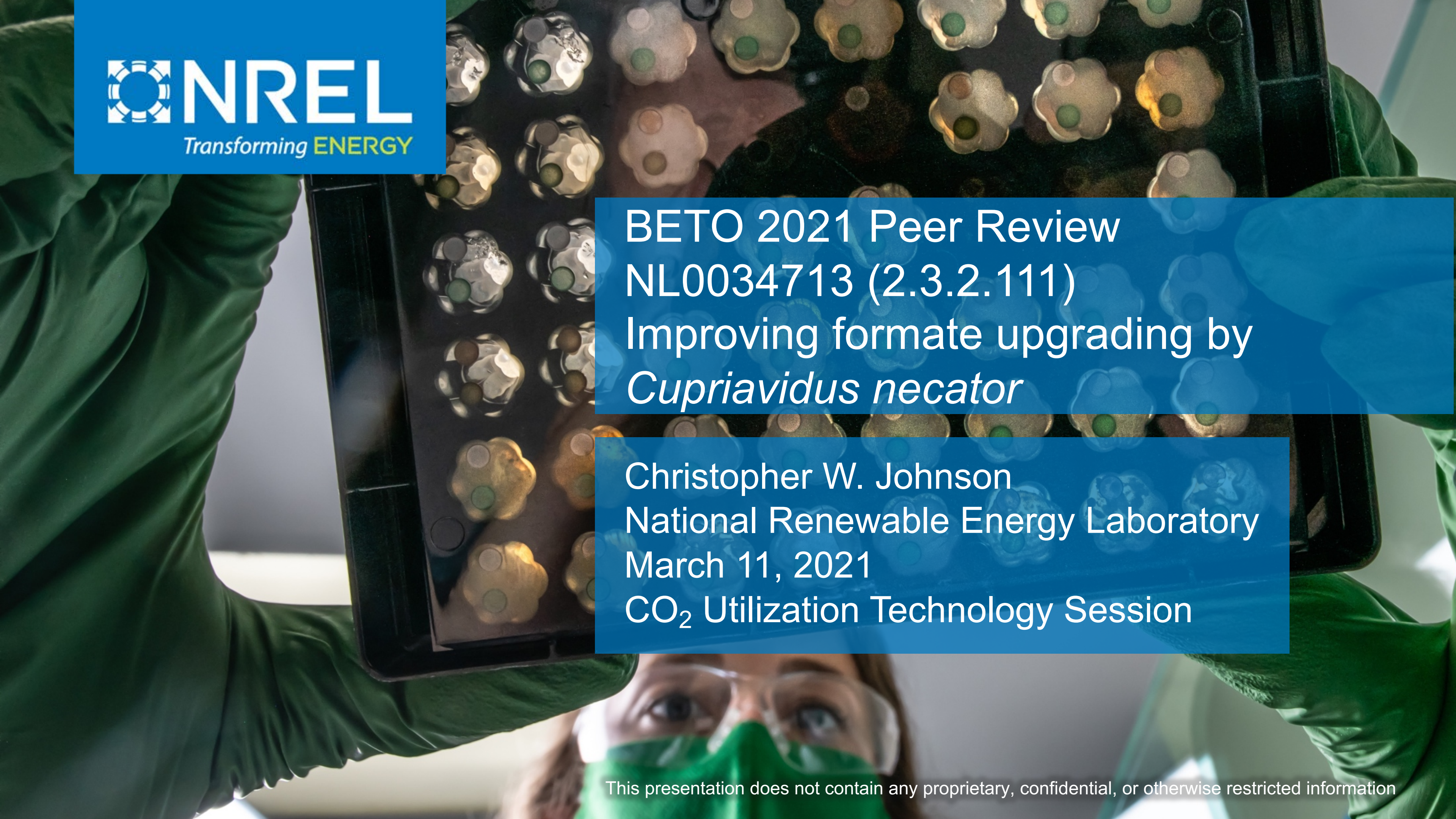


BETO 2021 Peer Review
NL0034713 (2.3.2.111)
Improving formate upgrading by *Cupriavidus necator*

Christopher W. Johnson
National Renewable Energy Laboratory
March 11, 2021
CO_2 Utilization Technology Session

Quad chart

Timeline

- Active Project Duration: 10/1/2018 – 9/30/2021
- Total Project Duration: 10/1/2018 – 9/30/2021

	FY20	Active Project (FY19-21)
DOE Funding	$0 Project was fully funded in FY19	$930,000

Project Partners

Barriers addressed

- Ct-H Gas Fermentation Development
- Ct-D Advanced Bioprocess Development
- Ct-J Identification and Evaluation of Potential Bioproducts

Project Goal

Develop the natural formatotroph, *C. necator*, as a robust microbial chassis for efficient formate conversion to value-added products in support of CO_2 capture.

End of Project Milestone

Demonstrate production of 2 g/L (14 mM) of 2-hydoxymuconate semialdehyde in *C. necator* using formate as the sole source of carbon and energy.

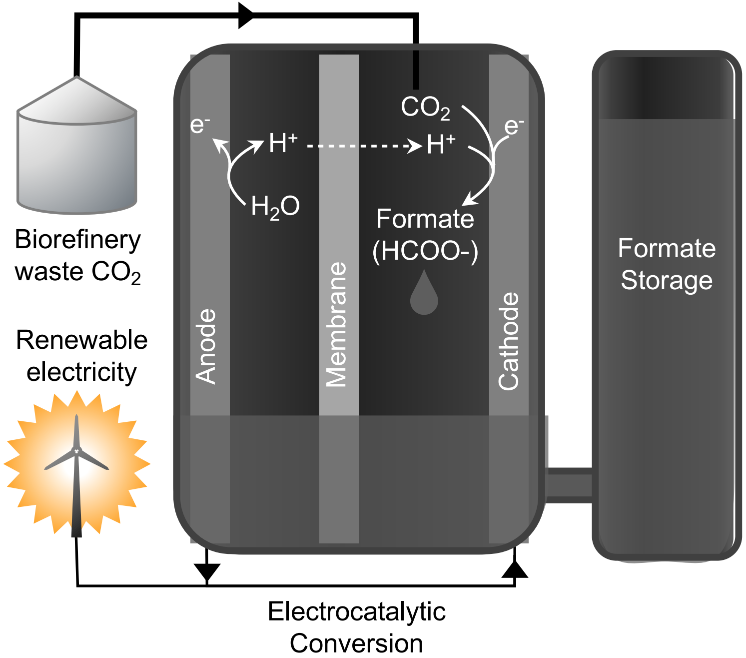
Funding Mechanism

Bioenergy Technologies Office FY19 AOP Lab Call (DE-LC-000L060) – 2018

Project Overview

Background:

- Waste CO_2 is generated by petroleum-based and biobased processes
- CO_2 can be electrocatalytically reduced to generate formate/formic acid
- Renewable energy could enable low cost, low GHG production of formate



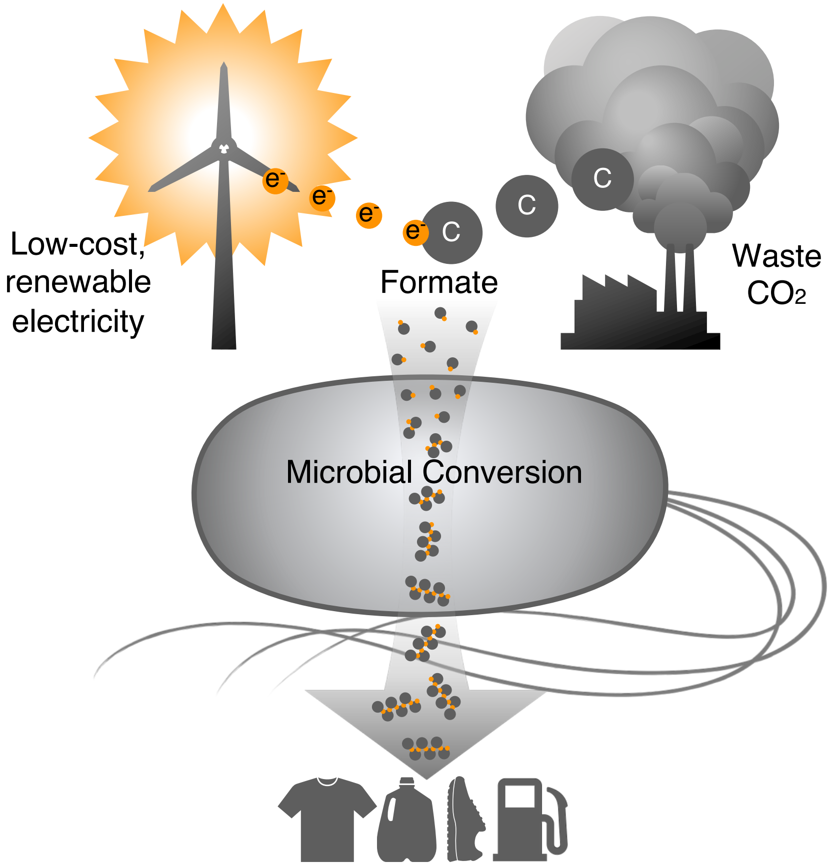
- Formate has been proposed as a soluble intermediate compound to store carbon and energy that overcomes challenges associated with gaseous intermediates (solubility, mass transfer, safety, storage, transport…)

Project Overview

Background:

- A microbial host capable of robust conversion of formate would enable the production of fuels and chemicals and bring value to CO_2

- ***Cupriavidus necator***
  - Natural formatotroph
  - Robust formate assimilation
  - High cell densities (≥200 g/L)
  - Well studied
  - Amenable to genetic manipulation
  - Proven industrial host (≥200,000 L)



Project Overview

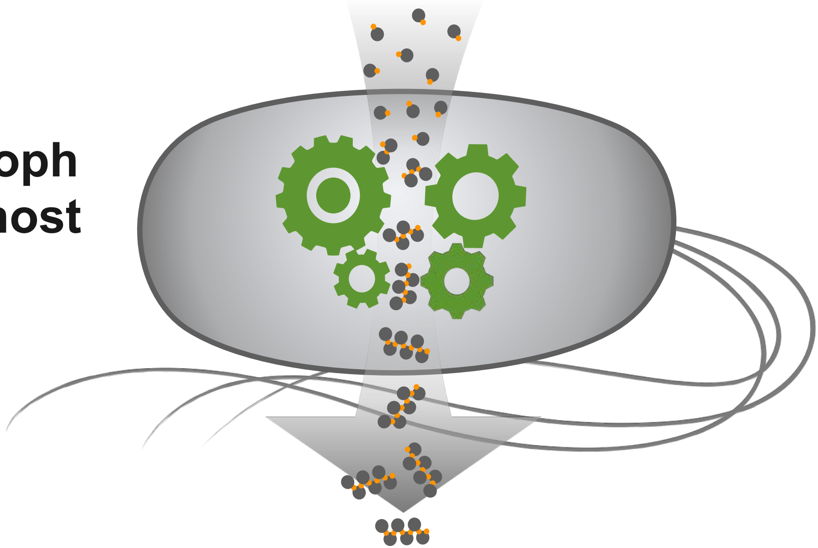
History:

- This project was funded by a “Rewiring the Bioeconomy: Biological Formate Upgrading Lab Call” issued by BETO in response to guidance from EERE leadership and congress.
- The focus of the call was on strain development for formate conversion:

> *“This Lab Call is **targeting the development of platform organisms** that can:*
> *1) use formate as their sole carbon source, and*
> *2) subsequently be leveraged to produce fuels and chemicals.”*

- While conversion of CO_2 to formate is ultimately important to this concept, the FOA did not specify or encourage a specify a specific source of formate
- Part of BETO focus on C1 intermediates for CO_2 conversion, but less developed (lower TRL) than other technologies such as CO fermentation

Project Overview



Project Goal: Develop the natural formatotroph *Cupriavidus necator* as a robust microbial host for conversion of formate to value-added products

Aims:

- Improve formate assimilation via the native pathway (≥ 1.2X)
- Improve formate assimilation via more efficient synthetic pathway (≥ 1.1X)
- Demonstrate improved conversion of formate to an exemplary product, the polymer precursor 2-hydroxymuonate semialdehyde (2HMS) (≥ 2 g/L)
- Perform techno-economic analysis (TEA) and life-cycle assessment (LCA)

Project Overview

Heilmeier Catechism:

- What are you trying to do?
  - Develop a robust microbial host for conversion of CO_2-derived formate to value-added products
- How is it done today and what are the limits?
  - Formate bioconversion not performed at scale today
- Why is it important?
  - Future carbon emissions *must go negative* to avoid global warming beyond 1.5°C
- What are the risks?
  - Scale up, toxicity of formate/formic acid

Market Trends

Product
- Anticipated decrease in gasoline/ethanol demand; diesel demand steady
- Increasing demand for aviation and marine fuel
- Demand for higher-performance products
- Increasing demand for renewable/recyclable materials

Feedstock
- Sustained low oil prices
- Decreasing cost of renewable electricity
- Sustainable waste management
- Expanding availability of green H_2
- Closing the carbon cycle

Capital
- Risk of greenfield investments
- Challenges and costs of biorefinery start-up
- Availability of depreciated and underutilized capital equipment

Social Responsibility
- Carbon intensity reduction
- Access to clean air and water
- Environmental equity

NREL's Bioenergy Program Is Enabling a Sustainable Energy Future by Responding to Key Market Needs

Value Proposition

- Enable conversion of formate generated from low-cost renewable energy and waste CO_2 to myriad fuels and chemicals
- Decarbonize or reduce the carbon intensity of processes that generate CO_2

Key Differentiators

- Biology can generate myriad products with high selectivity compared to catalytic routes
- *C. necator* is a natural formatotroph and a proven industrial host, making it an excellent starting point for formate conversion

1. Management

Management
- Christopher Johnson: PI
- Michelle Reed: Project Mgmt

Strain Development
- Christopher Calvey, Postdoc
- Carrie Eckert, Scientist

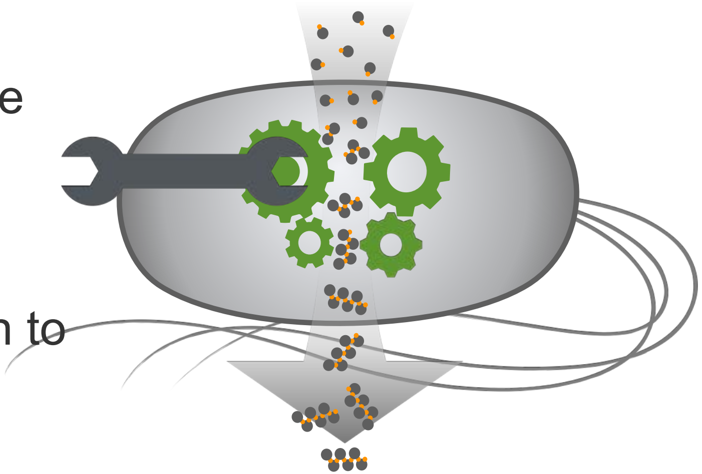
Bioprocess Development
- Violeta Sanchez, Scientist
- Colin Kneucker, Technician

TEA/LCA
- Ling Tao, Engineer

Coordination
- Strain development meetings biweekly
- Postdoc one-on-one biweekly
- Meetings with other formate conversion projects biannually
- *Ad hoc* meetings with TEA/LCA team

Risks
- Low transformation efficiency
  - Sufficiently improved (ORNL)
- Native pathway already optimal
  - Improvement demonstrated
- Synthetic pathway insufficient
  - No-Go redirection

2. Approach



Overall approach

- Use evolution and rational engineering to improve assimilation of formate by *C. necator*
- Engineer *C. necator* to convert formate to 2-hydroxymuconate semialdehyde (2HMS)
- Apply strategies to improved formate assimilation to improve production of 2HMS

Challenges

- Formate/formic acid is relatively toxic, complicating cultivation
- Detection of 2HMS by mass spec is difficult

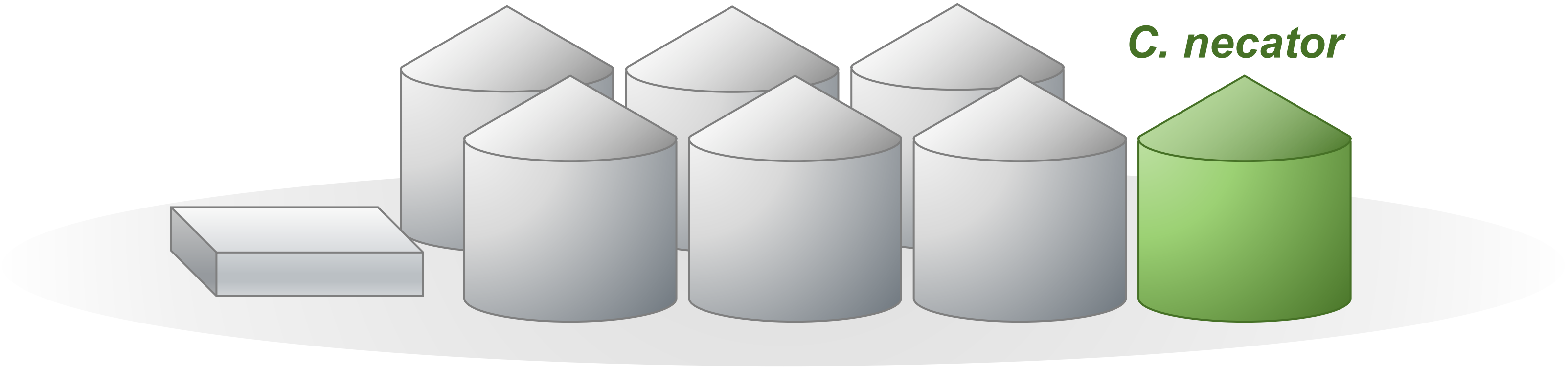
Go/No-Go Decision

- Demonstrate 1.1X benefit from incorporation of the synthetic formolase pathway or redirect effort toward improving assimilation via native pathway

3. Impact

CO_2 recycling in a biorefinery at scale

- A typical 40 million gallon/yr starch EtOH biorefinery generates around 15,000 kg CO_2/h
- 1,775,205 L of *C. necator* would be needed to assimilate formate at the same rate*
- A 1.2X improvement in formate assimilation would reduce this by 355,041 L



*Conditions and assumptions: *C. necator* consumes formate at a rate of 12 mmol/g cells/h and 16% is assimilated (Grunwald, et al., 2014. doi:10.1111/1751-7915.12149). Assumes 100% CO_2 is converted to formate. *C. necator* culture is 100 g/L cells. Fermenters are 2 million L.

3. Impact

Global Impact

- Decarbonizing or reducing the carbon intensity has the potential to reduce the environmental impact and improve the economics of a wide variety of CO_2 emitting processes

Industrial

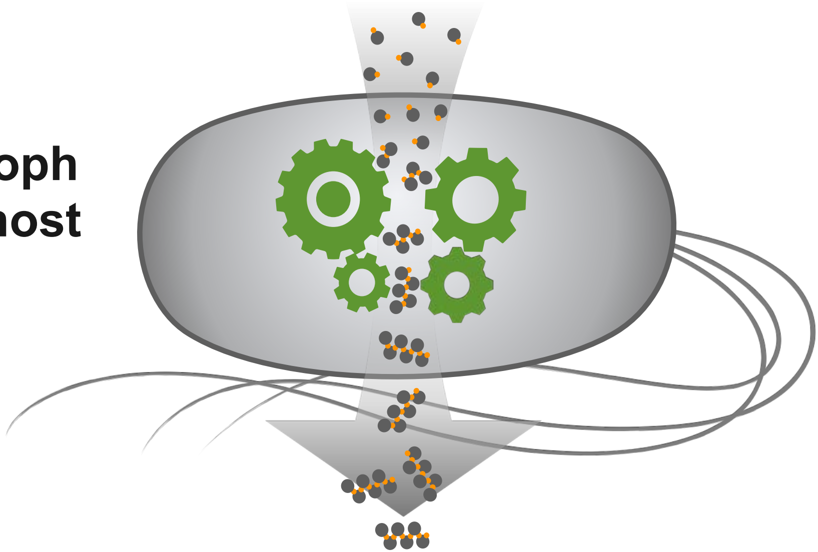
- Bring greater value to formate (and CO_2)
- Improve other processing using *C. necator*
- Anticipate at least one patent application resulting from this work

Scientific

- Anticipate at least two high-impact publications resulting from this work
  - In preparation: Calvey et al., Adaptive laboratory evolution of *C. necator* for improved formate utilization
- Contribute to our understanding of formatotrophy
- Potential integration with CO_2 reduction projects

4. Progress and Outcomes

Project Goal: **Develop the natural formatotroph *Cupriavidus necator* as a robust microbial host for conversion of formate to value-added products**



Aims:

- Improve formate assimilation via the native pathway (≥ 1.2X)
- Improve formate assimilation via more efficient synthetic pathway (≥ 1.1X)
- Demonstrate improved conversion of formate to an exemplary product, the polymer precursor 2-hydroxymuonate semialdehyde (2HMS) (≥ 2 g/L)
- Perform techno-economic analysis (TEA) and life-cycle assessment (LCA)

Two proposed routes for formate assimilation

Native
Calvin-Benson-Bassham (CBB) cycle

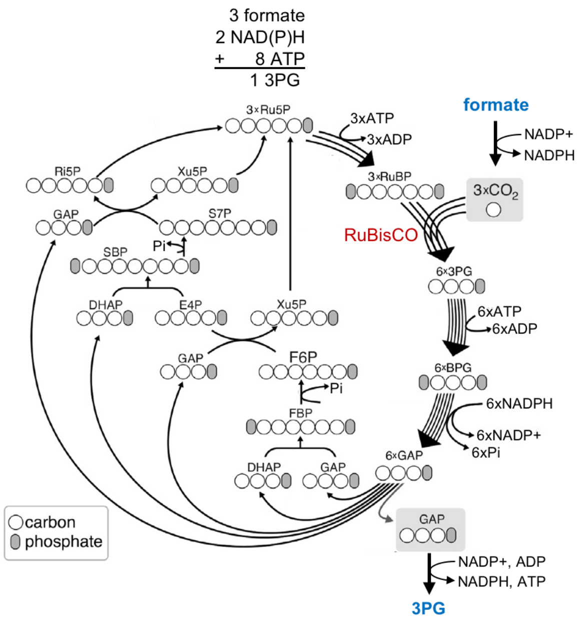


Figure adapted from Antonovsky, N., et al., 2017. Curr. Opin. Biotechnol. 47, 83–91. doi:10.1016/j.copbio.2017.06.006

Synthetic
Formolase pathway

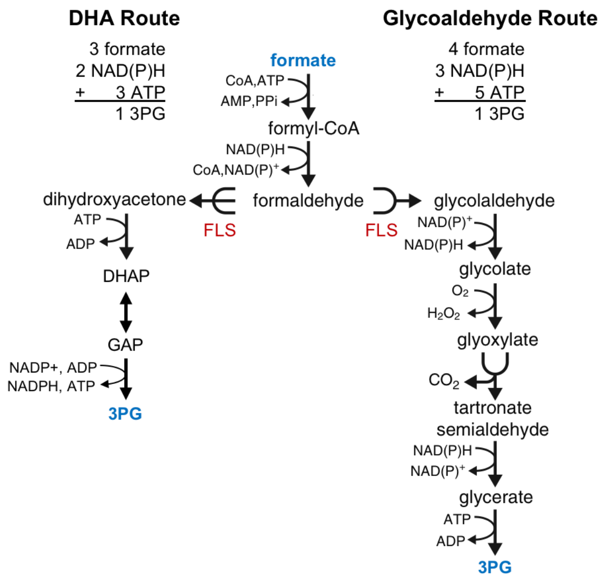
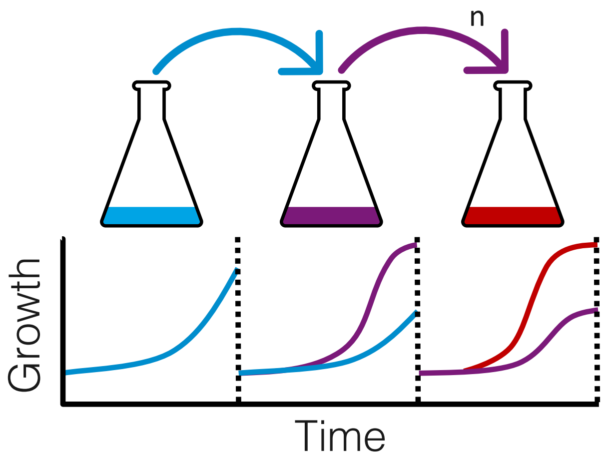
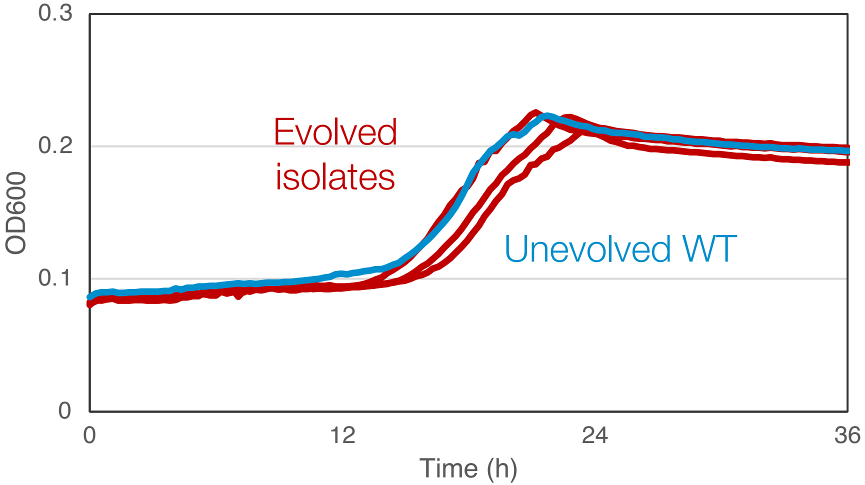


Figure adapted from Yishai, O., et al., 2016. The formate bio-economy. Curr Opin Chem Biol 35, 1–9. doi:10.1016/j.cbpa.2016.07.005

The formolase pathway did not improve formatotrophy

- Genes encoding the formolase pathway were integrated into the genome of *C. necator* and adaptive laboratory evolution was performed by serial transfer of three parallel lineages in minimal media containing 50mM sodium formate.
- Repeated 24-hour growth cycles, for 40+ generations.

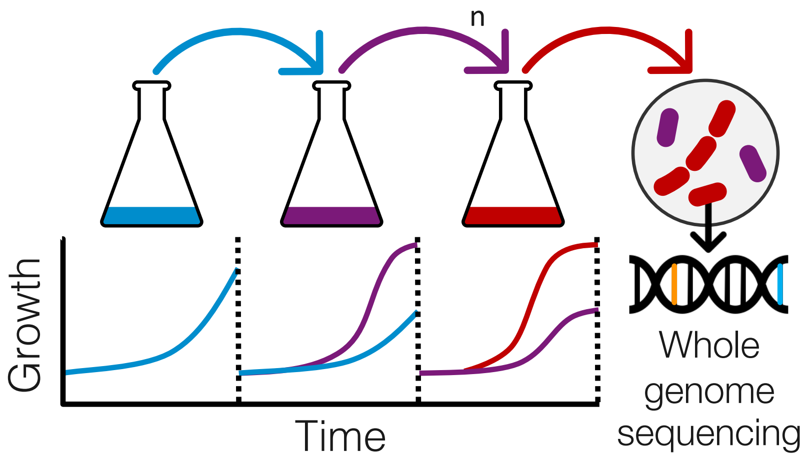


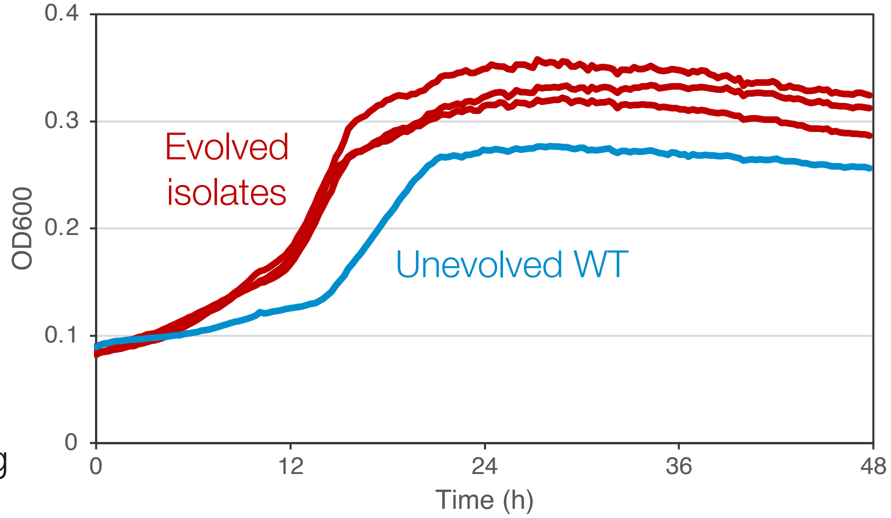


No benefit was observed from the incorporation of the formolase pathway (No-Go), so resources were redirected toward improving the native pathway.

Evolution improved formate assimilation

- Adaptive laboratory evolution (ALE) was performed using wild-type *C. necator* (WT) by serial transfer of six parallel lineages in minimal media containing 50mM sodium formate for 400+ generations.





Evolved isolates were identified that exhibit a ~1.2X rate of growth *and* biomass yield on formate relative to the WT parent.

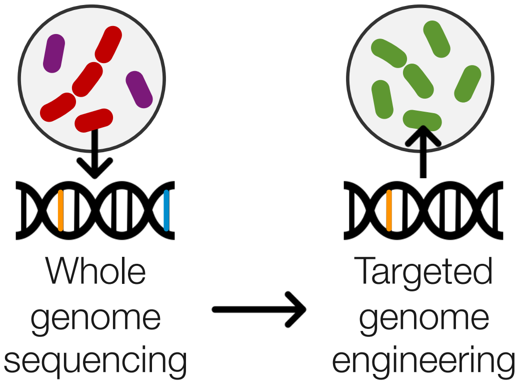
Potentially causative mutations identified

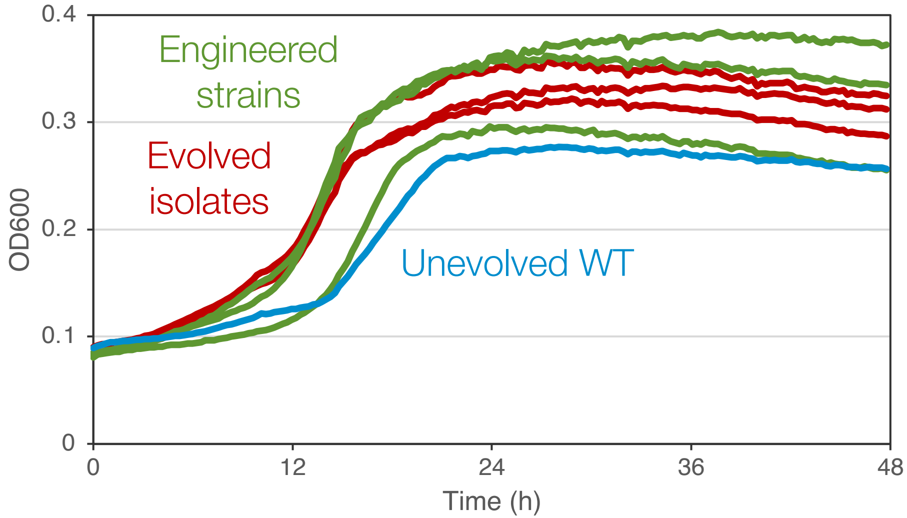
- Whole genome sequencing of 6 independently evolved isolates revealed that they shared several similar mutations, suggesting they were likely to be causative.

Lineage	Genome section 1	Genome section 2	Genome section 3	Transcription factor 1	Total deletion (bp)
HA6				Mutation	0
HB3	Deletion		Deletion	Mutation	42,177
HC8	Deletion	Deletion	Deletion		124,302
GD2	Deletion	Deletion	Deletion	Mutation	120,753
GE7	Deletion	Deletion	Deletion	Mutation	120,730
GF4	Mutation		Deletion		12,282

Rational engineering improved formate assimilation

- To evaluate the potential causation of these mutations and develop a genetically defined strain that recapitulates the performance of the evolved isolates, individual and combinations of these mutations were engineered into the otherwise WT strain.



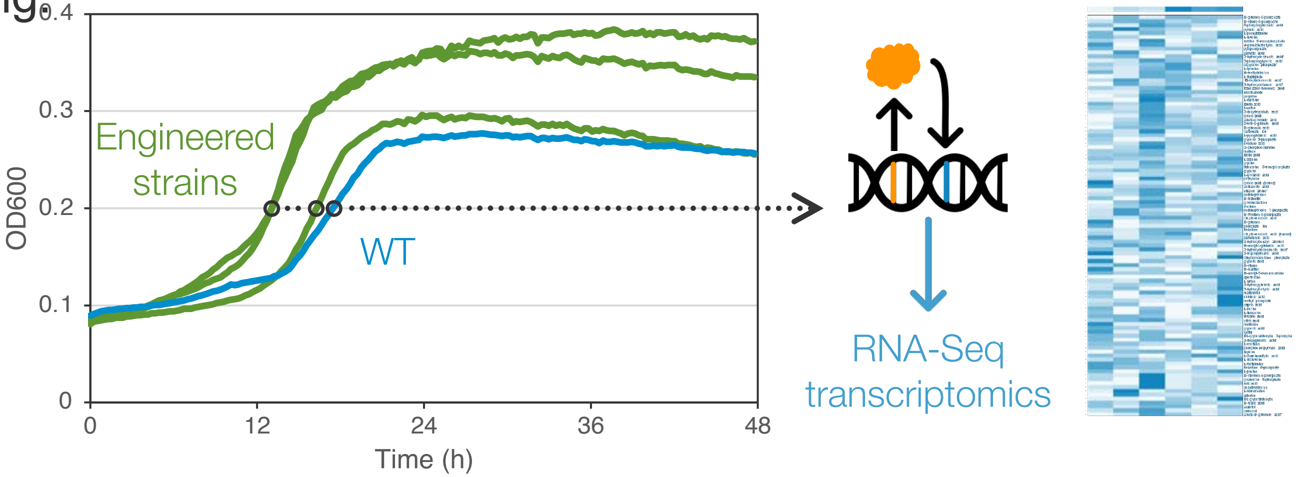


Genetically defined strains were developed that exhibit a ~1.2X rate of growth *and* biomass yield on formate relative to the WT parent.

- *This achieves our overall project goal of improving the growth rate or biomass yield of C. necator on formate by ≥ 1.2X*

Toward a mechanistic understanding

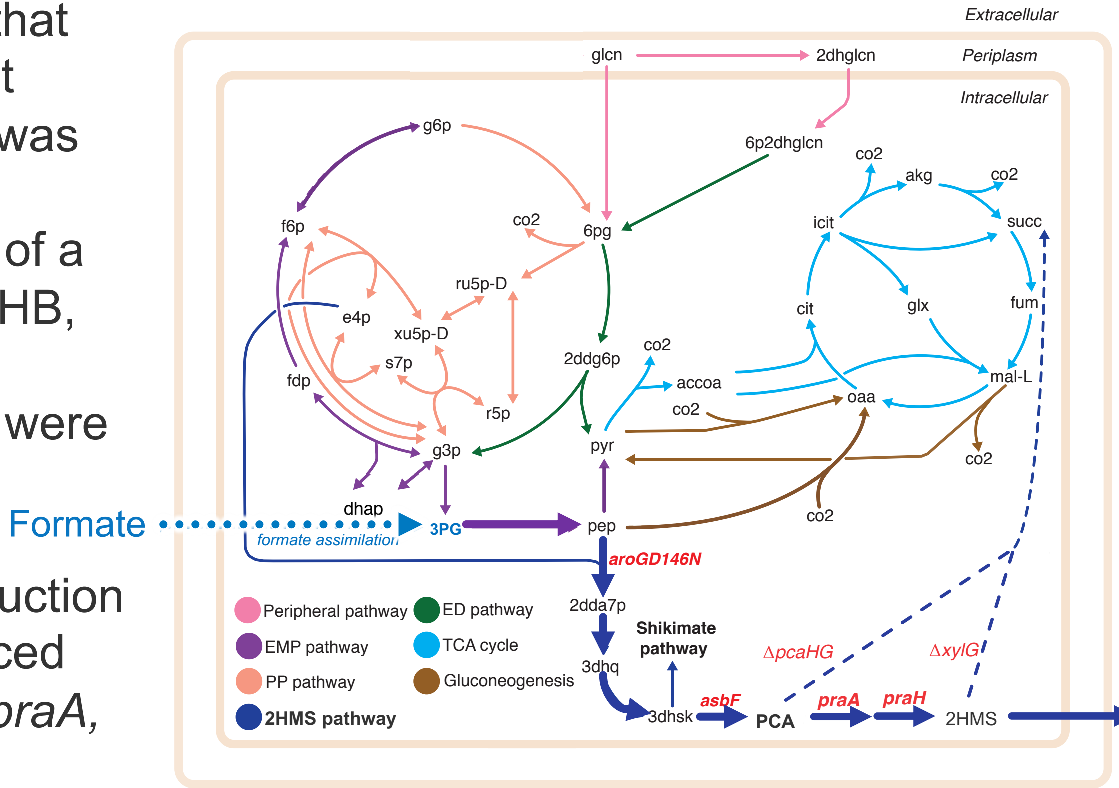
- The deletion of several genetic elements, in particular mutations in a single transcription factor, were shown to improve formate assimilation.
- RNA-Seq transcriptomics to identify the targets of this transcription factor and elucidate the metabolic changes resulting from these deletions are ongoing.

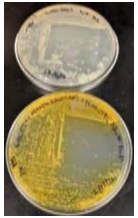


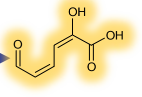
The results of this analysis will inform our basic understanding of formatotrophy and how it can be improved.

Engineering for production of 2HMS

- A restriction enzyme that enabled more efficient genome engineering was deleted (ORNL)
- Genes for production of a competing product, PHB, were deleted
- Competing pathways were deleted
  - *ΔpcaHG, ΔxylG*
- The pathway for production of 2HMS was introduced
  - *aroG*^{D146N}*, asbF, praA, praH*





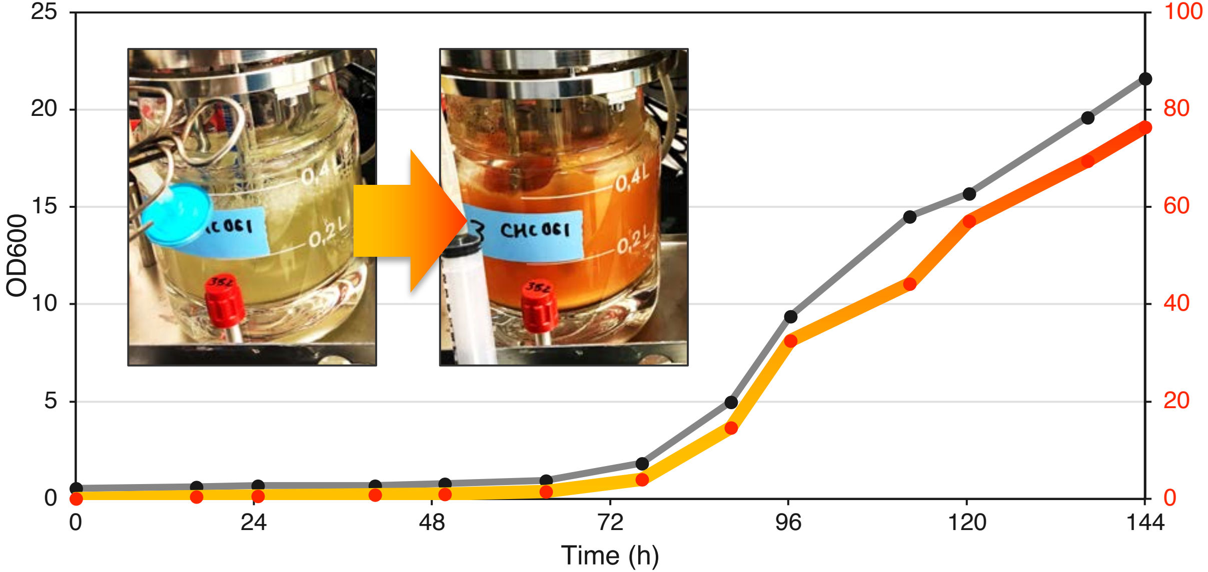


2HMS

Adapted from a figure by Jon Magnuson *et al.*, PNNL

pH-state Bioreactor production of 2HMS

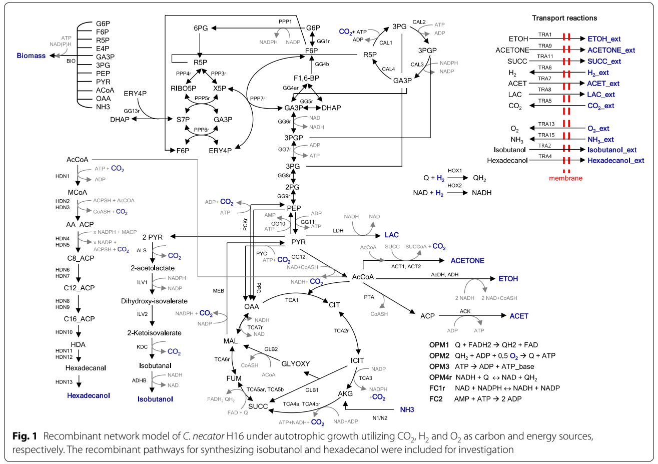
- A strain engineered for production of 2HMS was cultivated in a 0.5 L bioreactor using pH-stat feeding of 25% formic acid.
- The OD reached >20 and 76 mg/L 2HMS was produced



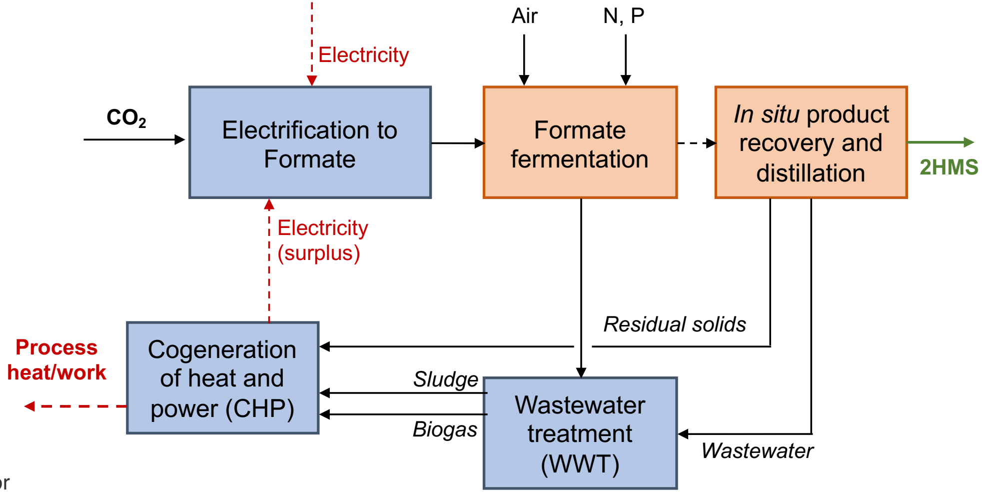
2HMS production and robust growth were demonstrated using pH stat control, which also mitigates toxicity of formate.

TEA/LCA

- A previously published metabolic model can be used to derive the governing equations for conversion of formate to 2HMS.



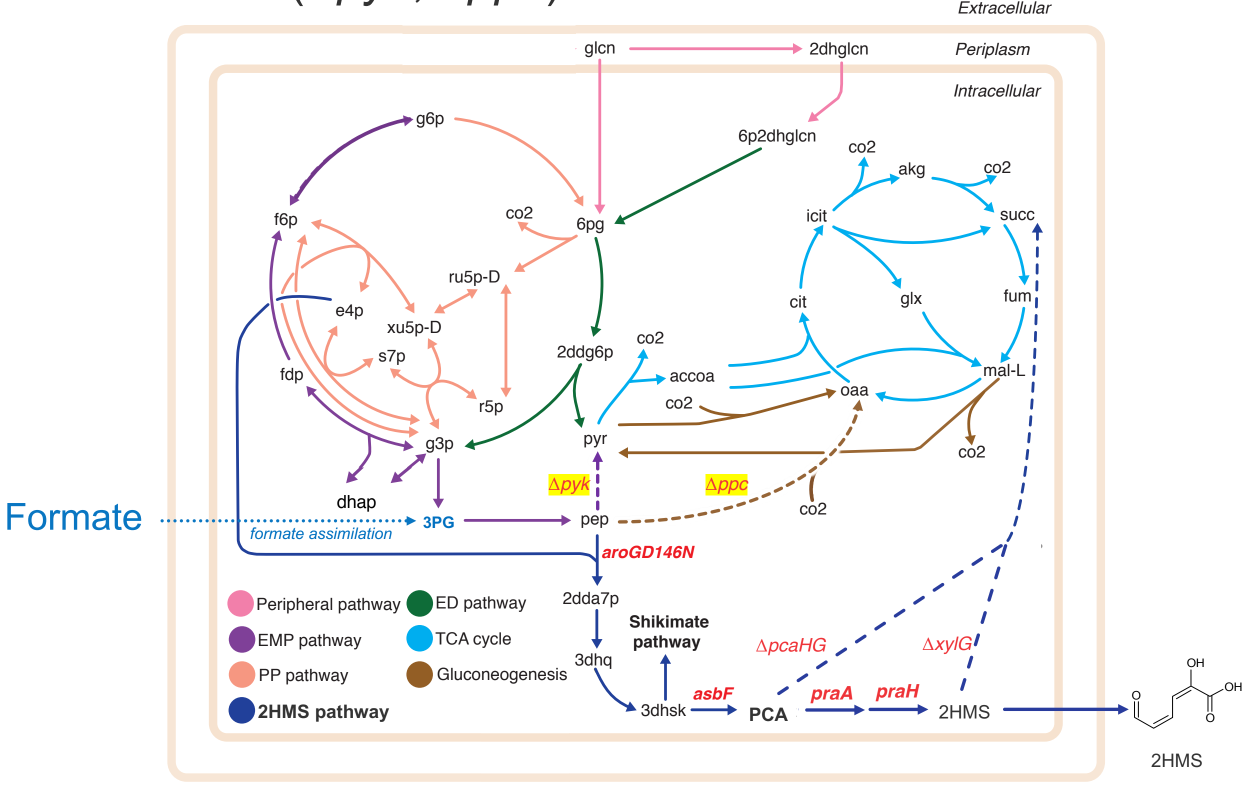
Unrean, P., Tee, K.L., Wong, T.S., 2019. Metabolic pathway analysis for in silico design of efficient autotrophic production of advanced biofuels. Bioresources and Bioprocessing 1–11. doi:10.1186/s40643-019-0282-4



- Final TEA/LCA on biological production of 2HMS from formate in FY21 will enable the DOE and other stake holders to evaluate the economic and environmental impact of this project.

Future Work

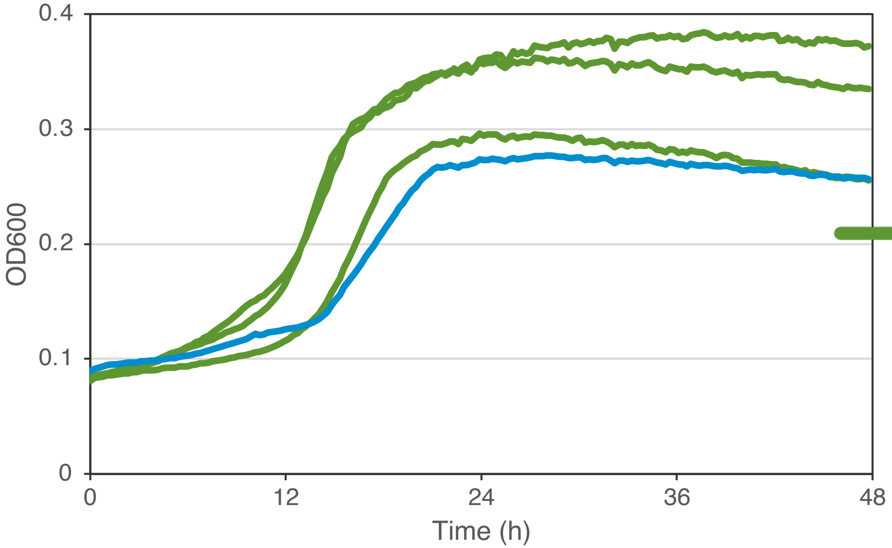
- Additional strain engineering and bioprocess development to improve production of 2HMS (Δ*pyk*, Δ*ppc*)

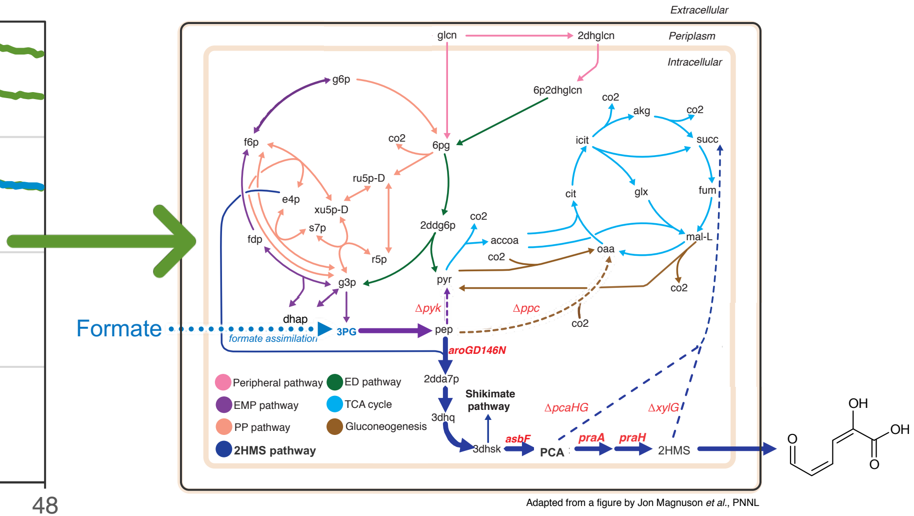


Adapted from a figure by Jon Magnuson *et al.*, PNNL

Future Work

- Additional strain engineering and bioprocess development to improve production of 2HMS (Δ*pyk*, Δ*ppc*)
- Improvements to formate assimilation will be leveraged to improve conversion of formate in strains engineered to produce 2HMS





- TEA and LCA will define the potential economic and environmental impacts of this and related technologies

Summary

Product
- Anticipated decrease in gasoline/ethanol demand; diesel demand steady
- Increasing demand for aviation and marine fuel
- Demand for higher-performance products
- Increasing demand for renewable/recyclable materials

Feedstock
- Sustained low oil prices
- Decreasing cost of renewable electricity
- Sustainable waste management
- Expanding availability of green H_2
- Closing the carbon cycle

Capital
- Risk of greenfield investments
- Challenges and costs of biorefinery start-up
- Availability of depreciated and underutilized capital equipment

Social Responsibility
- Carbon intensity reduction
- Access to clean air and water
- Environmental equity

NREL's Bioenergy Program Is Enabling a Sustainable Energy Future by Responding to Key Market Needs

Value Proposition
- Enable conversion of formate generated from low-cost renewable energy and waste CO_2 to myriad fuels and chemicals
- Decarbonize or reduce the carbon intensity of processes that generate CO_2

Key Accomplishments
- Demonstrated production of 2HMS and robust growth on formate
- Improved growth rate and biomass yield of *C. necator* on formate by ≥1.2X
- Contributing to our understanding of how formate conversion can be improved

Team Members

- Christopher Calvey
- Carrie Eckert
- Violeta Sanchez
- Colin Kneucker
- Ling Tao
- Michelle Reed

Q&A

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BIOENERGY TECHNOLOGIES OFFICE

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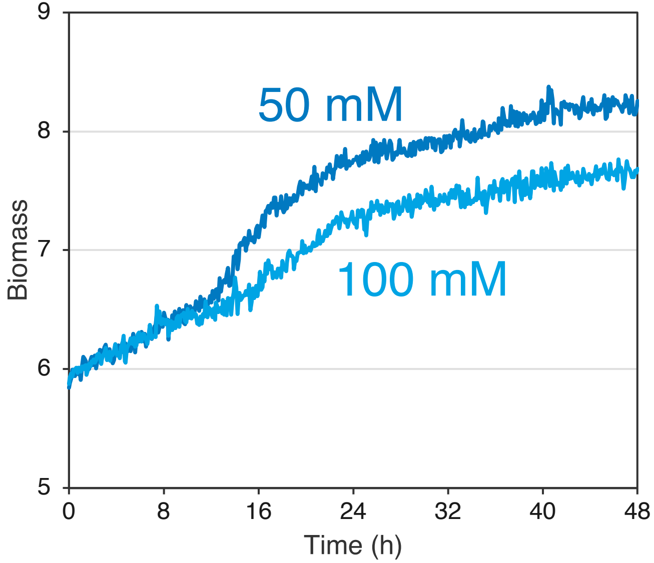
Additional Slides

Responses to Previous Reviewers' Comments

- "The performers are advised to deprioritize work on formolase (and focus on CBB) unless this enzyme's activity can be significantly improved." and "The performers should validate if they can demonstrate any flux using the extremely slow formolase enzyme and consider just focusing on improving the CBB pathway, though this will likely be extremely challenging and should be low priority. "
  - Introduction of the fomolase pathway into C. necator followed by adaptive laboratory evolution did not result in improved growth rate or biomass yield during growth on formate (**No-go**) so resources were redirected toward improving growth via the native CBB cycle.
  - Adaptive laboratory evolution resulted in the identification of mutations that improved the growth rate and biomass yield on formate via the native CBB cycle by 1.2X

Responses to Previous Reviewers' Comments

- "The safety and viability of the process can be better considered. Formate toxicity as an issue could be discussed."
  - We have demonstrated growth of C. necator on 100 mM and 50 mM formate, but the biomass yield on 100 mM was less than on 50 mM, suggesting toxicity negatively impacted growth. Accordingly, 50 mM has been used in most of our experiments.
  - In pH-stat bioreactors, formate is fed at the rate it is consumed, keeping the formate concentration low and, consequently, avoiding toxic effects.
  - Formic acid is caustic and solutions above 85% are flammable, but normal chemical safety measures can mitigate the risks this introduces.



Publications, Patents, Presentations, Awards, and Commercialization

- We anticipate at least two high-impact publications will result from this work.
  - Adaptive laboratory evolution of *C. necator* for improved formate utilization
  - Metabolic engineering of *C. necator* for upgrading of formate to 2-hydroxymuconate semialdehyde

- We also anticipate at least one patent application related to novel metabolic engineering strategies to improve formate assimilation will result from this work.