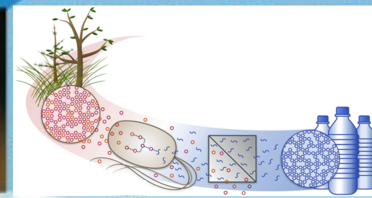
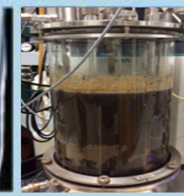







Purified Cell-Free Systems as a Metabolic Engineering and Biochemicals Production Platform

SIMB Annual Meeting

Joseph A. Rollin, Christopher W. Johnson, Peter St. John, Gregg T. Beckham

National Renewable Energy Laboratory

30 July 2017

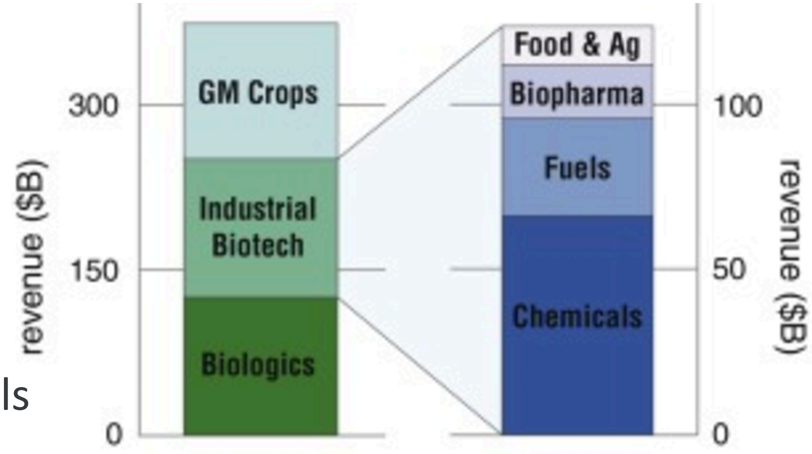
Outline

- The Promise of Industrial Synthetic Biology
- Cell-Free as a Bioconversion Platform
    - Lysate, Purified, and Hybrid Systems
    - Example Application: Biomass Sugars to H_2
    - Key Metrics
    - Challenges and Opportunities
- Cell-Free Metabolic Engineering

Promise of Industrial Synthetic Biology

Industrial applications

- Current:
  - Energy- and carbon-efficient conversions
  - Novel platform molecules
  - Waste stream utilization
    "vast difference: a mixture of valuable chemicals and a valuable mixture of chemicals"

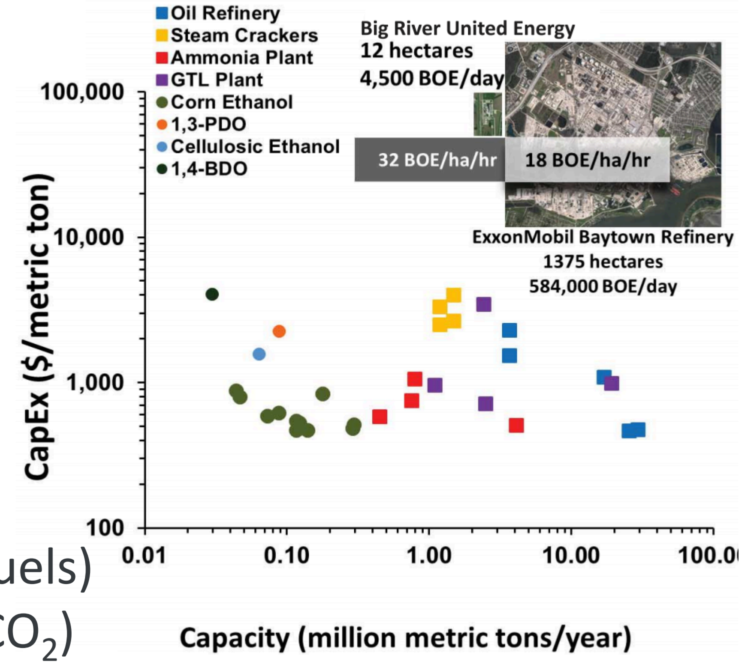


Rogers et al. (2016) Curr Op Biotechnol

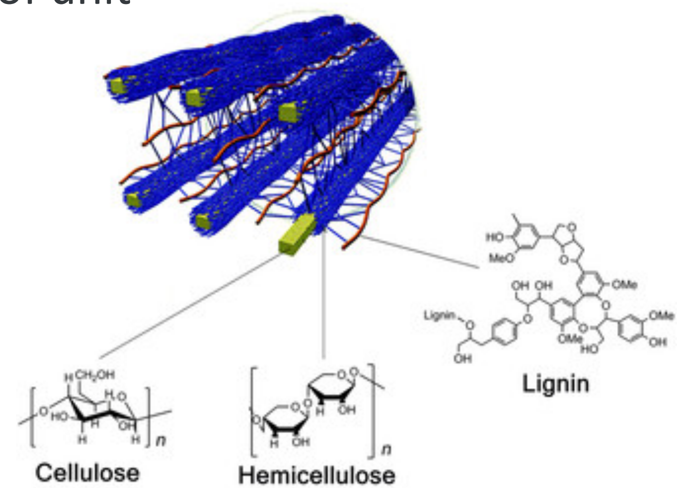
Promise of Industrial Synthetic Biology

Industrial applications

- Current:
  - Energy- and carbon-efficient conversions
  - Novel platform molecules
  - Waste stream utilization
    "vast difference: a mixture of valuable chemicals and a valuable mixture of chemicals"
- Long-term:
  - All C-based synthetic materials (including biofuels)
  - Renewable C is distributed (biomass, biogas, CO_2)
    - Biological conversions benefit from economy of unit number: CapEx efficient at small scale
  - Heterologous feedstocks
    - Selective removal of functionality required
    - Average oxidation state ~0



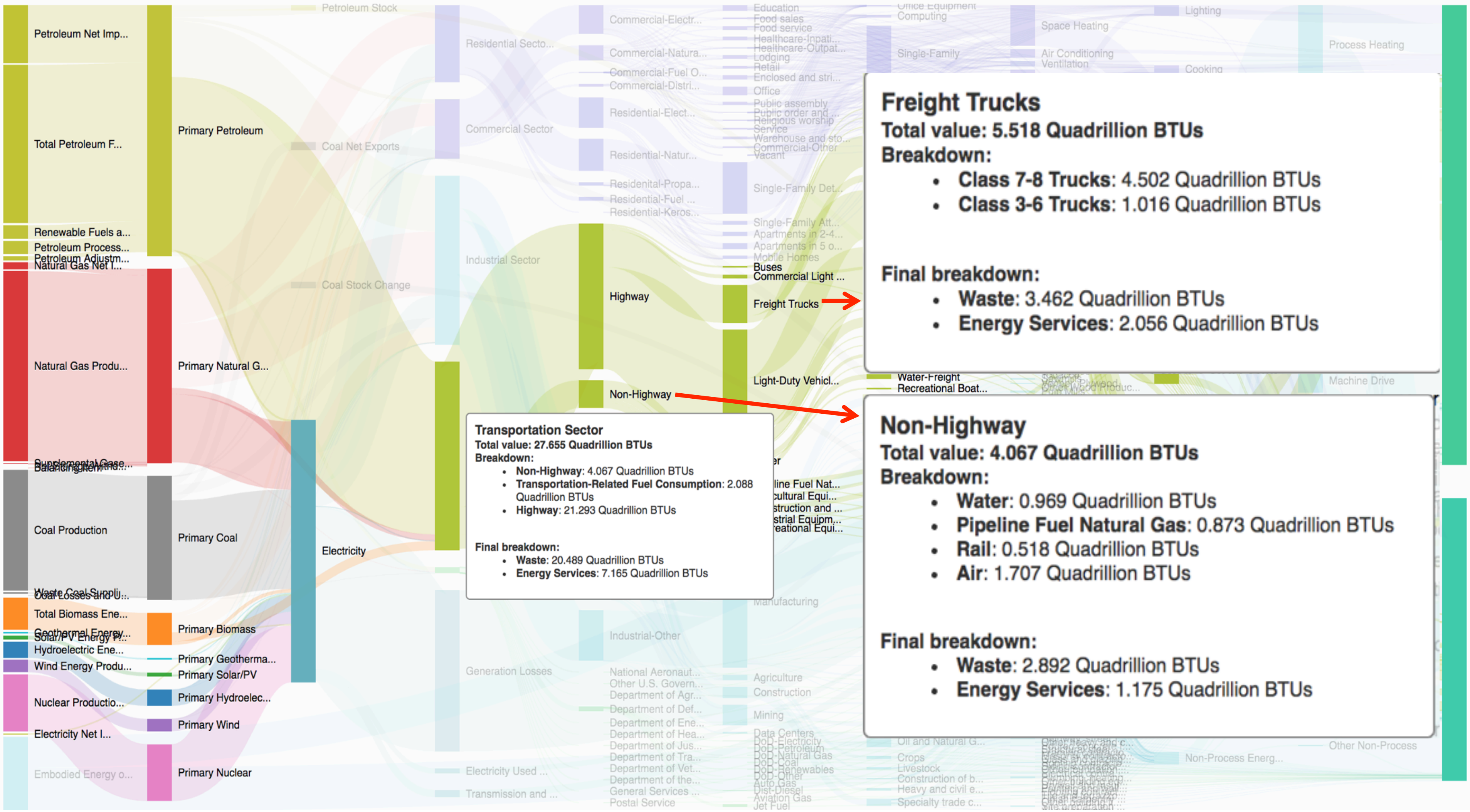
Clomburg et al. (2017) Science



Xu et al. (2016) Energy Technol

Enormous possible impact – potential to mitigate >15% of US emissions

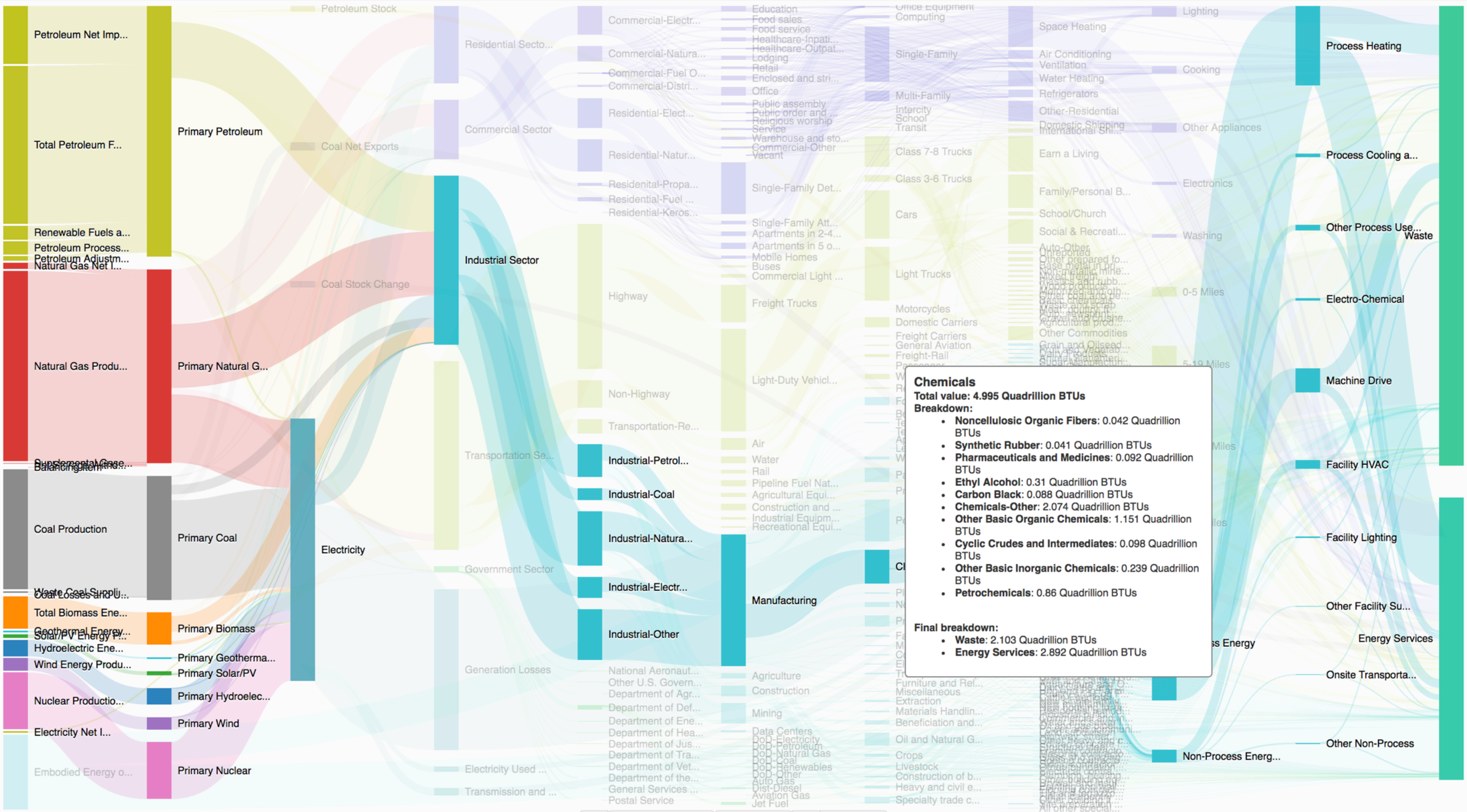
Transportation (Non-light duty): ~9% of US Energy Use



energyliteracy.com

Chemical Production: **5% of US Energy Use**

…plus 1% of additional emissions



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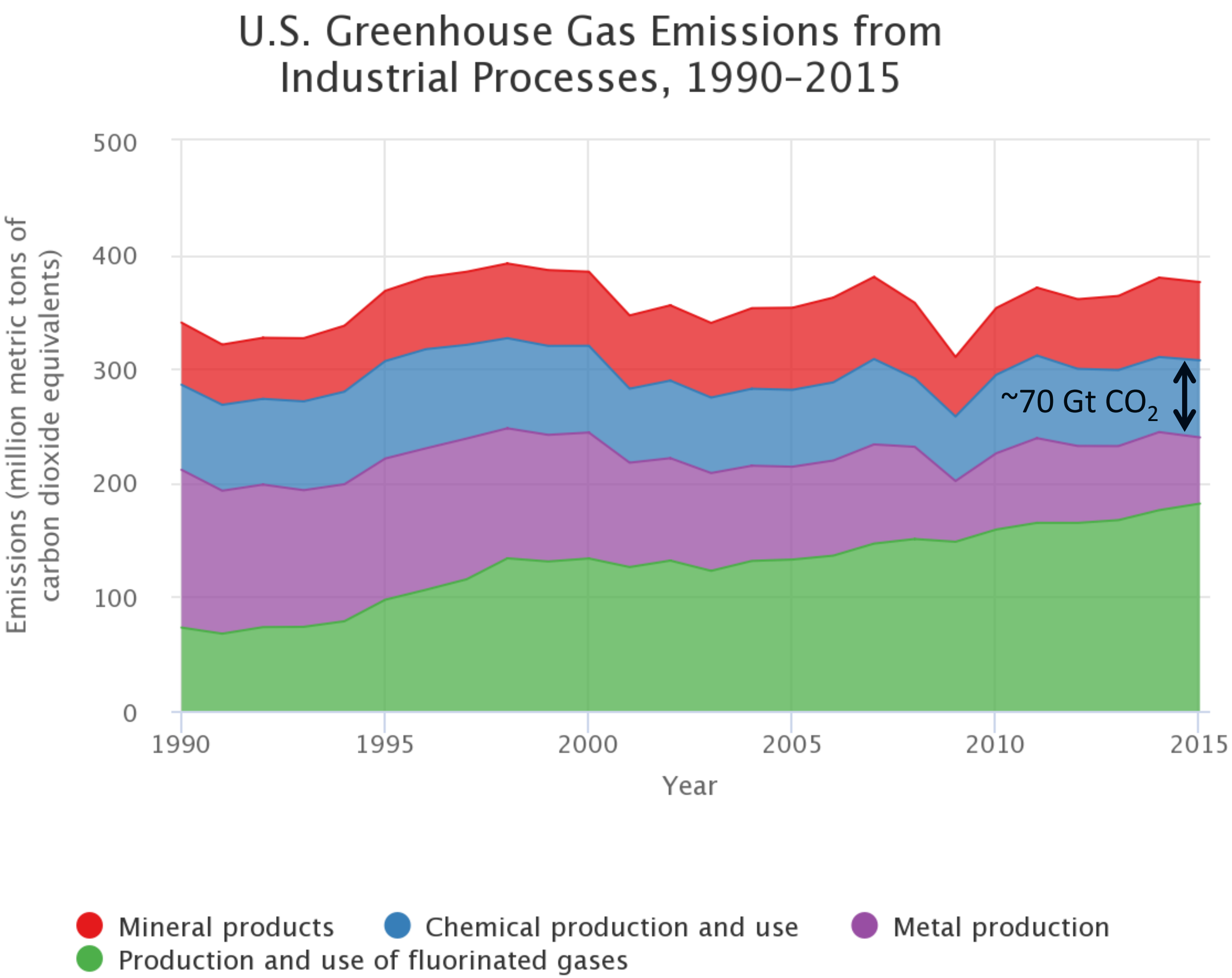
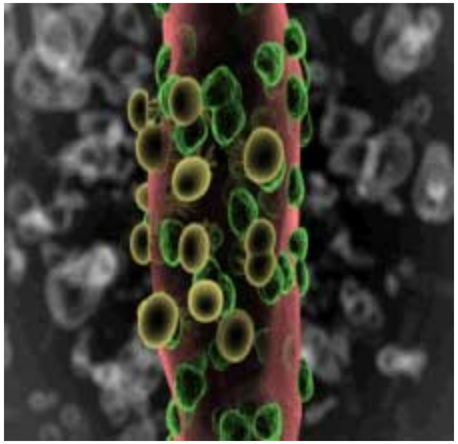


Source: US EPA's Inventory of US Greenhouse Gas Emissions and Sinks: 1999-2015
https://www.epa.gov/ghgemissions/inventory-us-greenhouse-gas-emissions-and-sinks

Cell-Free Bioconversion

Bioconversions – production of value-added compounds using biocatalysts

→ Microbial



Guedes et al. (2007) *Anim. Feed. Sci. Techno.*

Microbial Fermentation

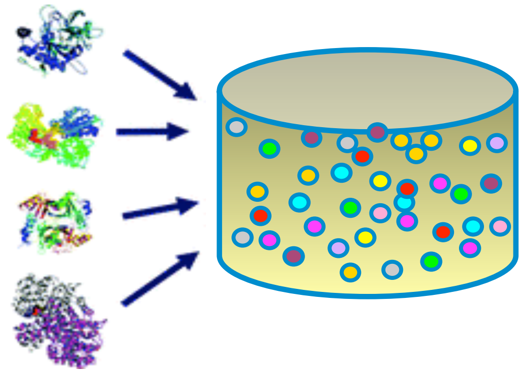
- Dominant biomanufacturing platform
- High engineering complexity
  - Self-replicating / self-repair
  - Natural system – evolves for maximum growth rate (opposed to engineering objective)
- Membrane-enclosed
  - Transport required
  - Solvent toxicity

Cell-Free Bioconversion

Bioconversions – production of value-added compounds using biocatalysts

Microbial

Cell-free



Zhang. (2011) ACS Catalysis 1: 998

Cell-Free Bioconversion

- Fast reaction rate
- Broad reaction conditions (solvent tolerance, reduced effect of toxins, temp., pH, etc.)
- Complete orthogonality
  –> high yield
- Challenge: unstable, expensive co-factors
  –> Pathway design
  –> Cofactor engineering

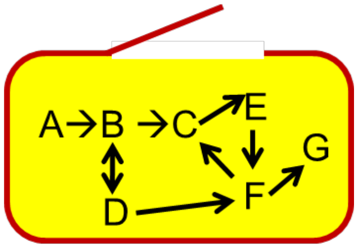
Cell-Free Bioconversion

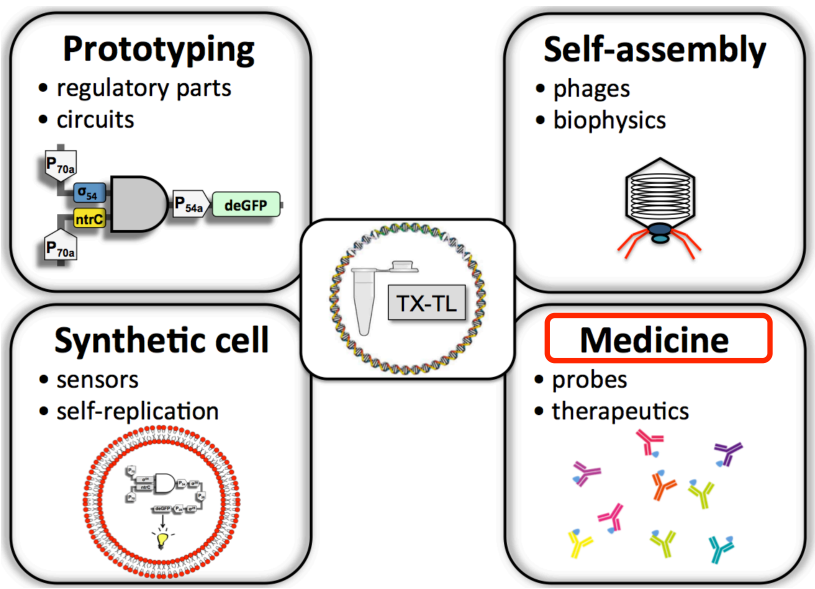
Bioconversions – production of value-added compounds using biocatalysts

Microbial

Cell-free

Cell extract-based





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SUTRO BIOPHARMA

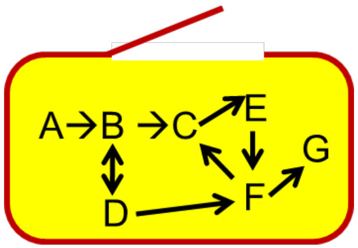
Cell-Free Bioconversion

Bioconversions – production of value-added compounds using biocatalysts

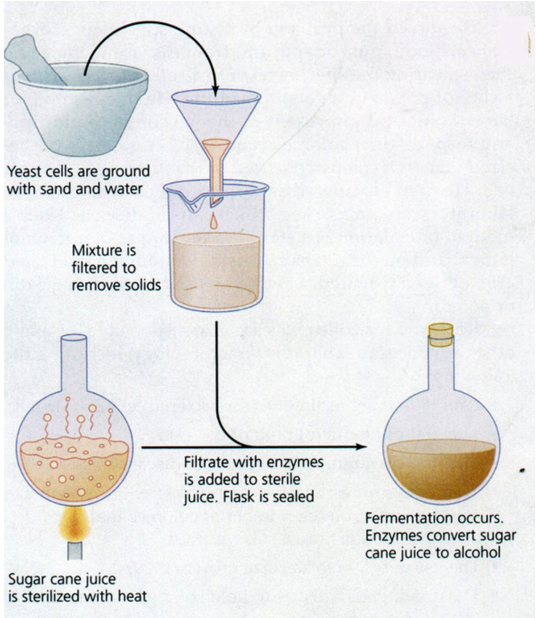
Microbial

Cell-free

Cell extract-based



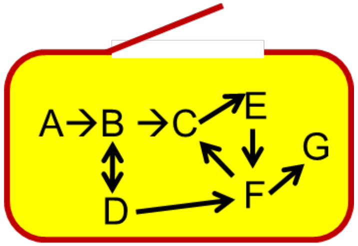
Cell-Free Ethanol Fermentation
Eduard Buchner
Nobel Prize in Chemistry, 1907

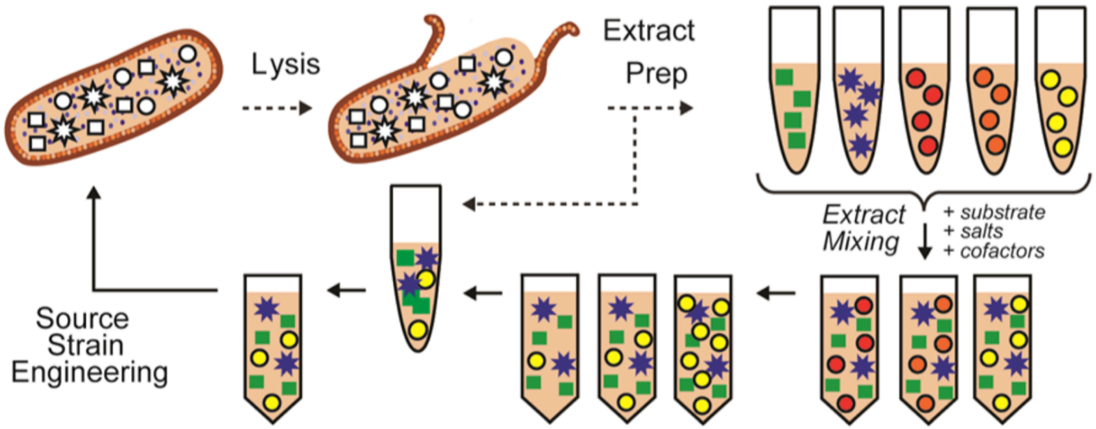


Cell-Free Bioconversion

Bioconversions – production of value-added compounds using biocatalysts

- Microbial
- **Cell-free**
  - Cell extract-based





greenlight BIOSCIENCES

Dudley QM et al. (2016) ACS Synth Biol 5, 1578-1588.

Cell-Free Bioconversion

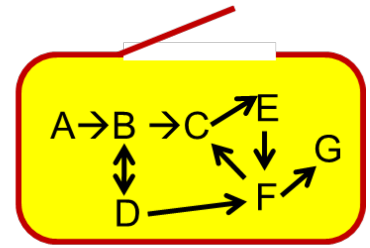
Bioconversions – production of value-added compounds using biocatalysts

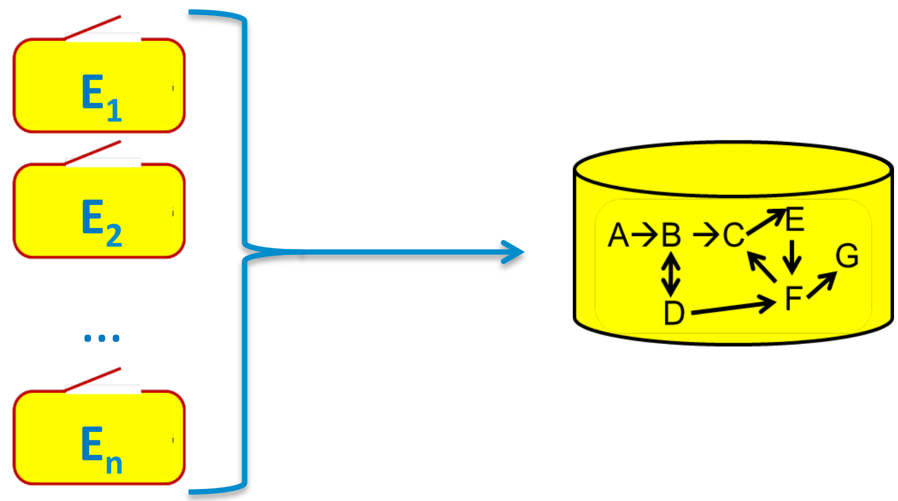
Microbial

Cell-free

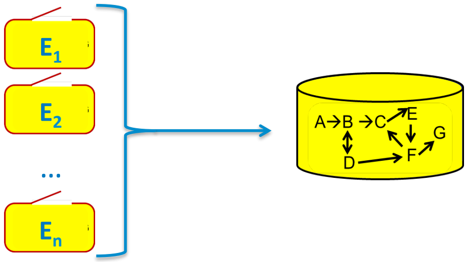
Cell extract-based

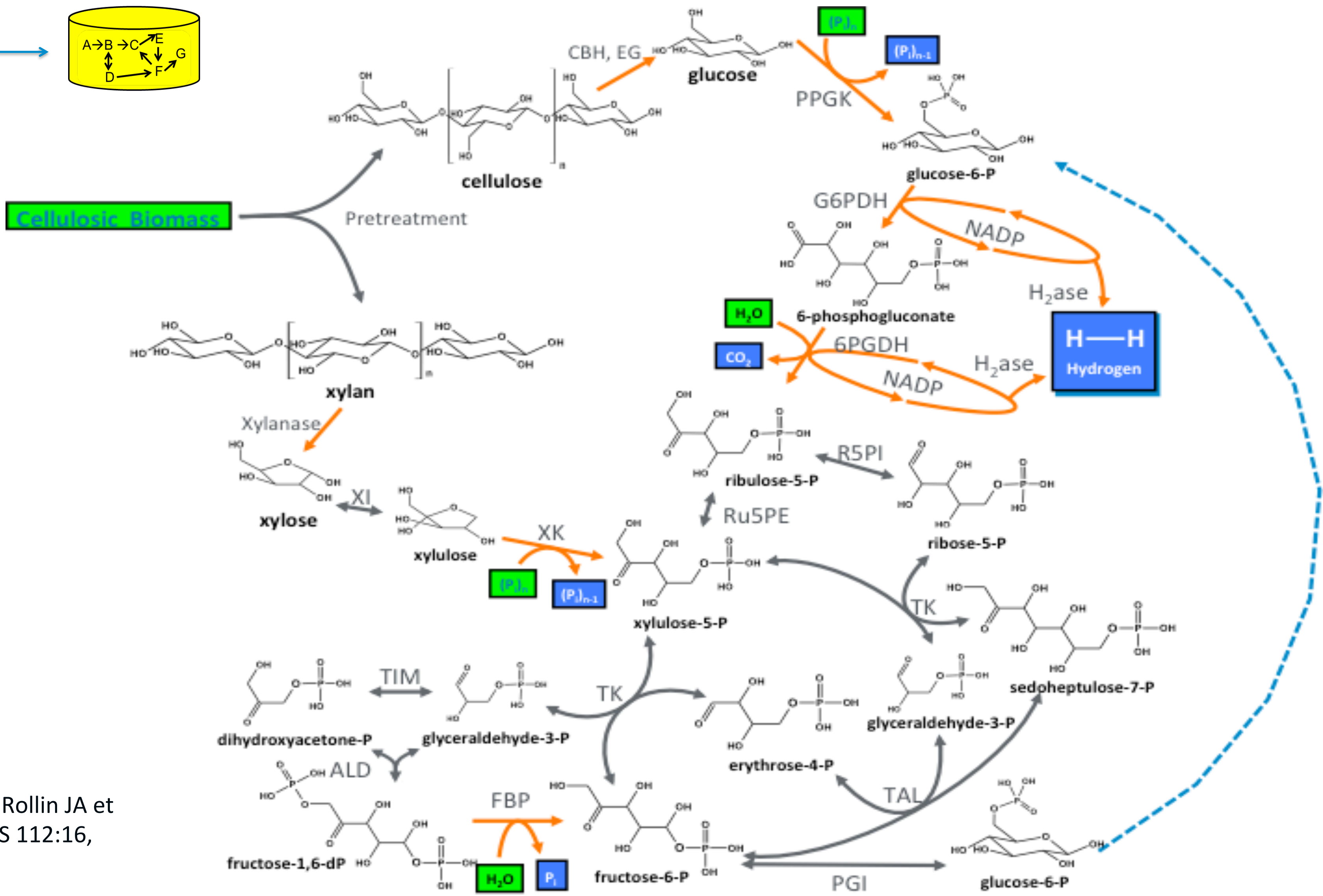
Purified enzyme-based





Purified Cell-Free Example: Biomass Sugars to H_2





Adapted from Rollin JA et al. (2015) PNAS 112:16, 4964-4969.

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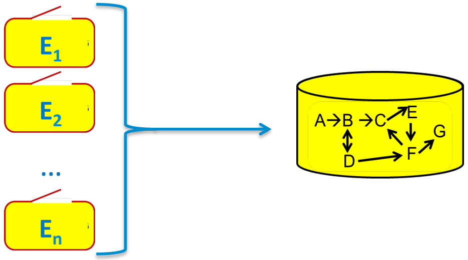
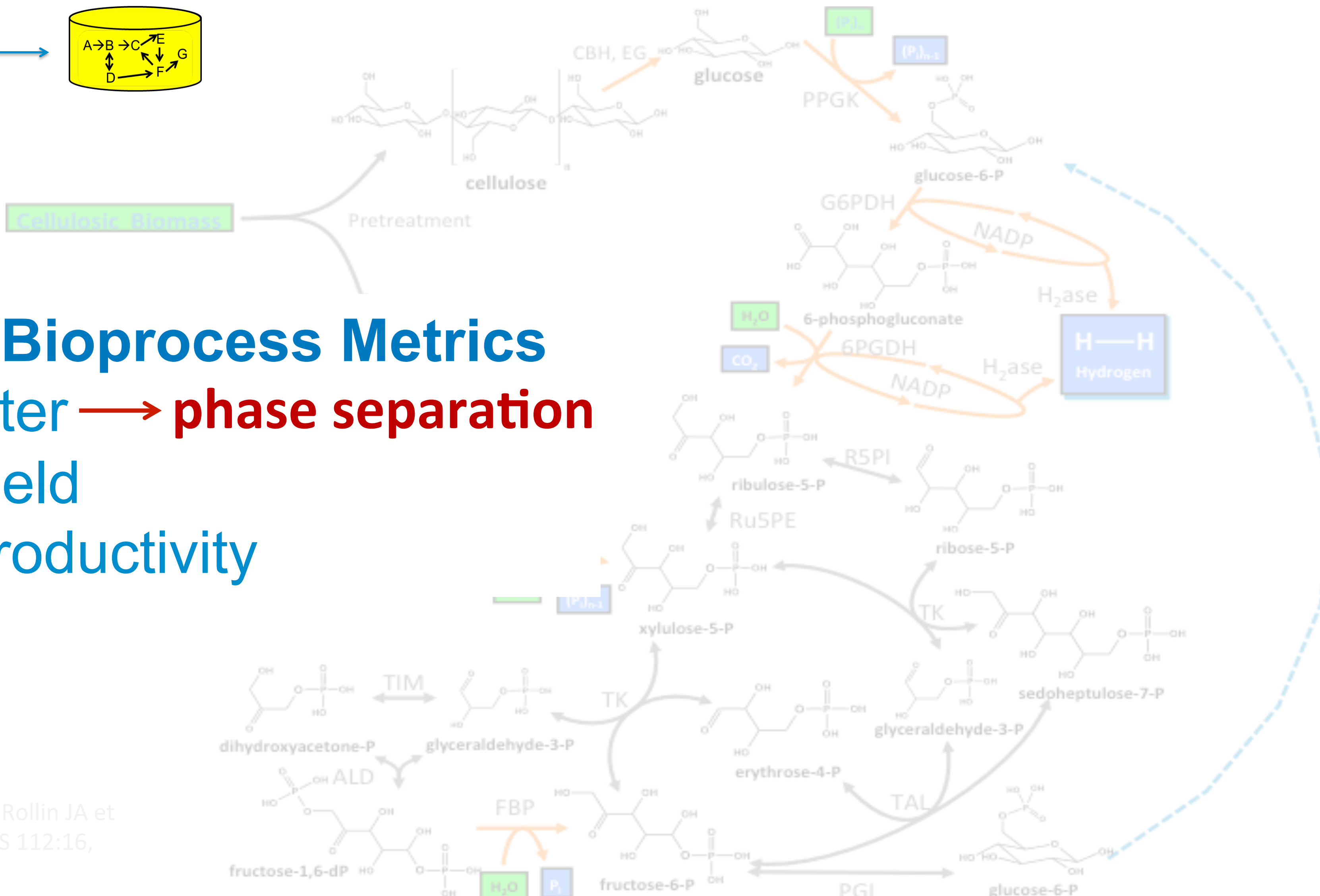


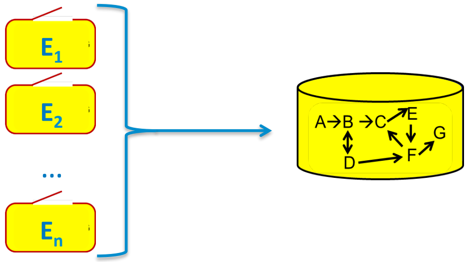


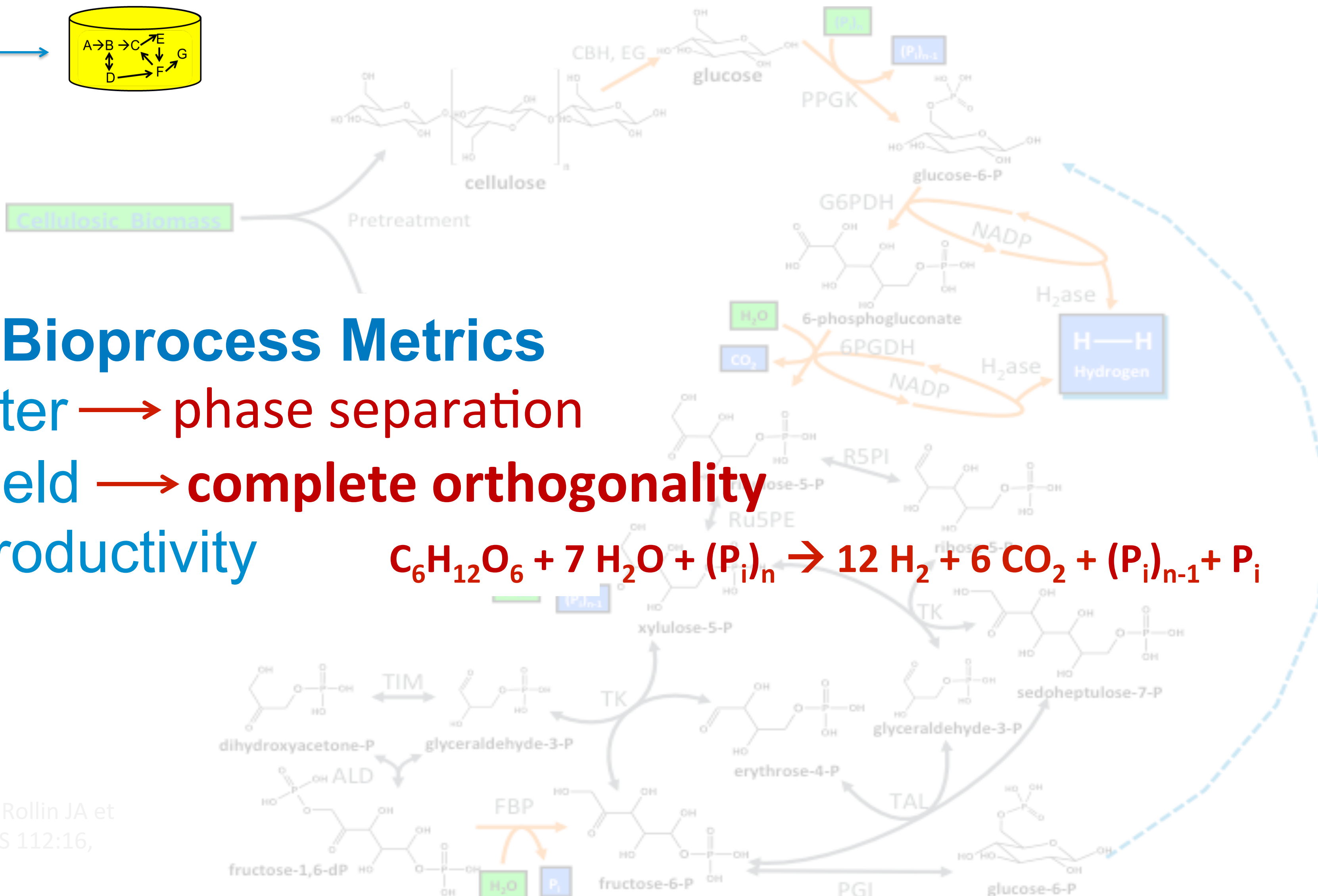
Key Bioprocess Metrics

- Titer ⟶ **phase separation**
- Yield
- Productivity

Adapted from Rollin JA et al. (2015) PNAS 112:16, 4964-4969.

Purified Cell-Free Example: Biomass Sugars to H_2





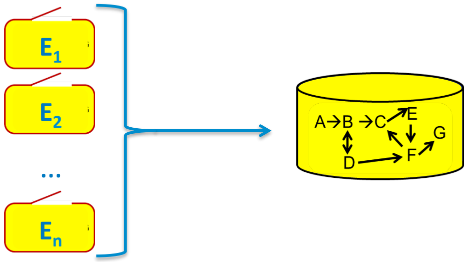
Adapted from Rollin JA et al. (2015) PNAS 112:16, 4964-4969.

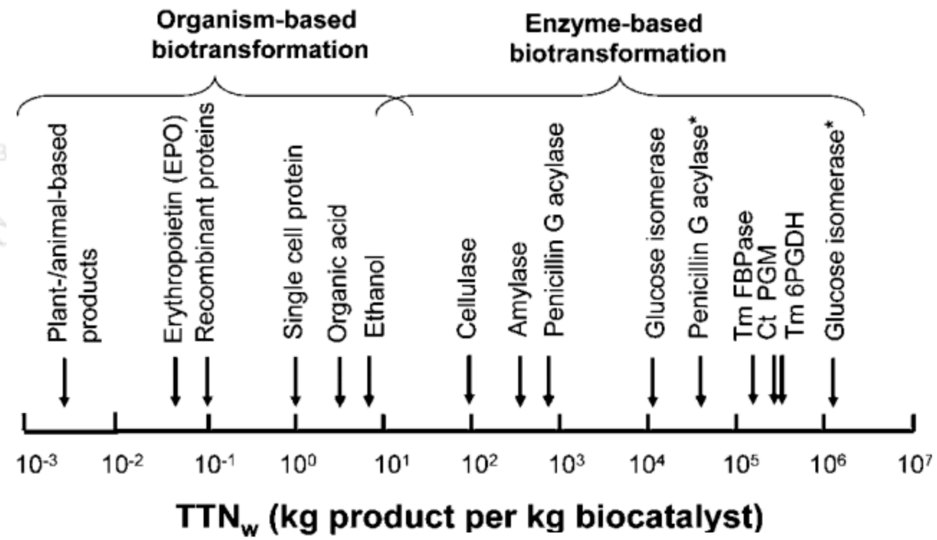
Key Bioprocess Metrics

- Titer ⟶ phase separation
- Yield ⟶ **complete orthogonality**
- Productivity

$$C_6H_{12}O_6 + 7\ H_2O + (P_i)_n \rightarrow 12\ H_2 + 6\ CO_2 + (P_i)_{n-1} + P_i$$

Purified Cell-Free Example: Biomass Sugars to H_2

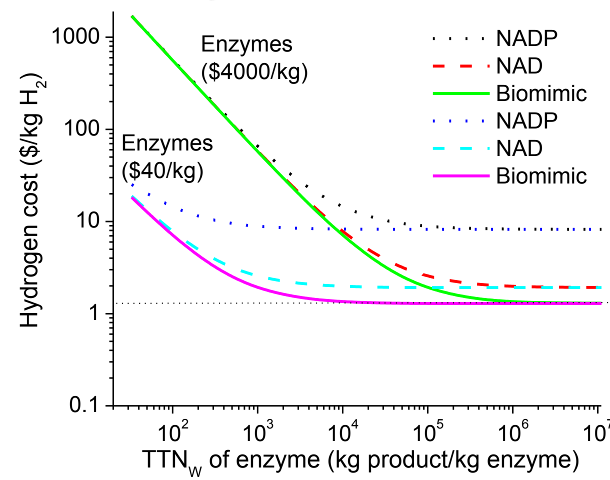




Zhang (2009) Biotechnol Bioeng

Key Bioprocess Metrics

- Titer ⟶ phase separation
- Yield ⟶ complete orthogonality; **TTN_W**
- Productivity



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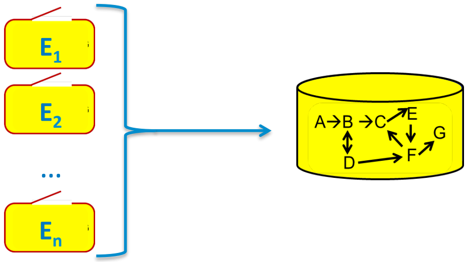

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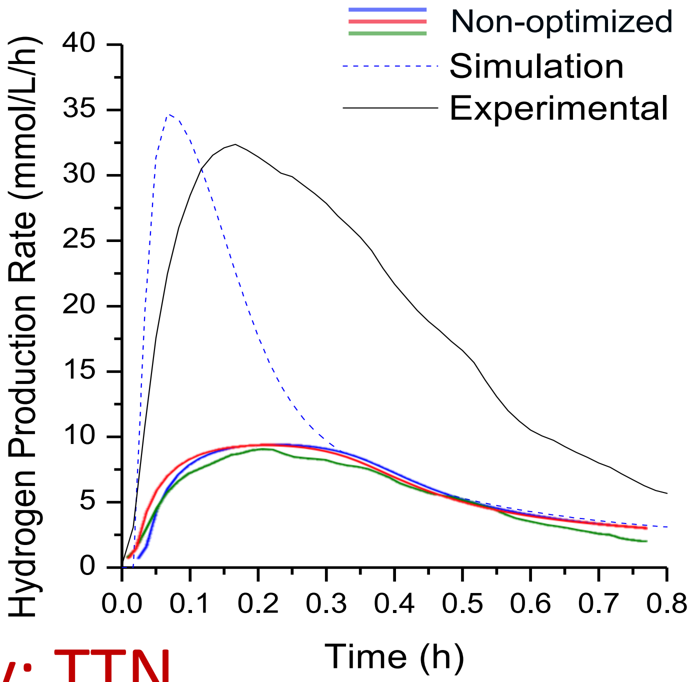
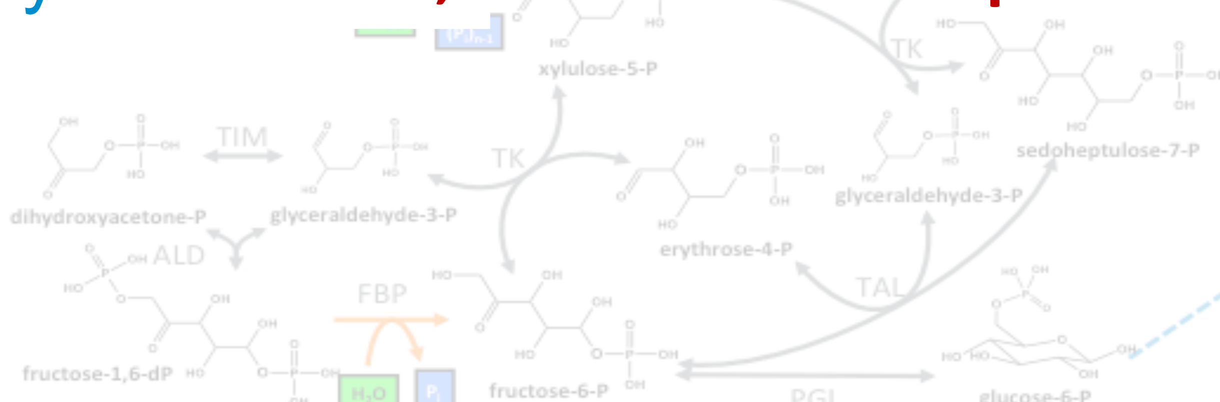
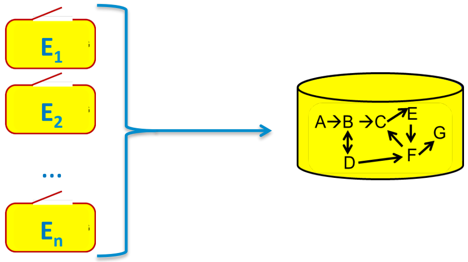


Key Bioprocess Metrics

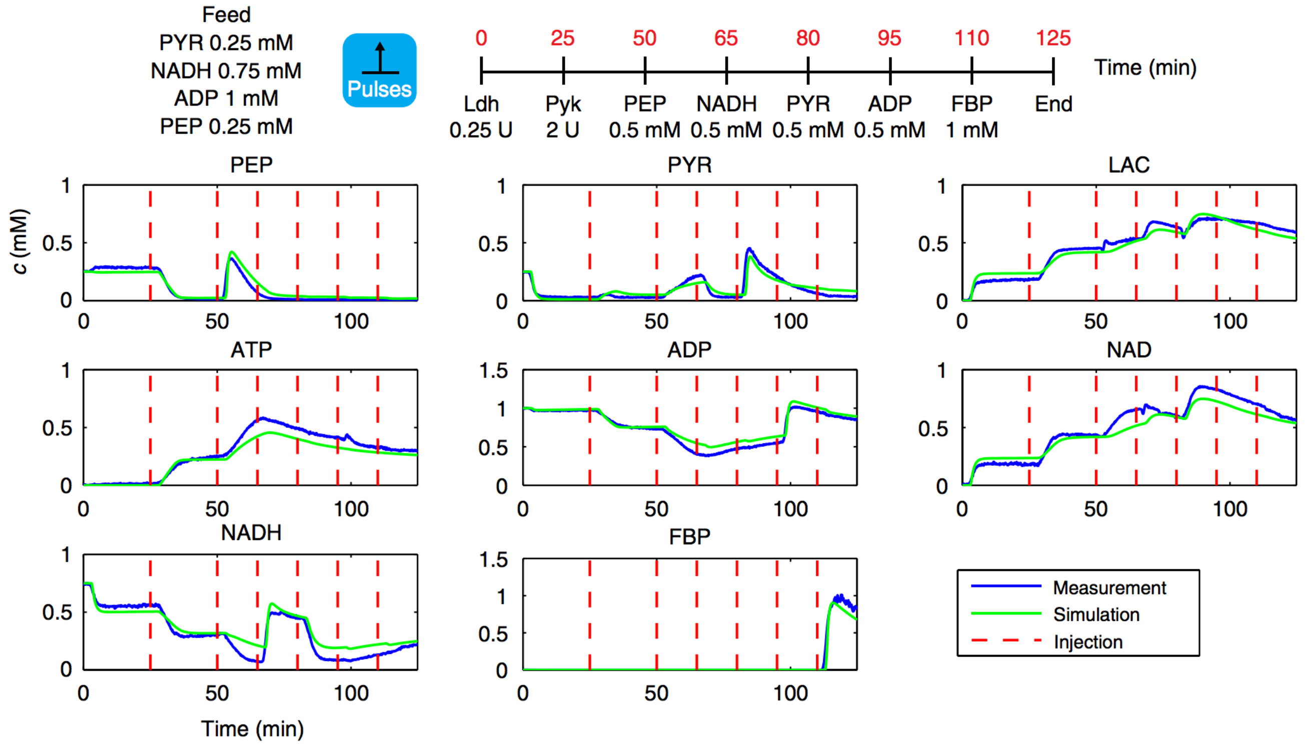
- Titer ⟶ phase separation
- Yield ⟶ complete orthogonality; TTN_W
- Productivity ⟶ **rational, model-driven improvement**



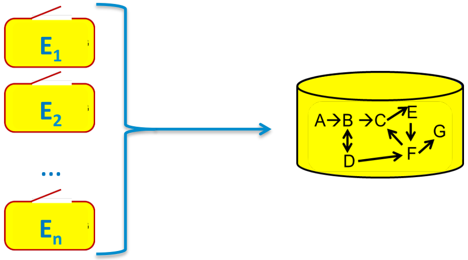
Purified Cell-Free Conversion – Challenges and Opportunities



- Advanced modeling
  - Ensemble approaches
  - Perturbations for robust parameterization
  - Alternative objectives (min [NADH], reduce inhibitory intermediates, modulation of ATP, PP_i recycle rates etc.)



Purified Cell-Free Conversion – Challenges and Opportunities



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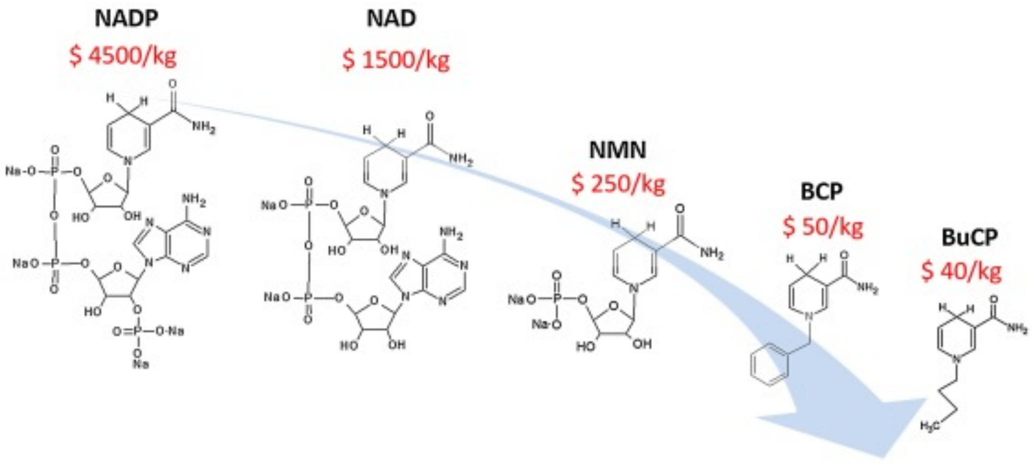
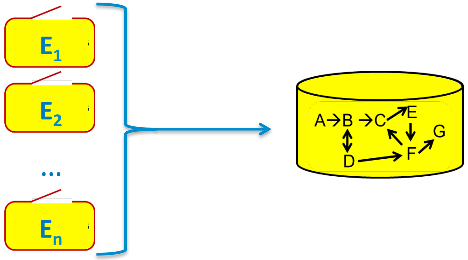
- Cofactor engineering
  - Replacement with biomimetics
  - Opportunities to harness external reducing power



You and Zhang (2017) Proc Biochem

Purified Cell-Free Conversion – Challenges and Opportunities



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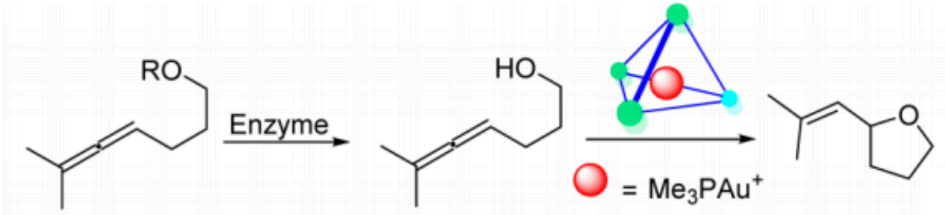
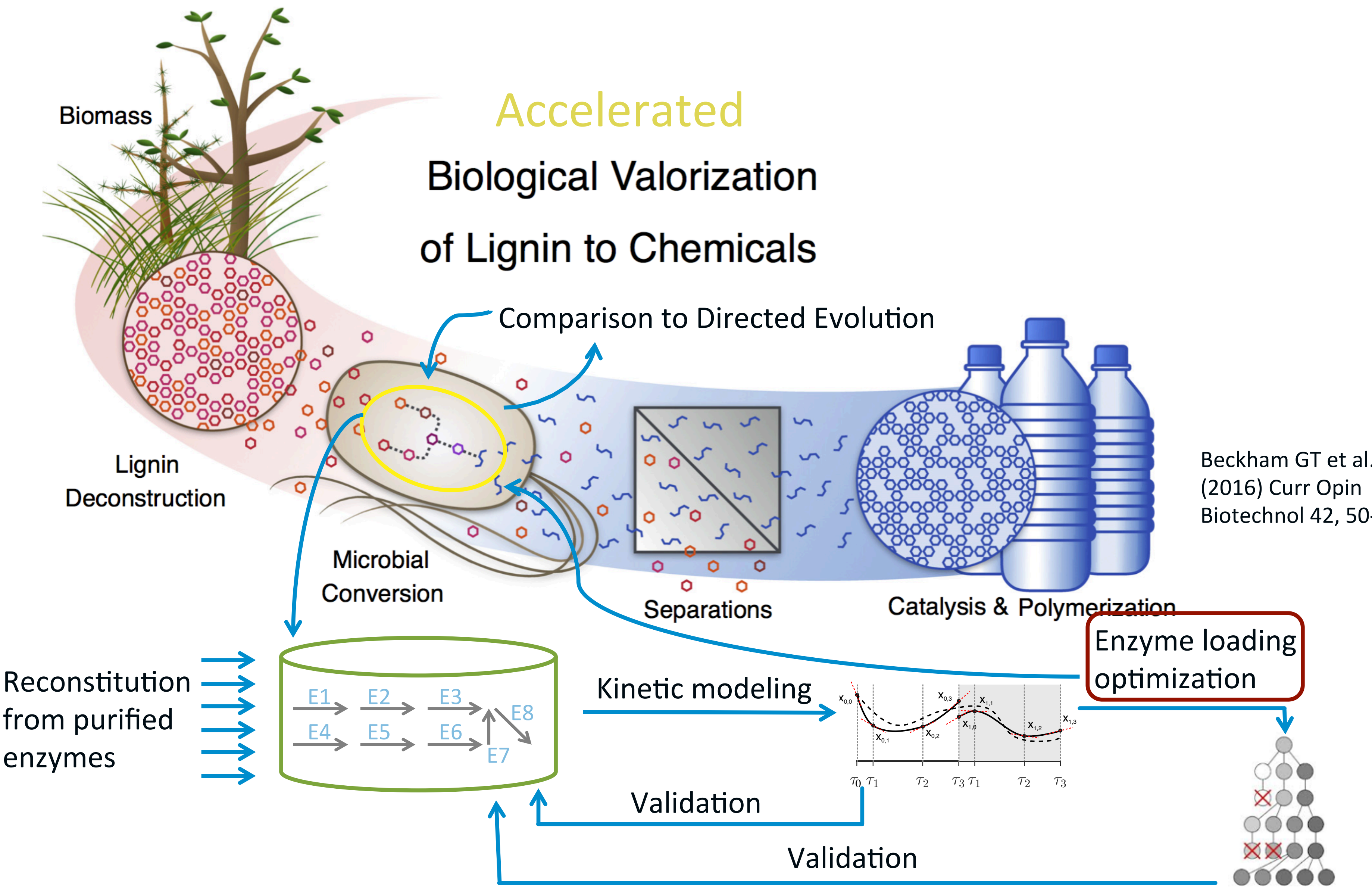
  - Alternative objectives (min [NADH], reduce inhibitory intermediates, modulation of ATP, PP_i recycle rates etc.)
- Cofactor engineering
  - Replacement with biomimetics
  - Opportunities to harness external reducing power
- Tunable pathway properties
  - Artificial actuators
  - Engineered allostery
- Compartmentalization / spatial control enabling for:
  - Substrate channeling
  - Hybridization with chemical catalysis



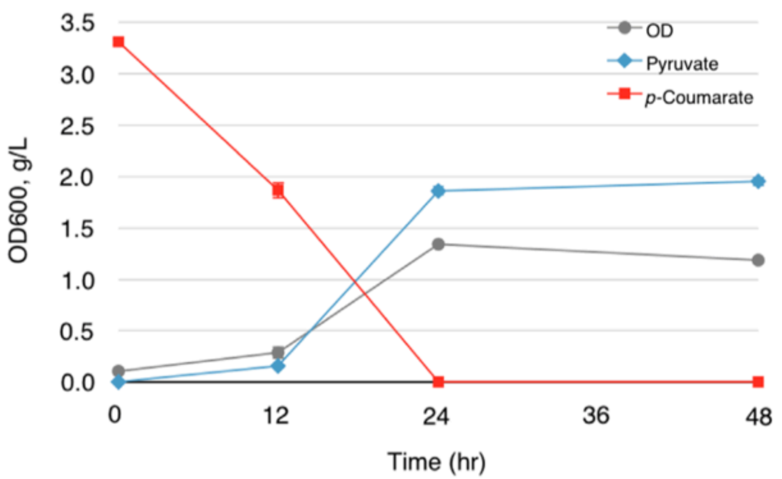
Denard et al. (2013) ACS Cat

Cell-Free Metabolic Engineering

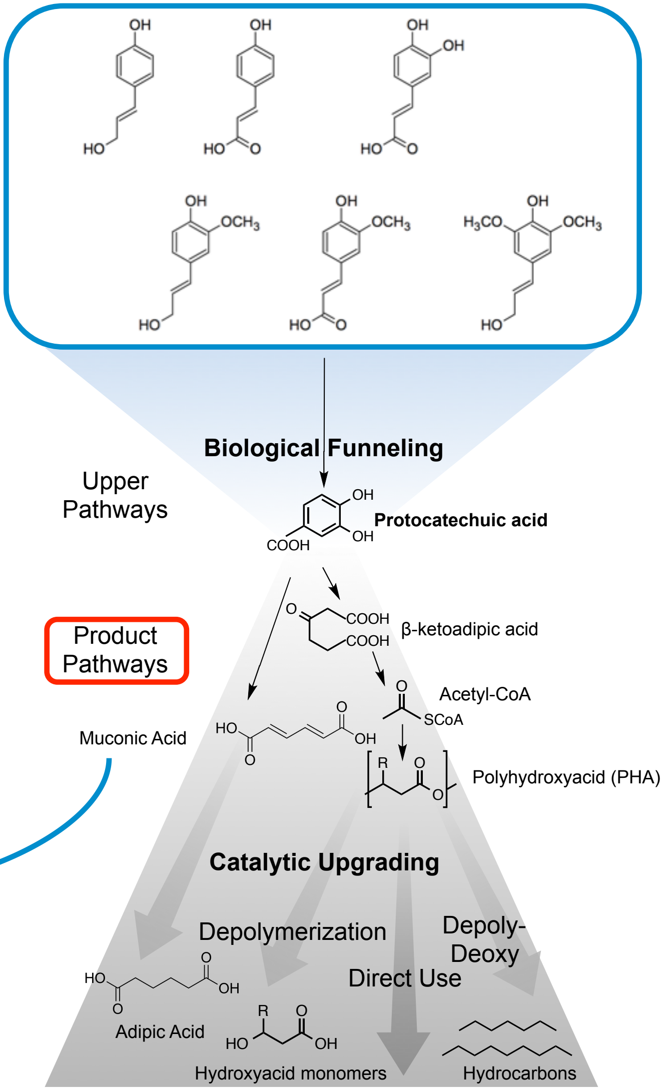


Beckham GT et al. (2016) Curr Opin Biotechnol 42, 50-53.

Pseudomonas putida KT2440 – Lignin Biological Funneling



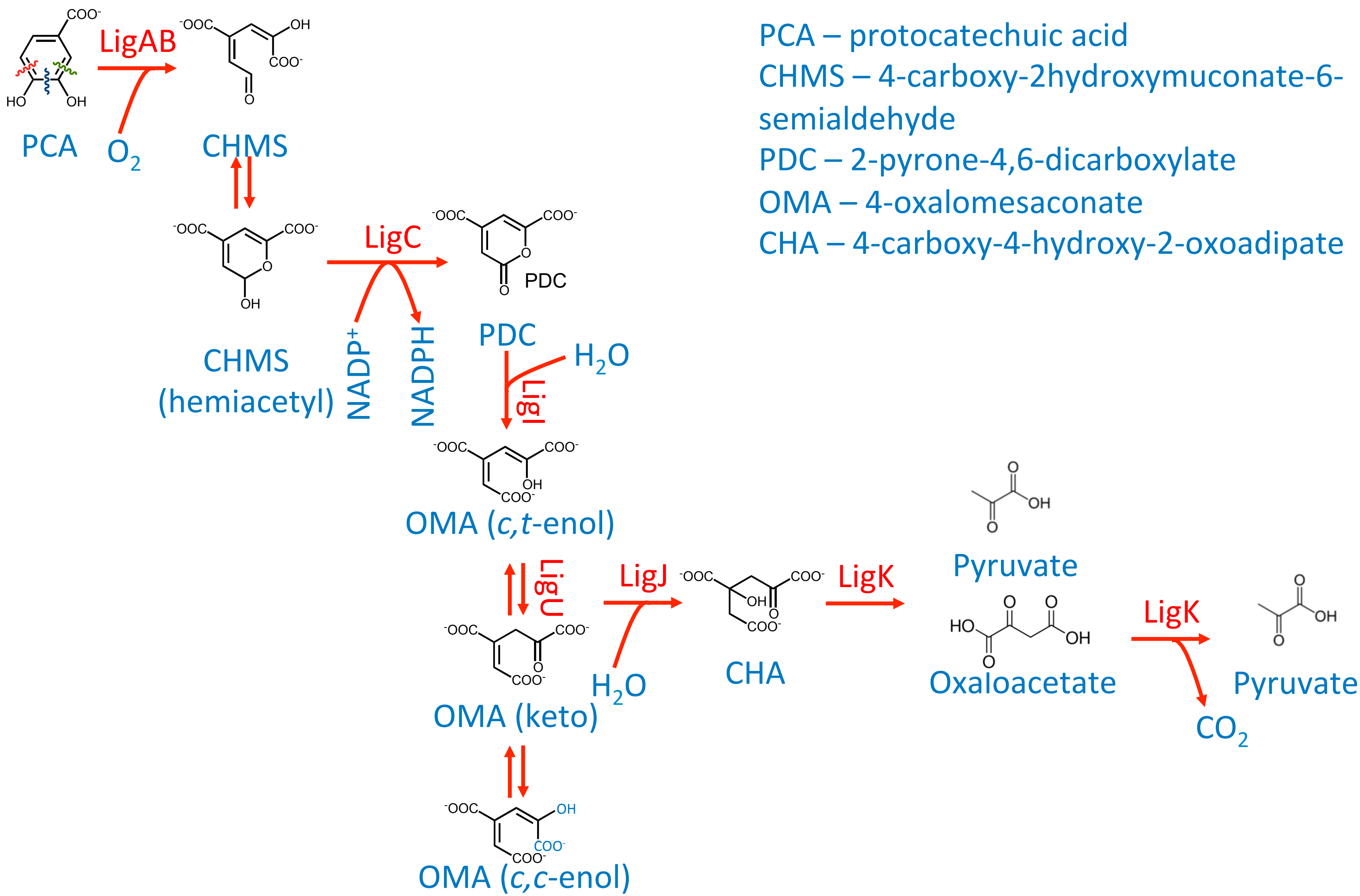
Johnson CW and Beckham GT. (2015) Metab Eng 28, 240-247.



Linger JG et al. (2014) PNAS 11:33, 12013-12018.

Pathway Selection: PCA 4,5-meta Cleavage

PCA – protocatechuic acid
CHMS – 4-carboxy-2hydroxymuconate-6-semialdehyde
PDC – 2-pyrone-4,6-dicarboxylate
OMA – 4-oxalomesaconate
CHA – 4-carboxy-4-hydroxy-2-oxoadipate



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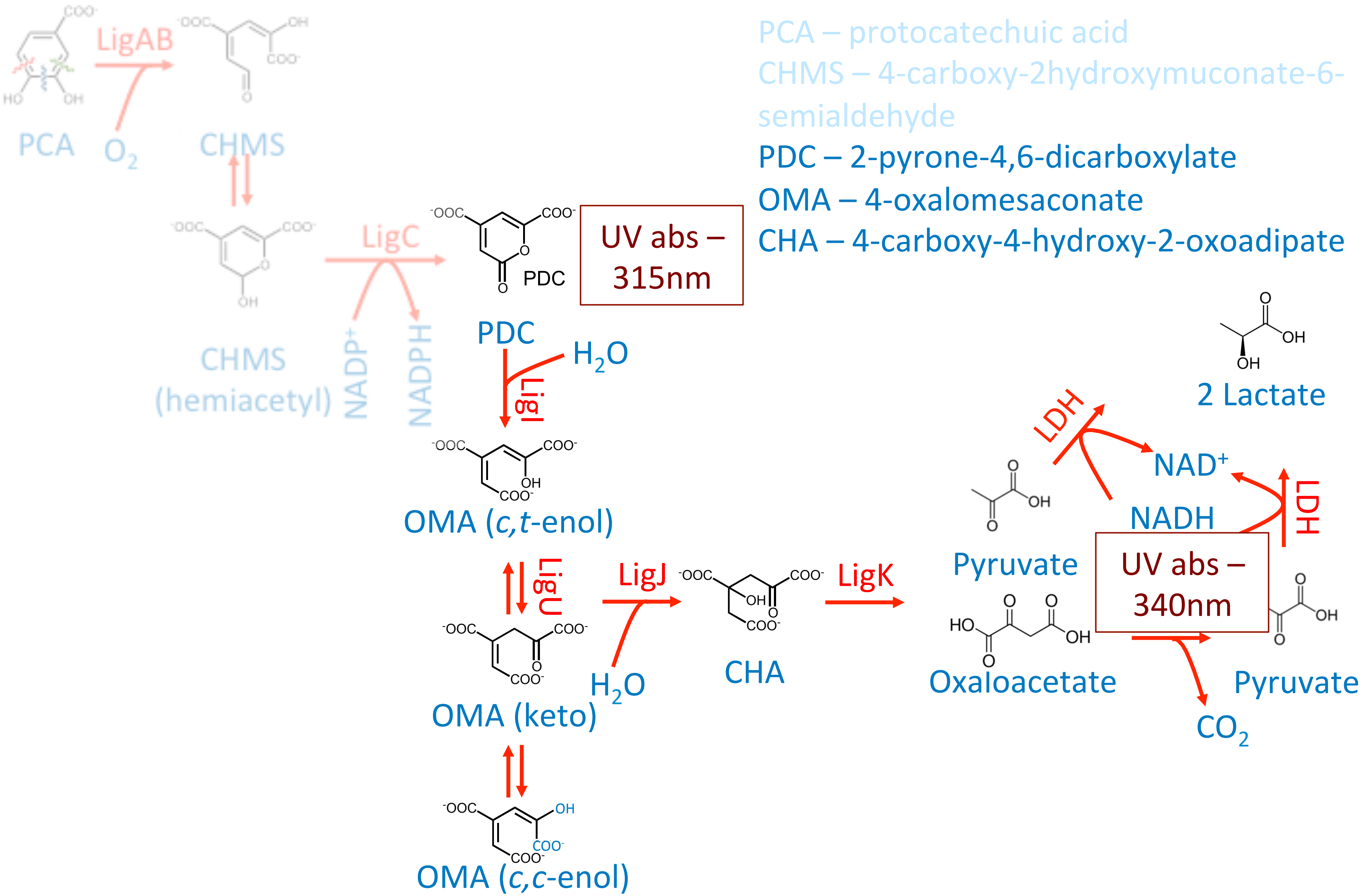
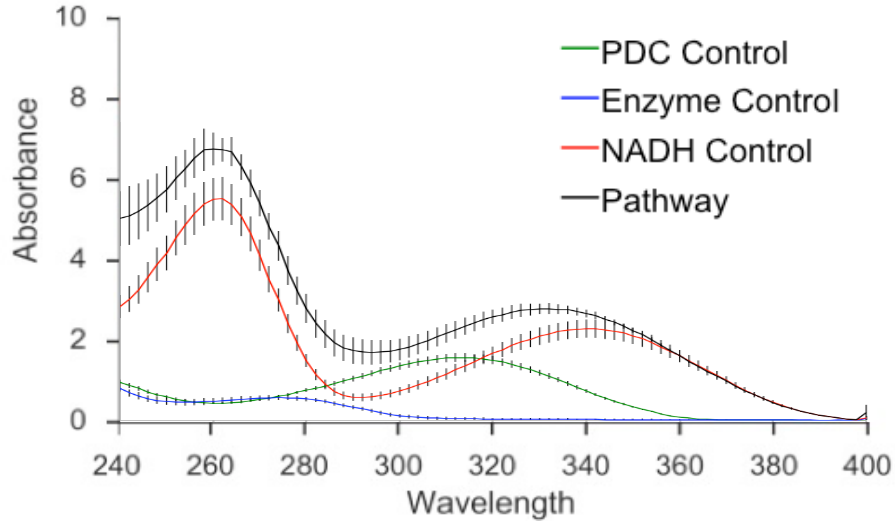
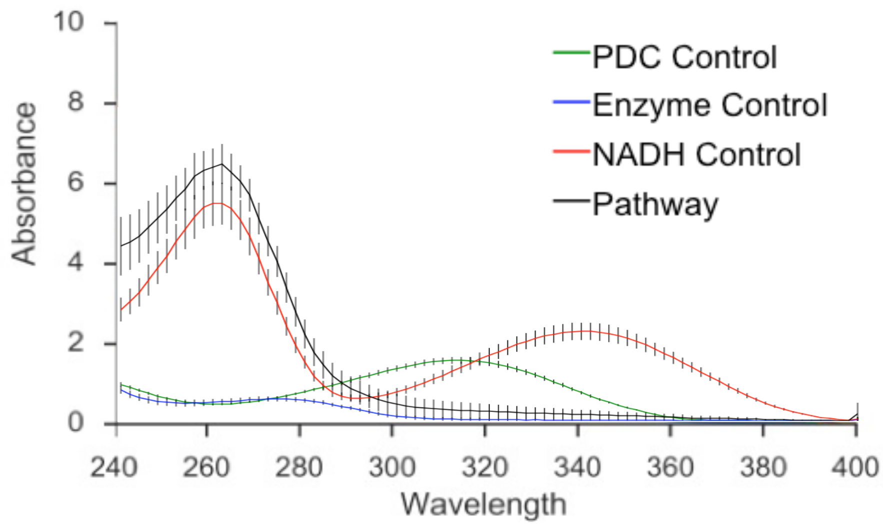


Confirmation of Pathway Activity

0 min

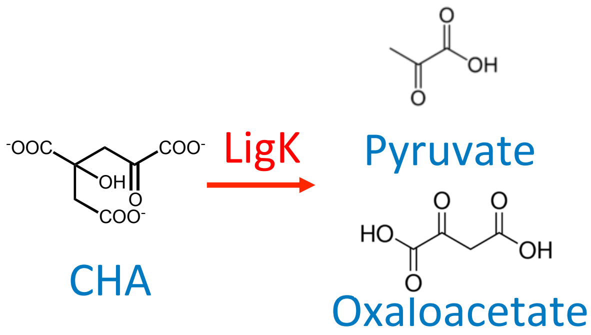


10 min



Pathway Kinetic Modeling

- Michaelis-menten kinetics rate equations built for each reaction



$$r_{LigK1} = \frac{d[OAA]}{dt} = \frac{d[Pyr1]}{dt} = \frac{k_{cat}^{LigK1}[LigK][CHA]}{K_{M,CHA}^{LigK} + [CHA]}$$

Pathway Kinetic Modeling

- Michaelis-menten kinetics rate equations built for each reaction
- Parameters determined for enzymes collectively
    - Orthogonal collocation for rapid curve-fitting
    - Bootstrap method to determine probability density for each variable
- Additional possible parameters: inhibition constants, inactivation terms

$$r_{LigI} = \frac{d[OMA_{enol}]}{dt} = \frac{k_{cat}^{LigI}[LigI][PDC]}{K_M^{LigI} + [PDC]}$$

$$r_{LigU} = \frac{d[OMA_{keto}]}{dt} = \frac{k_{cat}^{LigU}[LigU][OMA_{enol}]}{K_M^{LigU} + [OMA_{enol}]}$$

$$r_{LigJ} = \frac{d[CHA]}{dt} = \frac{k_{cat}^{LigJ}[LigJ][OMA_{keto}]}{K_M^{LigJ} + [OMA_{keto}]}$$

$$r_{LigK1} = \frac{d[OAA]}{dt} = \frac{d[Pyr1]}{dt} = \frac{k_{cat}^{LigK1}[LigK][CHA]}{K_{M,CHA}^{LigK} + [CHA]}$$

$$r_{LigK2} = \frac{d[Pyr2]}{dt} = \frac{k_{cat}^{LigK2}[LigK][OAA]}{K_{M,OAA}^{LigK} + [OAA]}$$

$$P = \begin{bmatrix} K_M^{LigI} \\ k_{cat}^{LigU} \\ K_M^{LigU} \\ k_{cat}^{LigJ} \\ K_M^{LigJ} \\ k_{cat}^{LigK1} \\ K_{M,CHA}^{LigK} \\ k_{cat}^{LigK2} \\ K_{M,OAA}^{LigK} \end{bmatrix}$$

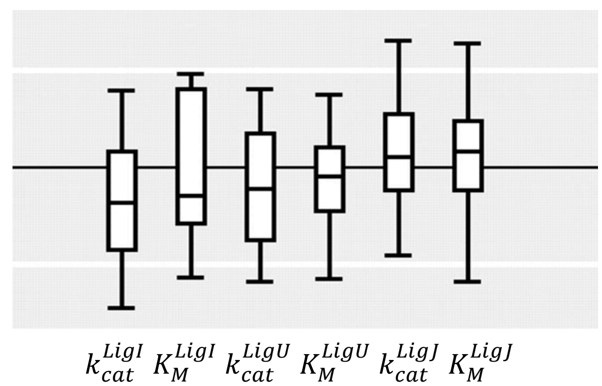
Pathway Kinetic Modeling - Experimental Validation and Iteration

- Input at least 10 different conditions to inform 9-parameter model

[E] (μM)

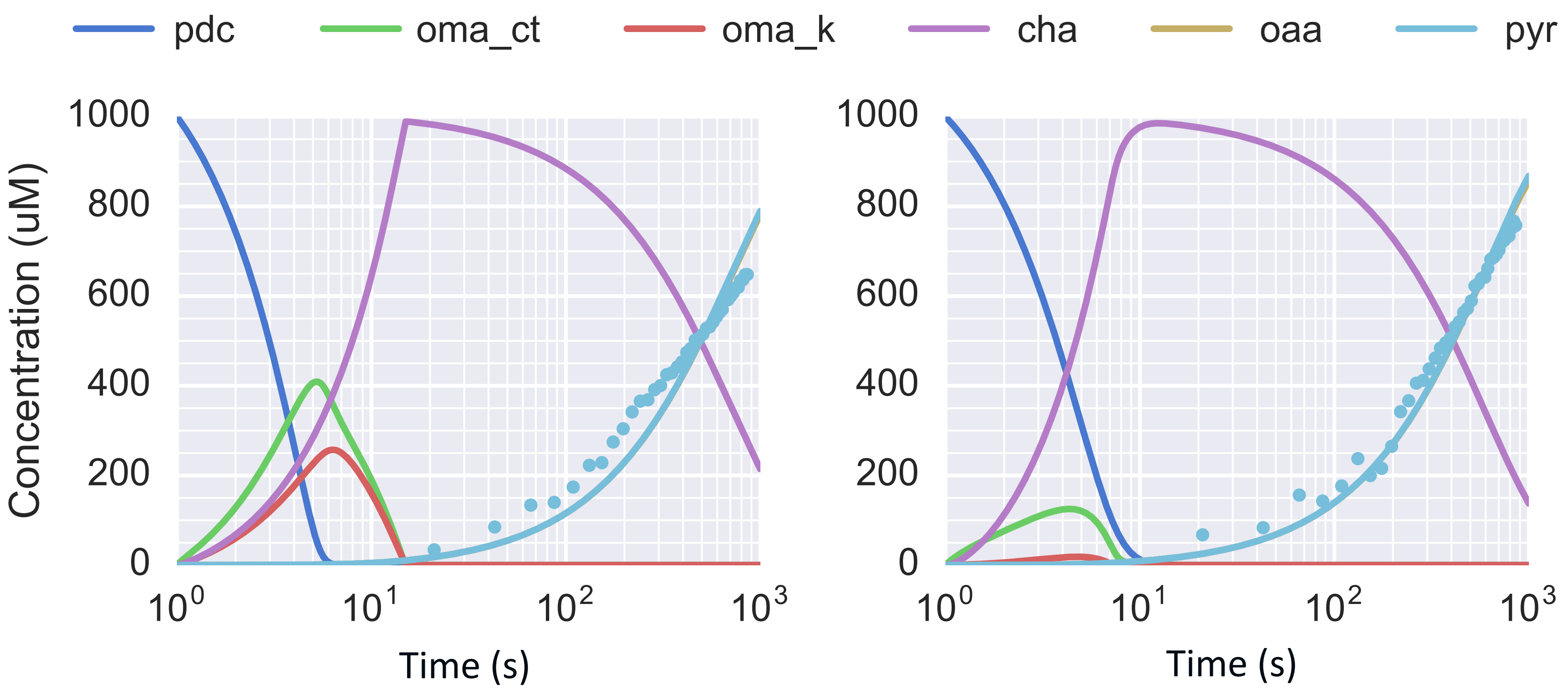
$$\begin{bmatrix} E1 \\ E2 \\ E3 \\ E4 \end{bmatrix} = \begin{bmatrix} 0.1 \\ 0.1 \\ 0.1 \\ 0.1 \end{bmatrix}, \begin{bmatrix} 0.5 \\ 0.5 \\ 0.5 \\ 0.5 \end{bmatrix}, \begin{bmatrix} 1 \\ 1 \\ 1 \\ 1 \end{bmatrix}, \begin{bmatrix} 0.1 \\ 1 \\ 1 \\ 1 \end{bmatrix}, \begin{bmatrix} 1 \\ 0.1 \\ 1 \\ 1 \end{bmatrix}, \begin{bmatrix} 1 \\ 1 \\ 0.1 \\ 1 \end{bmatrix}, \begin{bmatrix} 1 \\ 1 \\ 1 \\ 0.1 \end{bmatrix}, \ldots$$

- Output parameter range estimation



- Iterative improvement by varying enzyme ratio, substrate loading

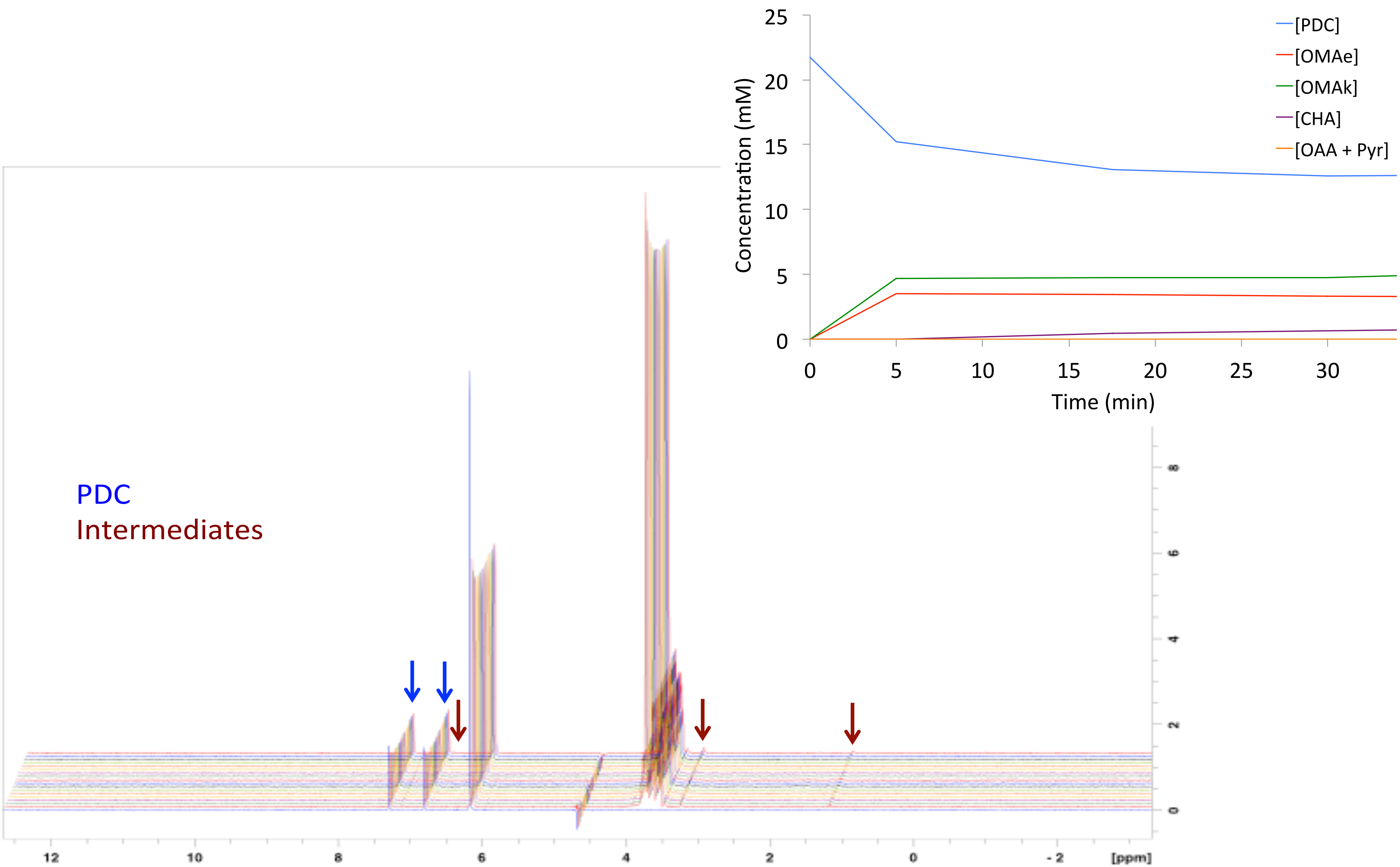
Preliminary Model Results (Peter St. John)



$[E]_t$ = 2 mg/L **$[E]_t$ = 4 mg/L**

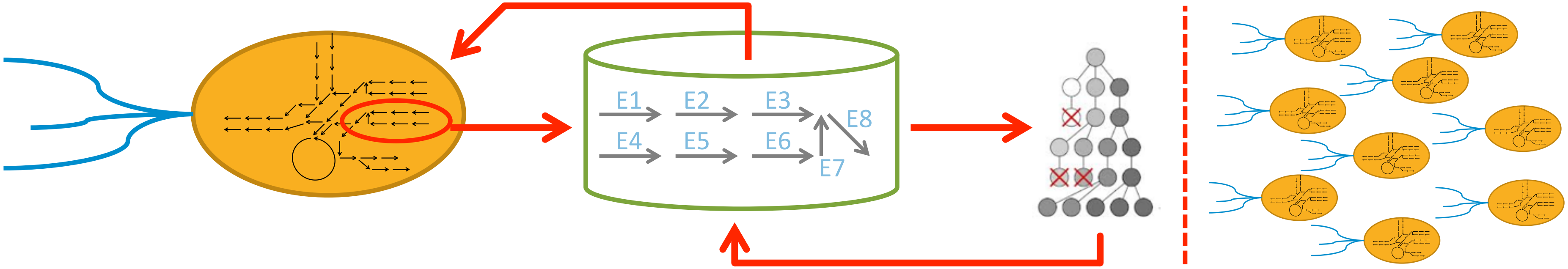
- Good initial fit of [Pyr] based on literature kinetic parameters
- **Intermediates detection required for robust model**

NMR Metabolomics – Initial Data



Cell-Free Metabolic Engineering Project Summary

1. Cell-free replication of lignin biological funneling pathways of interest
2. Characterization of the enzymes in this system
   - Kinetic parameters for each reaction: K_M, k_{cat}, K_I
   - Simultaneous system parameter estimation
3. Predict improvements that may be used in the production strain
   - Expression levels
   - Enzyme engineering targets / variant candidates
4. Use RBS calculator (or alternative methods) to translate optimal enzyme loadings into genetic elements
5. Directed evolution of this pathway to compare this approach to genetic, whole-cell optimization methods



Thanks to my mentors and collaborators:
Gregg Beckham and Chris Johnson
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