1. Rate-limiting step in ethanol production is most likely the conversion of Fd_{red} to NAD(P)H
2. PFL functions as a redox valve
3. Stall of H2 and formate fluxes caused growth cessation due to redox imbalance.

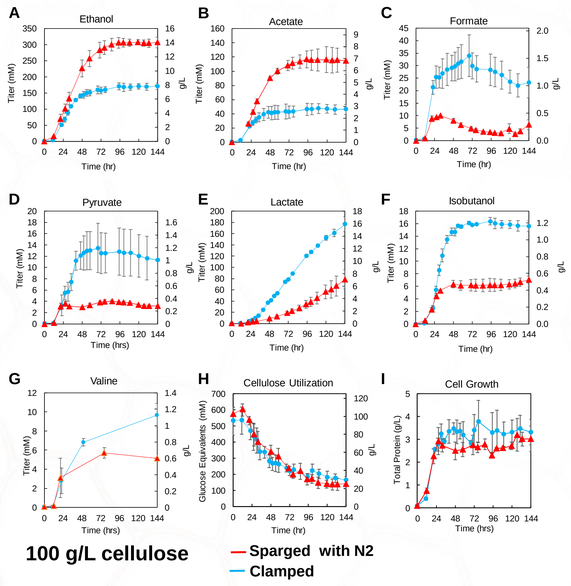
4. Experimental Validation of Modeling Inspired Hypotheses
Hydrogen Accumulation Dramatically Alters the Fate of Pyruvate

Effect of formate addition



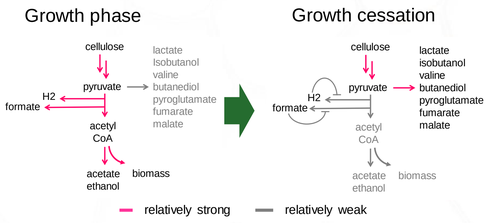
- The end concentrations of formate demonstrate the thermodynamic inhibition of PFL.
- To further validate the proposed mechanism, we seek to demonstrate the enhanced conversion of pyruvate to acetyl CoA.
- A simple experiment to perform is to relieve H2 accumulation by operating fermentation under the sparged conditions.

Enhance conversion of pyruvate to acetyl CoA by relieving redox imbalance



- Sparged: Increase conversion of pyruvate to acetyl CoA, leading to higher ethanol and acetate.
- Clamped: Decrease conversion of pyruvate to acetyl CoA, leading to formate, pyruvate, lactate, isobutanol, and valine.

Proposed mechanism of growth cessation and overflow metabolism



Highlights

- Metabolic flux and thermodynamic analyses revealed pyruvate formate lyase (PFL) inhibition and redox imbalance caused inefficient conversion of pyruvate to acetyl-CoA.
- Limited conversion of pyruvate to acetyl-CoA led to overflow metabolism and growth cessation in *C. thermocellum.*
- Pyruvate formate lyase (PFL) acts as a redox valve and is inhibited by formate accumulation.
- Nitrogen sparging to relieve hydrogen accumulation enhanced conversion of pyruvate to acetyl-CoA and hence biosynthesis of ethanol and acetate.
- Without sparging, wild-type *C. thermocellum* was able to produce 15.7 g/L lactate from cellulose.

References

1. Holwerda, Evert K., et al. "The exometabolome of Clostridium thermocellum reveals overflow metabolism at high cellulose loading." *Biotechnology for biofuels* 7.1 (2014): 155.
2. Thompson, R. Adam, et al. "Exploring complex cellular phenotypes and model-guided strain design with a novel genome-scale metabolic model of *Clostridium thermocellum* DSM 1313 implementing an adjustable cellulosome." *Biotechnology for biofuels* 9.1 (2016): 194.
3. Thompson, R. Adam, et al. "Elucidating central metabolic redox obstacles hindering ethanol production in *Clostridium thermocellum*." *Metabolic engineering* 32 (2015): 207-219.
4. http://equilibrator.weizmann.ac.il/
5. Thompson and Trinh, "Linking Overflow Metabolism and Growth Cessation in *Clostridium thermocellum* DSM1313 during High Cellulose Loading Fermentations "Biotechnol Bioeng, in press.

The BioEnergy Science Center (BESC) is a U.S. Department of Energy Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science.