PRocess Intensification for the reduced CommErcial CAPEX of Biofuels Production (PRICE CAP) using Dynamic Metabolic Control

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Overview

- Capex as a Key Barrier for Next Generation Bio-products
- 2-Stage Dynamic Metabolic Control Overview
- PRICE CAP Overview
- Review of TEA & Process Model
- Key Program Goals
- Progress to Date
  - High Cell Density Fermentations
  - Cellulosic Sugar Co-utilization
  - Terpene Production
  - Semi-continuous Process Development

Capex is a Key barrier to commercialization

Reported Capex for Various Processes		
Example Process	Capex ($ per gal annual capacity)	$ per 100Mgal/y facility
Petrochemical Plant	$3-$12	$300 M-$1.2 B
Cellulosic EtOH – NREL 2012	$6.92	$692 M
Cellulosic EtOH –Poet-DSM 2014	$13.75	$1.4 B
Cellulosic h-carbon–NREL-2013	$18.60	$1.8 B
Corn starch ethanol *	$1.10	$110M
Proposed Process	$0.50	$50 M

* Does not include corn milling costs

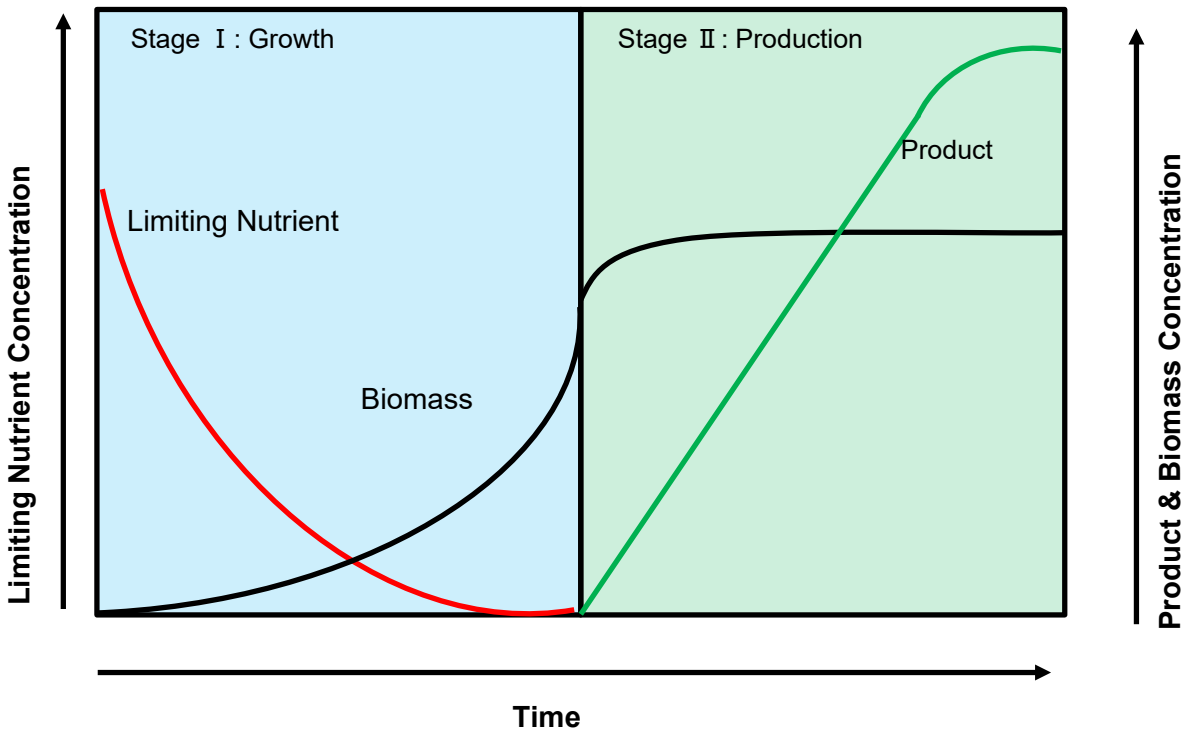
Large Capital Costs are a NO-GO Point on the commercialize bio-based processes.

- Often risky first of their kind plants
- Competitive Capital investments (petro) are proven
- Plant level ROI are not manageable even with low costs of capital

- For a $500M investment 20% minimal ROI and ten year payback period you would need to make a profit of $0.60/gallon on a 100M gallon/year facility, assuming 0 cost of capital. - No-one should put their money here.

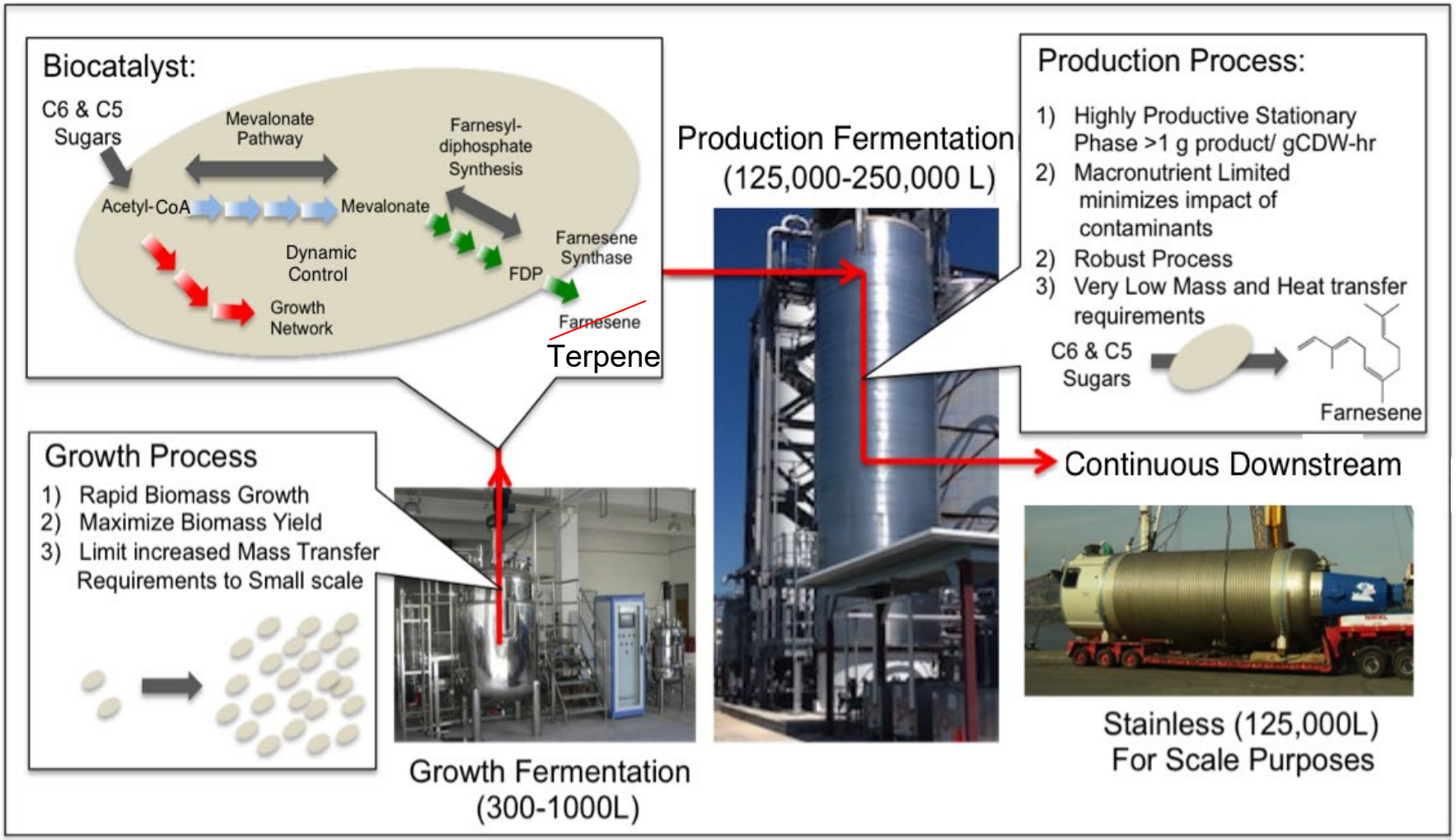
- A $50M investment only requires a $0.06/gallon profit.

Two Stage Dynamic Metabolic Control



PRICE CAP Approach

PRocess Intensification for the reduced CommErcial CAPEX of Biofuels Production (PRICE CAP)

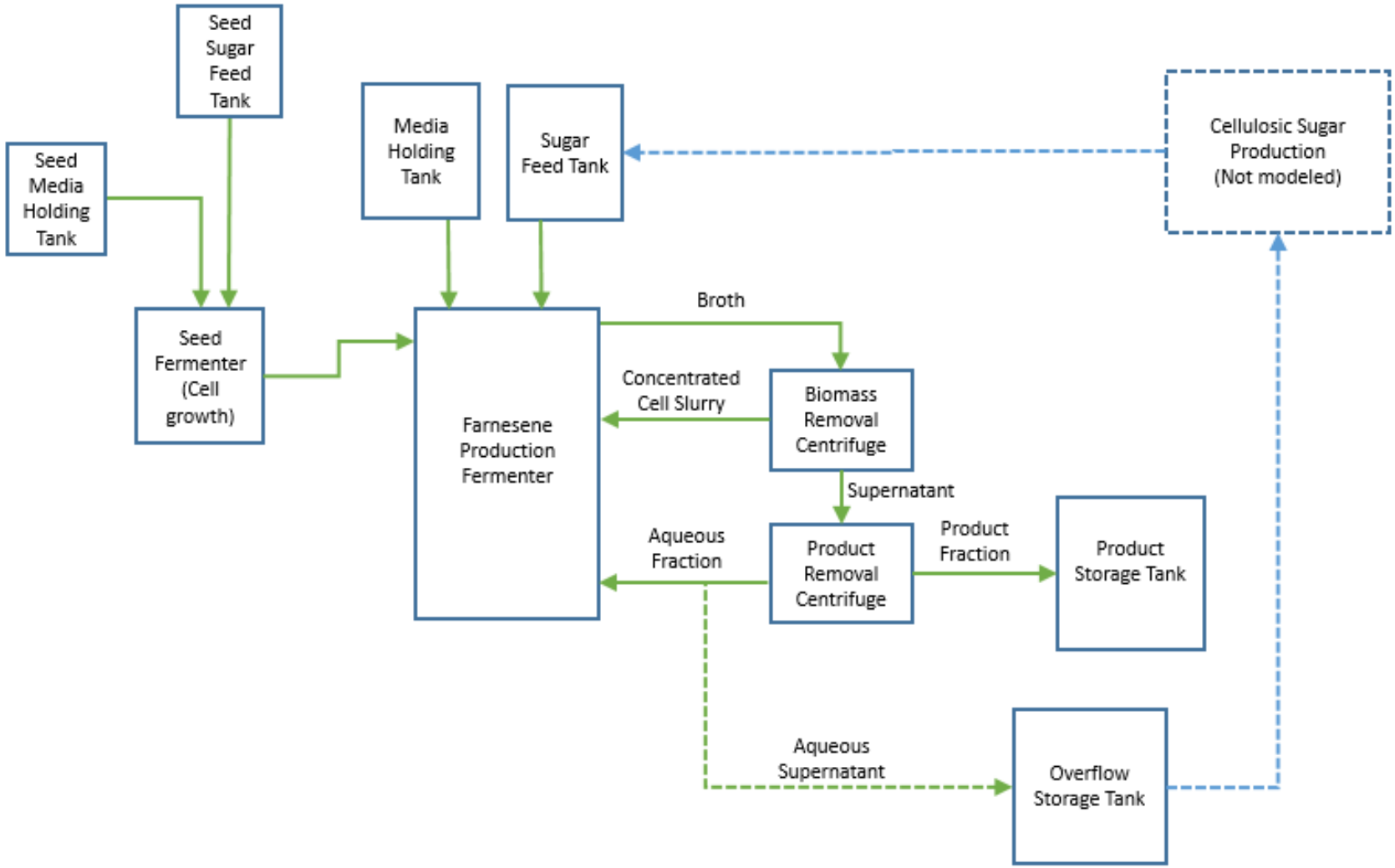


Key Program Goals

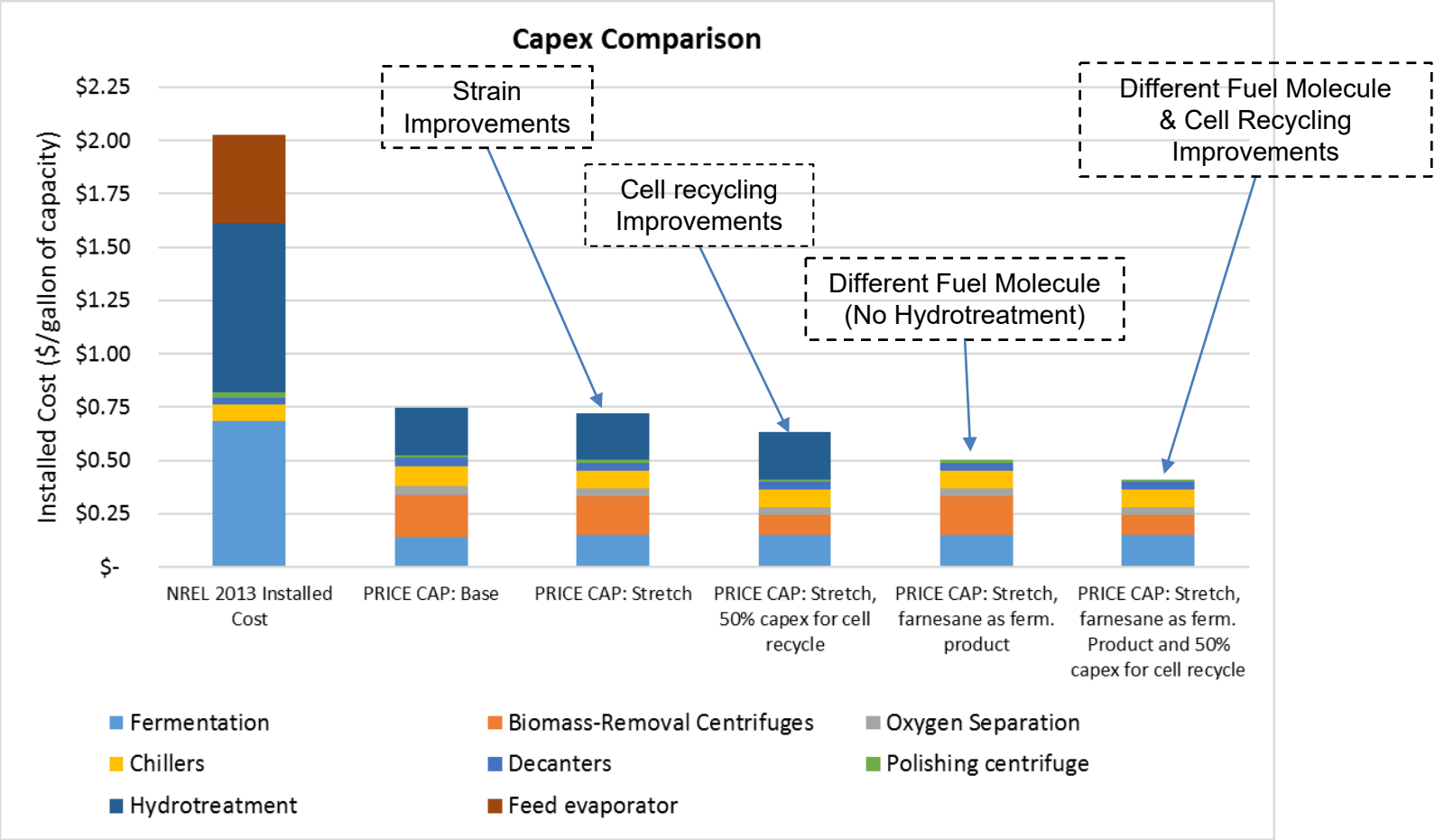
1) Techno-economic Analysis to validate the Price Cap Approach
2) Demonstrate cellulosic sugar co-utilization
3) Demonstrate high rates of terpene production
4) Demonstrate semi-continuous processing
5) Demonstrate system integration

TEA & Process Model

Process Flow Diagram



Capex Glide Path for Cost Reduction



- PRICE CAP addresses high cost of fermentation capex
  - Some DSP cost reductions as well, due to lower cost of hydrogenation vs. decarboxylation
- Further efforts needed to reduce cell recycle and downstream conversion costs

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Key Goals

1) Biomass Levels ~ 50 gCDW/L
2) Co-consumption of all cellulosic sugars
3) Terpene Production rates > 0.75g/gCDW-hr
4) Semi-continuous Processing
5) Process Integration

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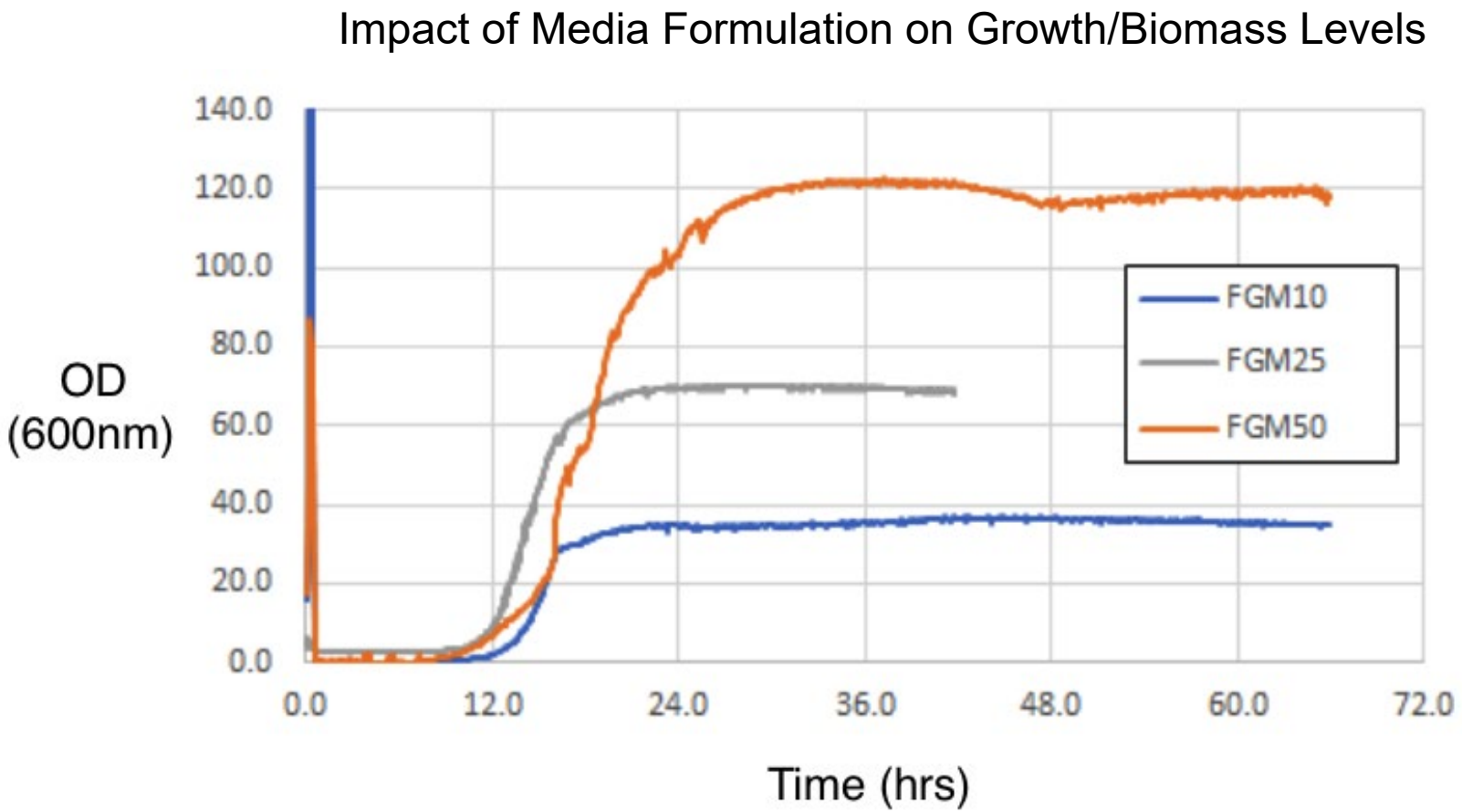
High Biomass Fermentations

High Biomass Fermentations



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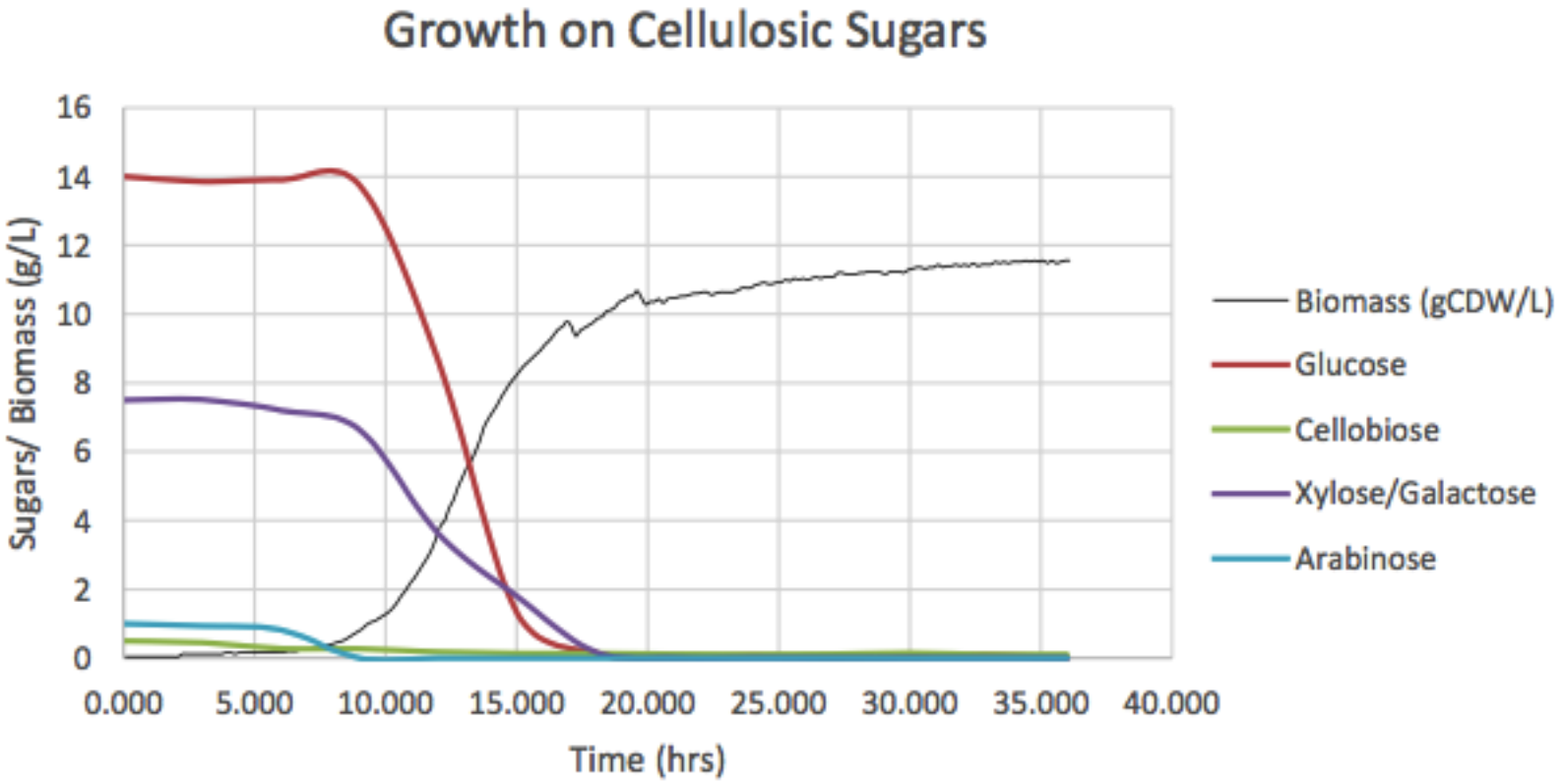
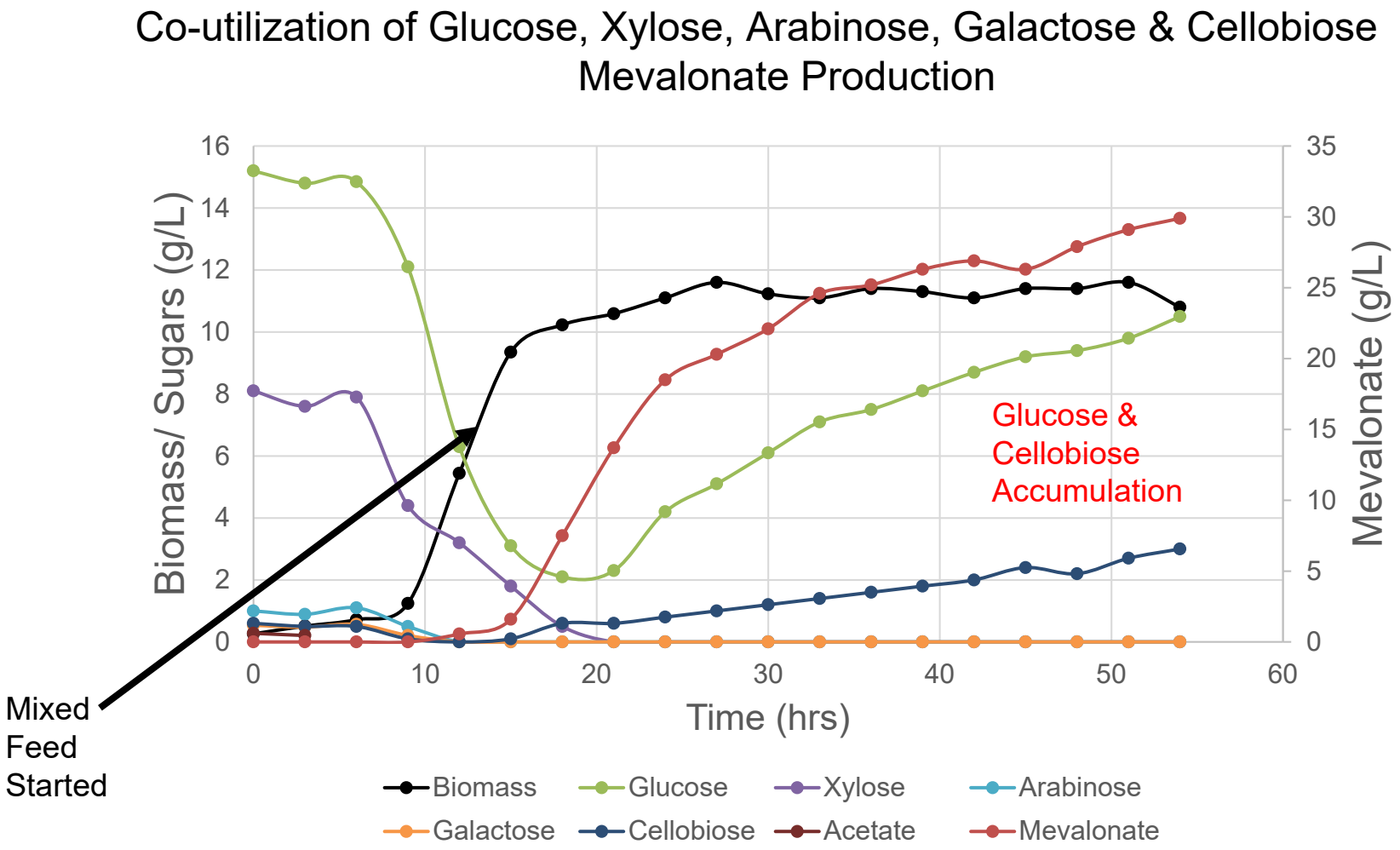
Cellulosic Sugar Utilization

Cellulosic Sugar Co-Utilization – Growth



Total Sugars ~ 25g/L
(14.1 g/L Glucose,7.5 g/L Xylose, 1.2 g/L Arabinose, 0.6 g/L Galactose, 0.6 g/L Cellobiose, 0.3 g/L Acetate)

Cellulosic Sugar Co-Utilization Stationary Phase Production



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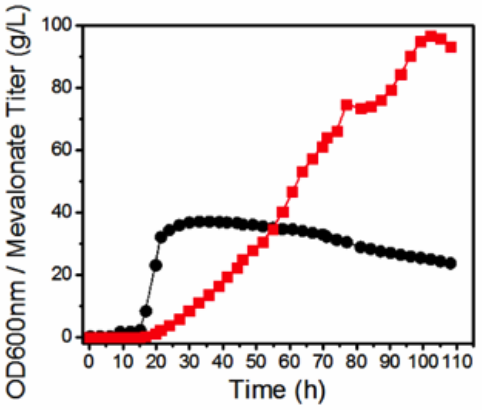
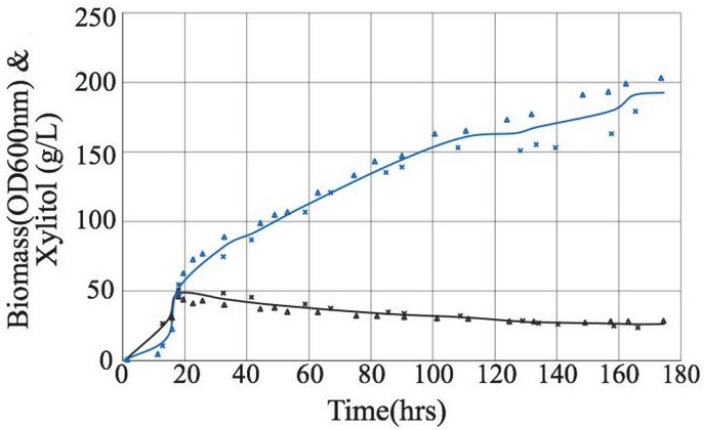
Cellulosic Sugar Co-Utilization Stationary Phase Production

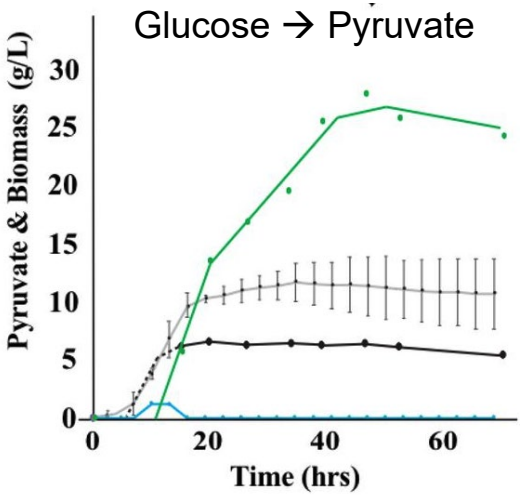
Glucose → Mevalonate



Xylose → Xylitol



Glucose → Pyruvate



- Orthogonal Work is consistent with glucose uptake “stalling” in a product dependent manner, whereas xylose uptake can be prolonged.
- The fact that glucose and cellobiose (both metabolized through upper glycolysis, other sugars are consumed through the PPP or lower glycolysis) both accumulated, led us to hypothesize that upper glycolysis was inhibited in certain metabolic states.
- Indeed we have engineered strains that can bypass this regulation and continue to consume glucose without “stalling”, testing these new strains with terpene products is pending. Patent filings pending for these new strains.

Key Goals

1) Biomass Levels ~ 50 gCDW/L
2) Co-consumption of all cellulosic sugars (Growth Complete/Stationary Phase In Process)
3) Terpene Production rates > 0.75g/gCDW-hr
4) Semi-continuous Processing

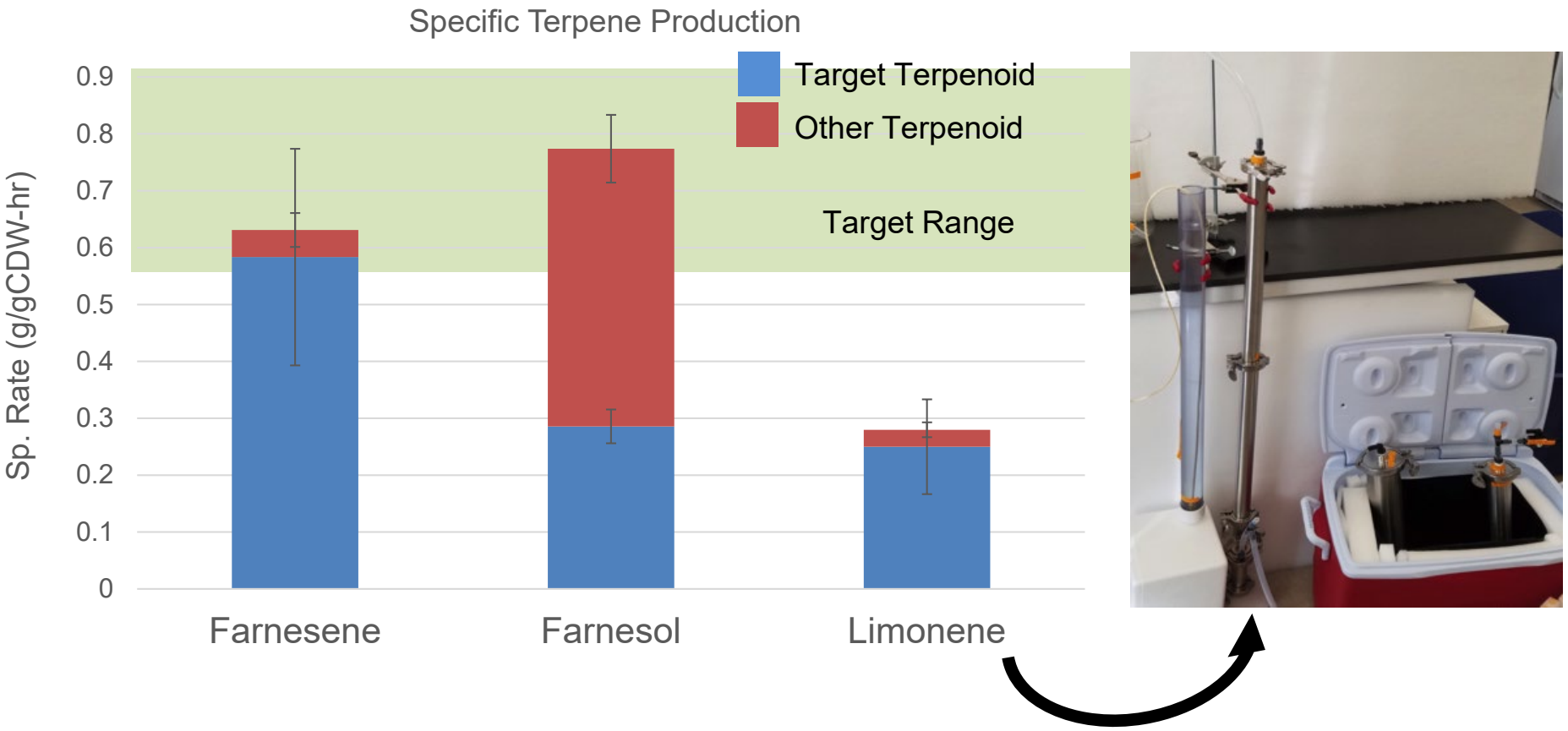
Terpene Production

Terpene Production – Strain Engineering

1. We previously reported 2-stage farnesene production, but encountered several challenges, several of which we have solved.
    a. High protein expression levels → Menacho Melgar et al. Biotech & Bioeng 2020, 117, 9, 2715-2727
    b. Plasmid/Strain Instability → Ye et al, *ACS Synth. Biol.* 2021, 10, 1, 29–37
    c. Promoter Robustness → Moreb et al. *ACS Synth. Biol.* 2020, 9, 6, 1483–1486

2. And a few in progress/pending
    a. Farnesene, large interphase at higher concentrations leading to very difficult accurate quantification and difficult separations.
    b. Alternative terpene options
        Farnesol
        Limonene – Separated in offgas

Terpene Production



Farnesol byproducts include other shorter chain alcohols, some esters and unknown products
Shorter chain alcohols lost in culture in vapor phase at high rates.

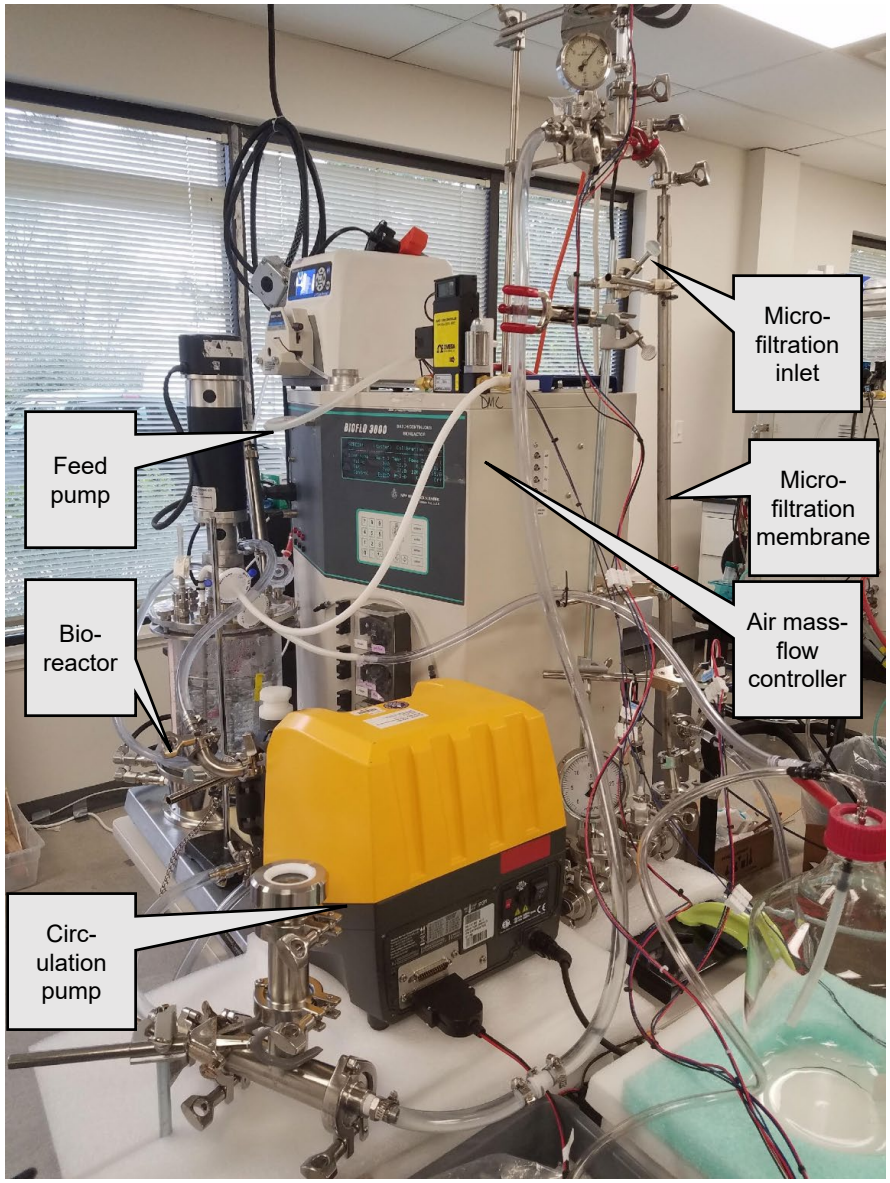
Limonene also lost from culture in vapor phase (depending on culture conditions)

Key Goals

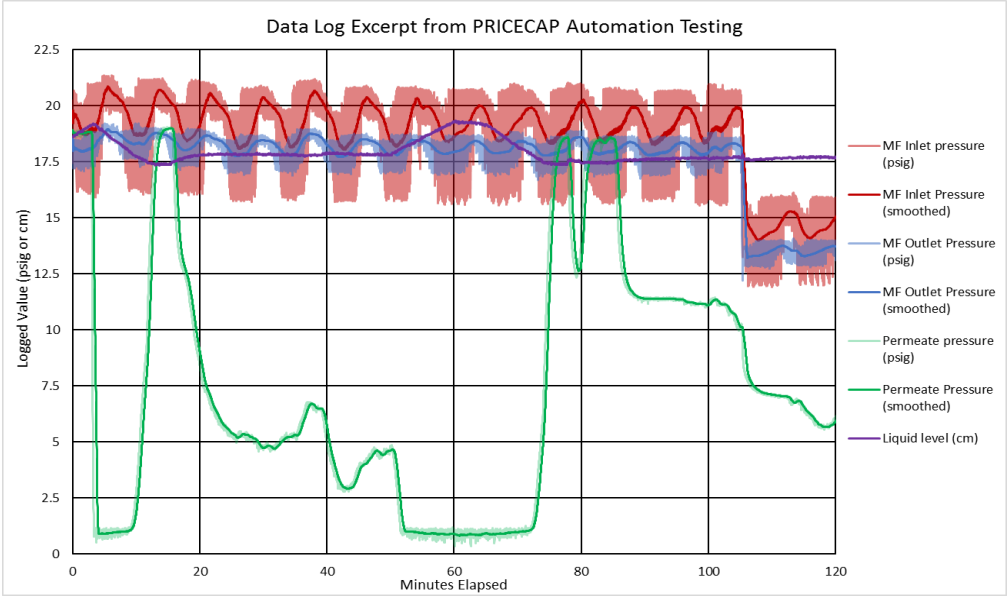
1) Biomass Levels ~ 50 gCDW/L
2) Co-consumption of all cellulosic sugars (Growth Complete/Stationary Phase Pending)
3) Terpene Production rates > 0.75g/gCDW-hr (MF Complete, Fermentation Pending)
4) Semi-continuous Processing

Semi-Continuous Processing

Semi-Continuous Process Automation



Hardware and control systems developed for automated semi-continuous fermentation



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Cell recycling @ 5L

Video Link 1

Cell Recycling – Moving Forward

1. Initial Cell recycling attempts with terpenes failed and so several fermentation attempts with soluble products were performed. These also had a higher failure rate, primarily due to lack of accuracy of process control , despite efforts described above.
2. Moving forward having only 1–2 tanks, each with significant effort to customize, is not amenable to the amount of work needed to optimize the system for 2-stage semi-continuous processing.
3. As result we designed and have initially validated low cost 3-D printed bioreactors (as well as auto-samplrs and recycling) that can be mass produced and rapidly altered to optimize future semi-continuous processes. Cost <$1500/reactor (not considering probes)
4. For auto-sampling refer to Efromson et al. https://www.sciencedirect.com/science/article/pii/S2468067221000067.

BioREACT Jr Prototype

Key Goals

1) Biomass Levels ~ 50 gCDW/L
2) Co-consumption of all cellulosic sugars (Growth Complete/Stationary Phase Pending)
3) Terpene Production rates > 0.75g/gCDW-hr (MF Complete, Ferm. Pending)
4) Semi-continuous Processing (Pending)
5) Process Integration (Pending)

Quad Chart

Timeline
Project start date
Project end date

	FY20 Costed	Total Award
DOE Funding	(10/01/2019 – 9/30/2020)	$1,691,595
Project Cost Share		$188,189

Project Goal
Demonstrate semi-continuous 20stage production of terpenoids from cellulosic sugars

End of Project Milestone
A Volumetric Rate of terpene in semi-continuous production > 25g/L-hr with cellulosic sugars as a feedstock.

Project Partners*
DMC Biotechnologies, Inc.

Funding Mechanism
BETO Incubator.

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Questions

Publications, Patents, Presentations, Awards, and Commercialization

Manuscripts In Press

11. Efromson, J.P., Li, S., and **Lynch M.D.** BioSamplr: An open source, low cost automated sampling system for bioreactors. HardwareX. April 2021. https://www.sciencedirect.com/science/article/pii/S2468067221000067

10. Li, S., Moreb, E.A., Ye, Z., Hennigan, J.N., Baez Castellanos, D. , Yang, T., and **Lynch M.D.** Dynamic control over feedback regulatory mechanisms improves NADPH fluxes and xylitol biosynthesis in engineered *E. coli*. Metabolic Engineering. March 2021. https://www.sciencedirect.com/science/article/pii/S1096717621000136

9. Moreb, E.A, Hutmacher, M., and Lynch, M.D.. CRISPR/Cas "non-target" sites inhibit on-target cutting rates. CRISPR Journal. 18 Dec 2020. https://www.liebertpub.com/doi/10.1089/crispr.2020.0065

8. Ye, Z., Moreb, E.A., Li, S., Lebeau, J., Menacho-Melgar, R., Munson, M., and **Lynch M.D.** *Escherichia coli* Cas1/2 Endonuclease Complex Modifies Self-Targeting CRISPR/Cascade Spacers Reducing Silencing Guide Stability. December 2020. https://pubs.acs.org/doi/10.1021/acssynbio.0c00398

7. Decker, J.S., Menacho-Melgar, R., and Lynch, M.D. Low-Cost, Large-Scale Production of the Anti-viral Lectin Griffithsin Frontiers in Biotechnology & Bioengineering. 2020. https://doi.org/10.3389/fbioe.2020.01020

6. Menacho-Melgar, R., Moreb, E.A, Efromson, J.P., and **Lynch, M.D**. Improved, two-stage protein expression and purification via autoinduction of both autolysis and auto DNA/RNA hydrolysis conferred by phage lysozyme and DNA/RNA endonuclease. Biotechnology & Bioengineering. 2020. https://doi.org/10.1002/bit.27444

5. Menacho-Melgar, R., Ye, Z., Moreb, E.A, Yang, T., Decker, J.S., Efromson, J.P., Wang, R., and **Lynch, M.D**. Scalable, two-stage, autoinduction of recombinant protein expression in *E. coli* utilizing phosphate depletion. Biotechnology & Bioengineering. 2020 https://doi.org/10.1002/bit.27440

4. Moreb, E.A, Ye, Z., Efromson, J.P., Hennigan, J.N., Menacho-Melgar, R., and **Lynch, M.D.**. Media robustness and scalability of phosphate regulated promoters useful for two-stage autoinduction in *E. coli.* ACS Synthetic Biology. 2020. https://doi.org/10.1021/acssynbio.0c00182

3. Lebeau, J., Efromson, J.P., and **Lynch, M.D.**, 2020. A review of the biotechnological production of methacrylic acid. Frontiers in Biotechnology & Bioengineering. 2020. doi: 10.3389/fbioe.2020.00207

2. Menacho-Melgar, R., Decker, J.S., Hennigan, J.N., and **Lynch, M.D.** A review of lipidation in the development of advanced protein and peptide therapeutics. Journal of Controlled Release. 2019. Vol. 295. p1-12. doi: 10.1016/j.jconrel.2018.12.032

1. Moreb, E.A, Hoover, B., Yaseen, A., Valyasevi, N., Roecker, Z., Menacho-Melgar, R. and **Lynch, M.D**. Managing the SOS Response for Enhanced CRISPR-Cas-based Recombineering in *E. coli* through Transient Inhibition of Host RecA Activity. ACS Synthetic Biology. 2017 Vol. 6(12). p 2209-2218. doi: 10.1021/acssynbio.7b00174

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Publications, Patents, Presentations, Awards, and Commercialization

Manuscripts Submitted or In Review.

5. Rios, J., Lebeau, J., Yang, T., Li, S., and **Lynch M.D.** A Critical Review on the Progress and Challenges to a More Sustainable, Cost Competitive Synthesis of Adipic Acid. (In Review)

4. Hennigan, J.N., Wagner, P.S., Burk C., Efromson, J.P., Ye, Z., Lipscomb, M.L., and **Lynch M.D.** A Technoeconomic Evaluation of the Potential of Industrial Biotechnology for the Competitive Production of Commodity and Bulk Chemicals (Nature Sustainability: In Review). https://doi.org/10.26434/chemrxiv.13238996.v1

3. **Lynch M.D.** The Bioprocess TEA Calculator: An online techno-economic analysis tool to evaluate the commercial competitiveness of potential bioprocesses. (Metabolic Engineering: In Review). BioRxiv 2020. https://www.biorxiv.org/content/10.1101/2020.10.08.331272v1

2. Ye, Z., Li, S., Hennigan, J.N., Lebeau, J., Moreb, E.A., Wolf, J., and **Lynch M.D.** Two-stage Dynamic Deregulation of Metabolism Improves Process Robustness & Scalability in Engineered E. coli. (Metabolic Engineering In Review.) BioRxiv 2020 https://doi.org/10.1101/2020.08.30.274290

1. Li, S.*, Ye, Z.*, Lebeau, J., Moreb, E.A., and **Lynch M.D.** Dynamic control over feedback regulation identifies pyruvate-ferredoxin oxidoreductase as a central metabolic enzyme in stationary phase *E. coli*. (In Review)* authors contributed equally to this work. BioRxiv 2020. doi: https://doi.org/10.1101/2020.07.26.219949

Manuscripts In Preparation

5. Li., S., Ye, Z., Moreb, E.A., Kalantari, A., and **Lynch M.D.** Dynamic, Scalable 2-Stage Terpenoid Production in Engineered *E. coli* . (In Preparation)

4. Wagner, P., and **Lynch M.D.** A technoeconomic evaluation of the potential of 2-stage semi-continuous fermentations in large scale bioprocesses. (In Preparation)

3. Li., S., and **Lynch M.D.** Robust 2-stage co-consumption of cellulosic sugars. (In Preparation)

2. Wolf N.W., Efromson, J.P., Hubman, C.D., Poplyk, M., and **Lynch M.D.** BioReact: An Open Source, Low Cost, 3D Printed Bioreactor System. (In Preparation)

1. Menacho-Melgar, R., Tang, T., and **Lynch M.D.** Eazymes (Easy Enzymes): Rapid Expression and Purification of Routine DNA modifying enzymes. (In Preparation)

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Patents & Patent Applications

Several Additional in Preparation.

10. Menacho-Melgar, R., Moreb, E.A., Efromson, J.P., and **Lynch, M.D.** Compositions and Methods for Auto-inducible Cellular lysis and nucleotide hydrolysis. PCT/US2021/012605

9. Menacho-Melgar, R., Moreb, E.A., Efromson, J.P., and **Lynch, M.D.** Compositions, Systems and Methods for High Level Expression of Recombinant Protein. PCT/US2020/057062

8. Menacho-Melgar, R., and **Lynch, M.D.** Robust Protein Expression Enabled by Dynamic Control over Host Proteases. US63/064501

7. Menacho-Melgar, R., Yan, T., and **Lynch, M.D.** Rapid Expression and Purification of Thermostable Proteins Including Taq Polymerase. US63/064217

6. Li, S. and **Lynch, M.D.** Methods and Compositions for the Production of Xylitol from Xylose Utilizing Dynamic Metabolic Control . US63/056085

5. Li, S. and **Lynch, M.D.** Methods and Compositions for the Production of Acetyl-CoA Derived Products. US63/056031

4. Li, S. and **Lynch, M.D.** Methods and Compositions for the Production of Acetyl-CoA Derived Products. US63/056031

3. Decker, J.S. and **Lynch, M.D.** Systems an Methods for the Production of Griffithsin and Related Proteins. US62/992557

2. Moreb, E.A., Lebeau, J., Ye, Z., and **Lynch, M.D.** Methods and Compositions for Improved Type I-E CRISPR Based Gene Silencing. US62/990172

1.Ye, Z., and **Lynch, M.D.** Compositions and Methods for the Production of Pyruvic Acid and Related Products Utilizing Dynamic Metabolic Control. US62/687874

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Commercialization

1. Several patents/patent applications have been licensed to DMC Biotechnologies, Inc.
2. A new company Roke Biotechnologies, LLC. was founded in 2020 to commercialize Heterologous Protein Expression Technology Developed in part by this funding.
3. Initial Commercial Deployment of a Bioprocess to produce GRFT, a broad spectrum antiviral, and potential SARS-CoV-2 preventative begun in 2020 using the protein expression technology developed in part by this funding. Technology Transfer from Duke to a team at the NIH/NCATS is underway to produce material for clinical trials.
4. Initial Commercial Deployment of a Bioprocess to produce a higher value terpenoid to an established chemical company have begun. The work to develop this strain was in part supported by this work.