DOE Bioenergy Technologies Office (BETO) 2019 Project Peer Review

A Novel Platform for Algal Biomass Production Using Cellulosic Mixotrophy

Advanced Algal Systems

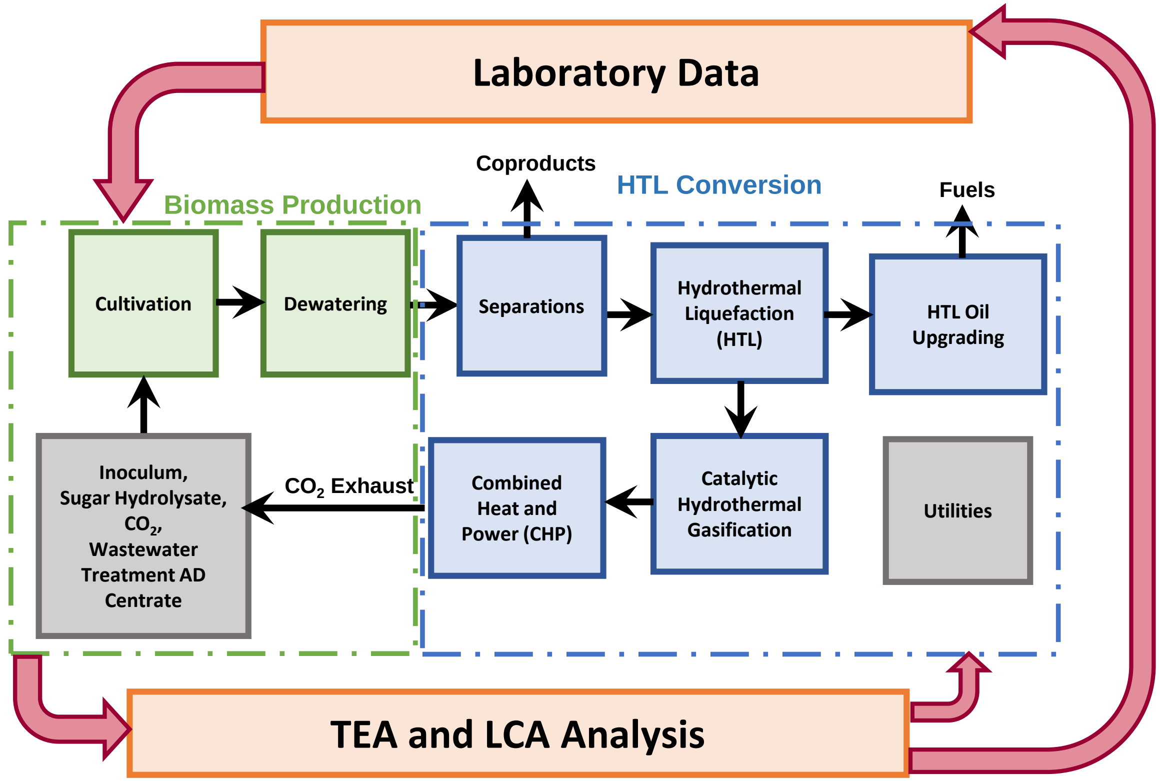
P.I. Peter J Lammers
Arizona State University

Approach

- The NREL cellulosic sugar hydrolysate process was used as a starting point for cultivation intensification (glucose, xylose, arabinose, galactose and β,1-4 linked cellobiose)
- Polyextremophilic red microalga ***Galdieria sulphuraria*** (heat and acid tolerant) employed to limit heterotrophic contaminants
- Arizona State University: (Prime) management, cultivation and reactor innovations, mixotrophic metabolism and catabolic repression (**John McGowen & Mark Seger, Colton Skavicus)**
- Heliae: phycocyanin co-product development (**Eneko Ganuza**)
- New Mexico State University: carbon partitioning, proteomics and cellulase/secretome analysis (**Omar Holguin**)
- Colorado State University and NREL: LCA & TEA modeling analysis (**NREL: Ryan Davis & Jenn Clippinger, CSU: Jason Quinn & Students J. Cruce, M. Sommers and S. Compton**)
- No scope for downstream processing – HTL assumed pathway based on several publications with **Shuguang Deng at ASU** and PNNL data.

Process Model

- Laboratory data feed into the cultivation process model
- Process model design feeds into TEA and LCA data and assumptions
- TEA and LCA results feed back to decisions on new laboratory experiments to run for more data



Project Milestones/Activities	FY17				FY18			
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Techno-Economic Analysis and Life Cycle Assessment								
Process Modeling Work								
Techno-economic Analysis				▼				⬣
Life Cycle Assessment				▼				⬣

▲= Milestone ▼ = Quarterly progress measure ⬣ = Go/no-go decision

Project Overview

Goals: Enhance volumetric algal biomass productivity 10-fold in support of biofuel production based on mixotrophic metabolism of cellulosic sugars in closed photobioreactors to reduce water consumption enabling deployment in arid regions

Outcomes Demonstrated:

- Volumetric biomass productivities >1.0 g L^{-1} day^{-1} on corn stover hydrolysate (3-fold higher than tubular PBR-based photoautotrophy, 20-fold higher than typical open raceway photoautotrophy)
- Substrate yields >0.7 – 1.1 g-biomass g-$sugars^{-1}$, 50-200% higher than heterotrophic substrate yields (0.42-0.45)
- Submitted patent (12/2018) for balanced mixotrophic cultivation with no requirement for external oxygen or carbon dioxide
- Identified cellulase activity in poly-extremophilic *Galdieria* strains; basis for *in situ* glucose+xylose release from acid-pretreated cellulose

Project Overview

Relevance: Abundant flat land in arid regions of the U.S. could support biofuel production in evaporation-resistant reactors coupled with thermotolerant, high-productivity mixotrophic algae.

Project outcomes:

- Methods for avoiding catabolic repression of photosynthesis by sugars have been identified (microaerobic conditions)
- Phycocyanin co-product levels are not compromised
- The *Galdieria sulphuraria* secretome contains a variety of polymeric hydrolases that could be intensified for *in situ* release of cellulosic sugars
- TEA results show photobioreactor cost reductions are needed.
- Lower cost PBR design criteria have been identified via demonstration that CO2 and O2 gas recycling via sugar respiration and photosynthesis eliminate the need for a supplying metabolic gases and the associated energy requirement for mass transfer

Quad Chart Overview

Timeline

- Start - Oct. 1, 2016
- End - Apr. 15, 2019
- Current - 99% complete

	Total Costs Pre FY17**	FY 17 Costs	FY 18 Costs	Total Planned Funding (FY 19-Project End Date)
DOE Funded	$169K	$676K	$676K	$1,689,791
Project Cost Share*	$28K	$110K	$112K	$277,374

•Partners: Arizona State University, NREL, Colorado State University, New Mexico State University

Barriers Addressed:

- Increase algal biomass yields and value
- Decrease water use and culture failures
- Lignocellulose/algae system integration

Objectives

- Demonstrate stable, mixotrophic cultivation on cellulosic sugars
- Evaluate acid-pretreated cellulose breakdown by *Galdieria sulphuraria*
- Heat-stable phycocyanin co-product development

End of Project Goals

- TEA/LCA models
- Roadmap to biorefinery process intensification

3 – Technical Accomplishments/ Progress/Results

Patents – Pending or In Preparation

- Lammers, P.J. Seger, M, Park, W., Csakan, N. Methods of Increasing Biomass Productivity in Algae Cultures. PCT/US 2018065822 (microaerobic mixotrophy with metabolic O_2 & CO_2 gas recycling - ASU)
- Ganuza, E. Sellers, Amezquita M., Locsin J. (2019) Methods to control the relative contribution of photo and heterotrophic metabolism in mixotrophic cultures. In preparation. (ammonium/pH auxostat culture method – Heliae)

Publications:

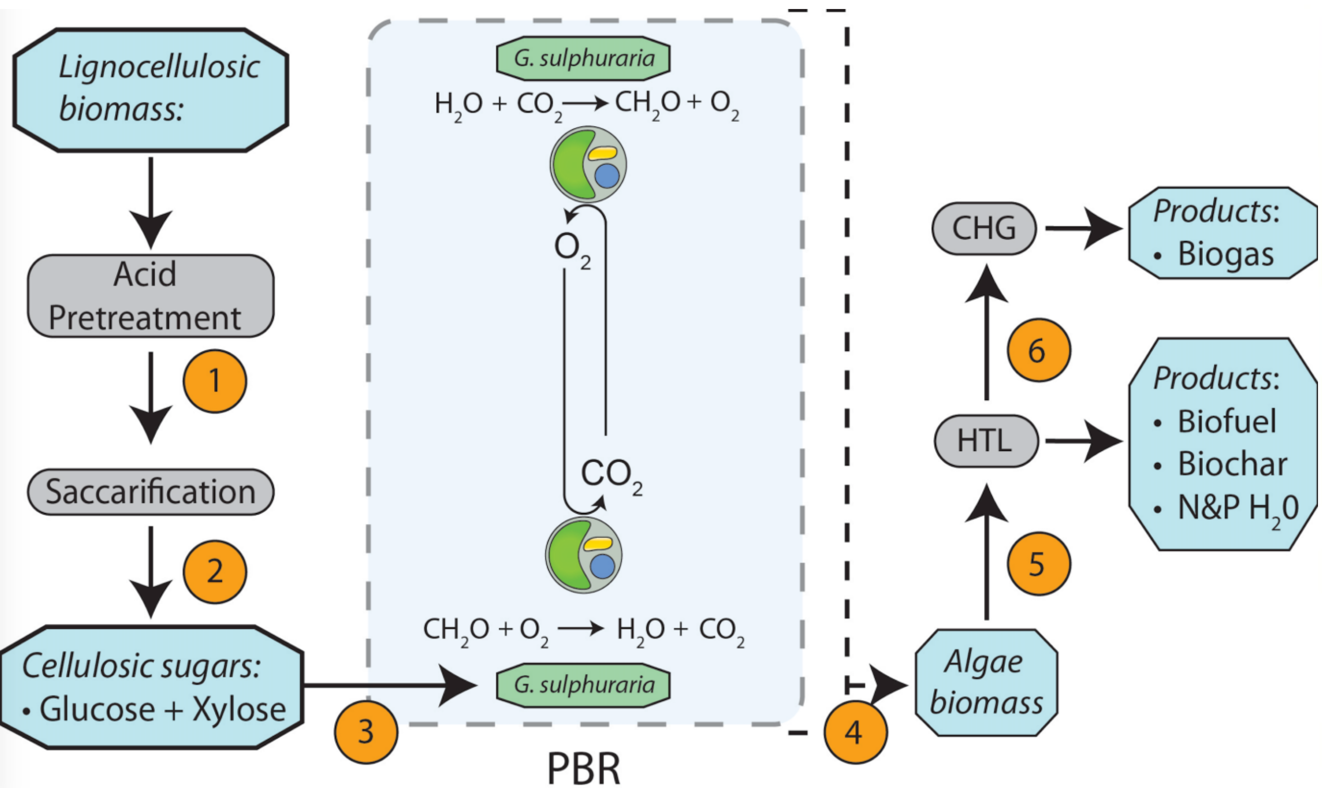
- Henkanatte-Gedera, S. M., Selvaratnam T., Karbakhshravari, M., Myint, M. Nirmalakhandan, N., Van Voorhies W. and Peter J. Lammers. (2018). Removal of dissolved organic carbon and nutrients from urban wastewaters by *Galdieria sulphuraria*: Laboratory to field scale demonstration. Algal Research 24 450–456.
- Cruce, J., Quinn, J.C., Economic viability of multiple algal biorefining pathways and the impact of public policies, 2019, Applied Energy, 233-234, 735-746
- Sommers, M., Quinn, J.C., Sustainability of Carbon Delivery to an Algal Biorefinery: A Techno-economic and Life-cycle Assessment, 2019, Journal of CO2 Utilization (in press)
- Compton, S., Lammers, P., Quinn, J.C., Bulk growth model of algal productivity in various outdoor cultivation platforms, 2019, Bioresource Technology Reports, (minor revisions peding)
- Park, W, Seger, M., Skavicus, C., and P.J. Lammers. Minimizing the effects of catabolic repression of photosynthesis during mixotrophic growth of *Galdieria sulphuraria*. (2019) New Phytologist, Manuscript in preparation.
- Mozaffari, K., Seger, M, Dungan, B, Hanson, D.T., Lammers, P.J. and F.O. Holguin. Alterations in photosynthesis and energy reserves in *Galdieria sulphuraria* during corn stover hydrolysate supplementation. Algal Research, Manuscript in preparation.
- Singh, A., and J. Clippinger. Strategies for demetallation of algae oil -a review of potential options. (2019) Green Chemistry, Manuscript in preparation.
- Compton, S., Quinn, J.C., A model based assessment of U.S. algae biomass potential in open ponds considerate of practical constraints, 2019, Algal Research, Manuscript in preparation.
- Braden, B., Chen, P., Cruse, J., Somers, M., Quinn, J.C., Driving towards sustainable algal fuels: a harmonization of techno-economic and life cycle assessments, 2019, Algal Research, Manuscript in preparation.

Meeting Presentations:

- 25 meeting presentations, oral and poster

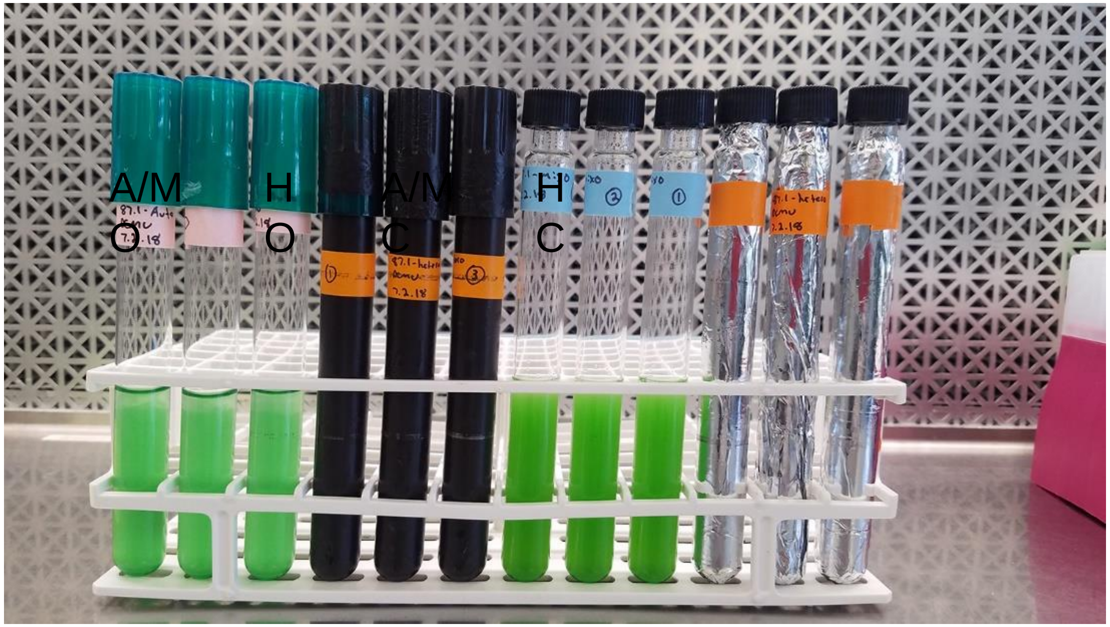
Microaerobic Mixotrophy in Tubular PBR
Forced metabolic gas exchange between and/or within cells

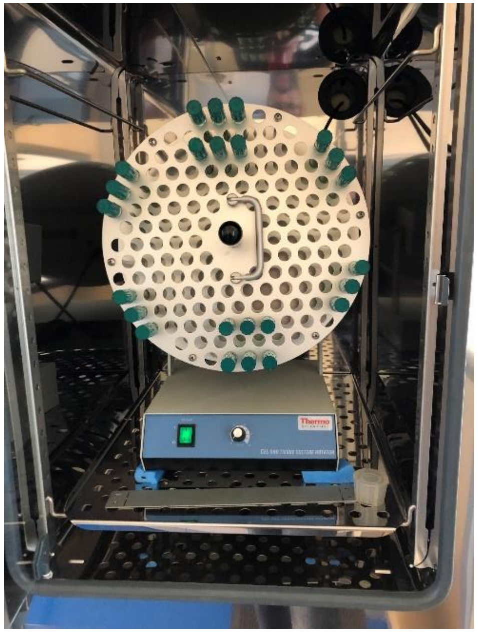
Respiratory substrates are glucose and xylose; *photosynthetic O_2 evolution is the only source of available O_2 for sugar oxidation*; heat and acid tolerant red algal extremophile *Galdieria sulphuraria;* biomass yields up to 10 g/L



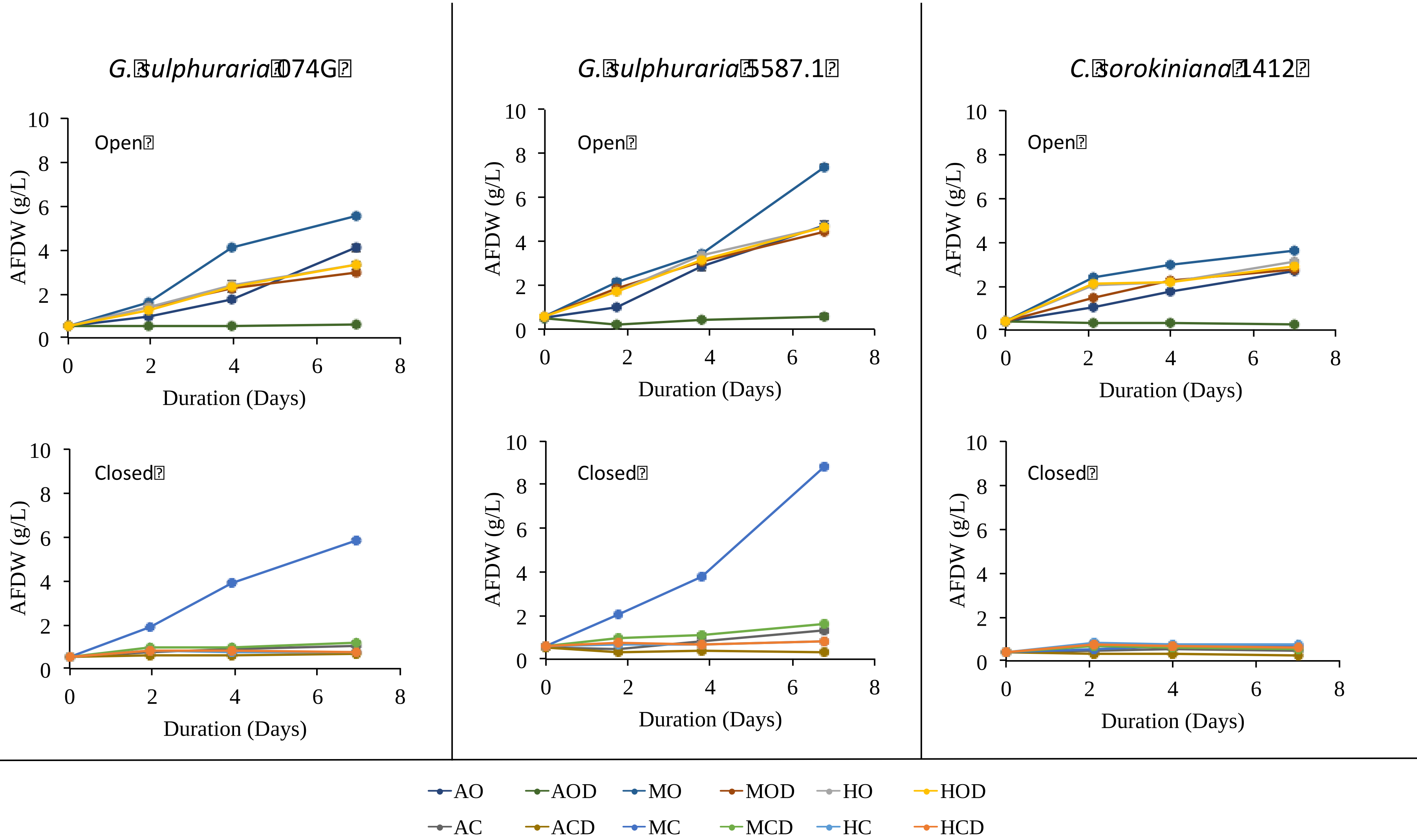
Metabolic Gas Exchange Test in Red and Green Algae

Effect of DCMU on the growth of *Galdieria sulphuraria* and *Chlorella sorokiniana* 1412 in open (external gas - 1% vol/vol CO_2) and closed (no external gas supply) tubes under autotrophic, mixotrophic, and heterotrophic conditions.





Metabolic Gas Exchange in Red but not Green Alga



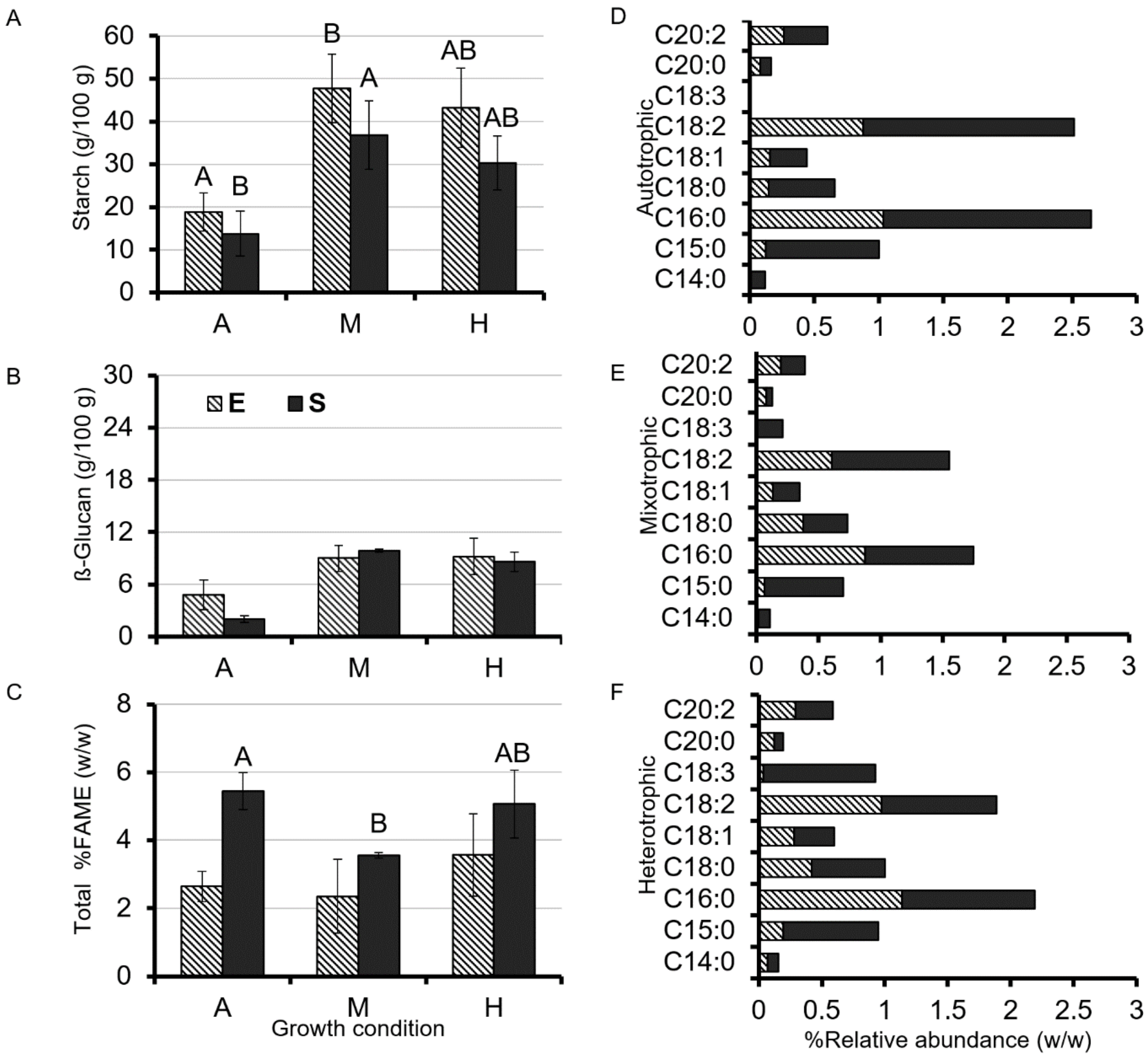
Microaerobic Mixotrophy Does it Scale Up?

500-L Helix Tubular PBR; Consecutive Fed-Batch, 24-hr data

Substrate yields determined from glucose consumption

Start Date	Starting Cell Density g/L	Ending Cell Density g/L	Average F_v/F_m	Productivity g/L/Day	Substrate Yield g-biomass/g glucose-consumed
Nov 14	2.03	2.42	0.57	0.81	0.7
Nov 15	2.24	3.65	0.6	1.61	1.06
Nov 16	2.48	3.81	0.76	1.52	1.14
Nov 17	2.38	3.23	0.85	0.97	0.7
Nov 18 to Nov 21	3.23	5.69	0.83	0.82	0.83

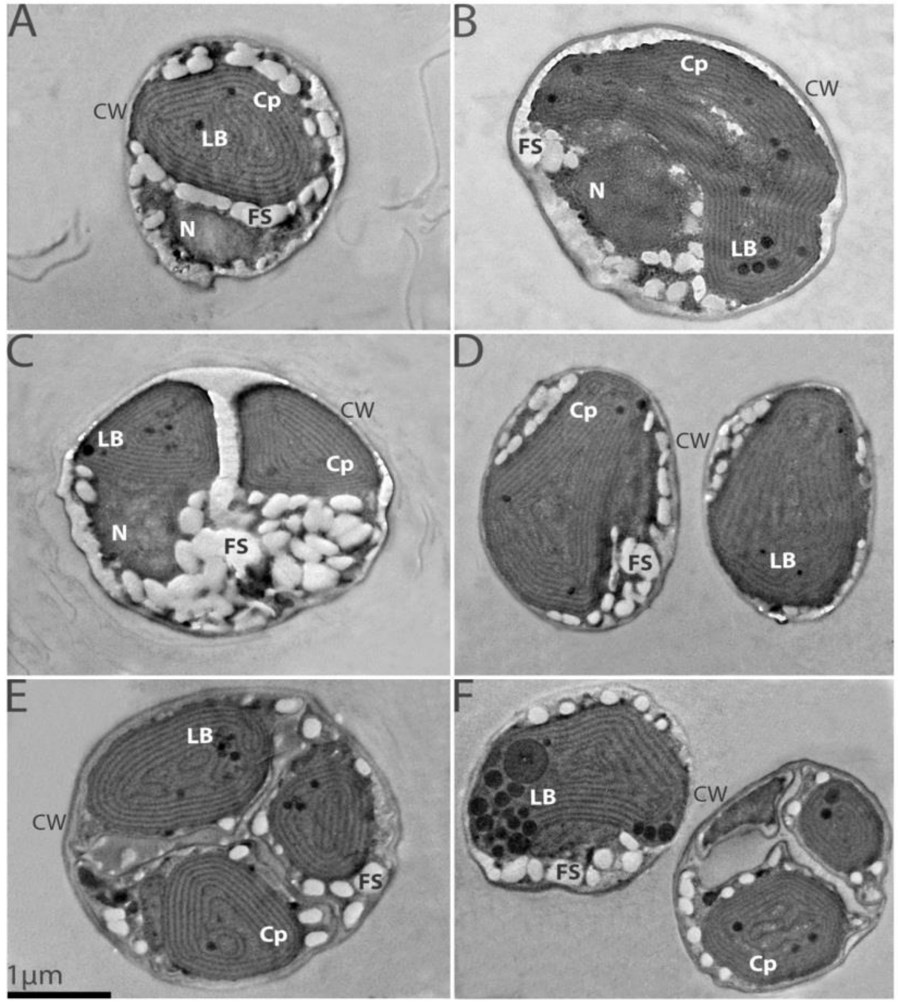
Biochemical Analysis of Carbon Allocation



A. Accumulation of starch and **B.** β-1-3, glucans in *G. sulphuraria*. **C.** Total fatty acids of *Galdieria,* during autotrophic (A), mixotrophic (M) and heterotrophic (H) cultivations. **D.** Fatty acid profile of photoautotrophic, **E**. mixotrophic and **F**. heterotrophic conditions. Cells were harvested in the exponential (E), stationary (S) phases and quantified per dry weight. Note the presence of C18:3 fatty acids in the M and H culture conditions but not present in the A. Error bars indicate SD (n=3).

Ultrastructure & Carbon Allocation

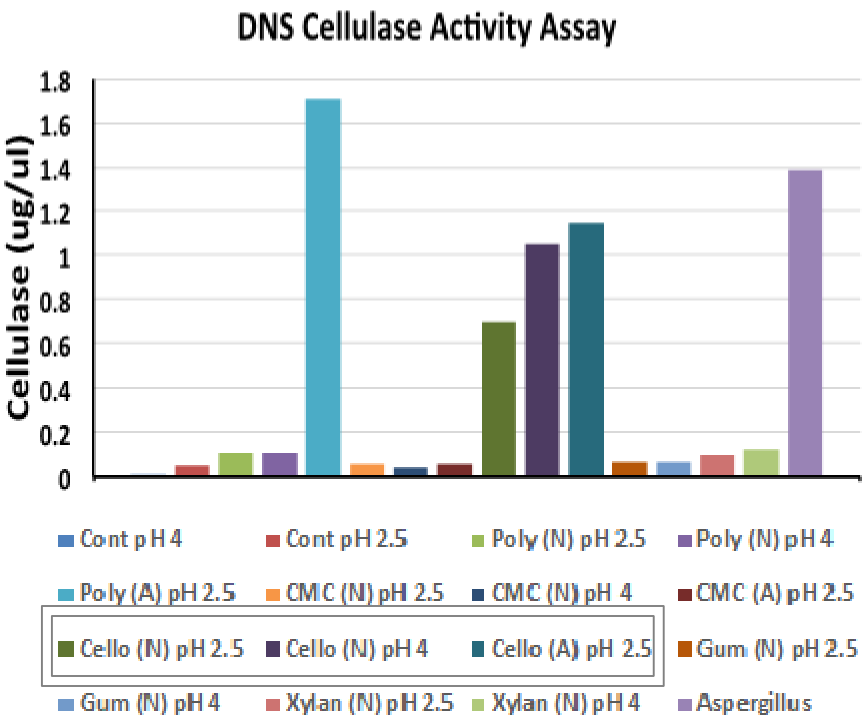
Mixotrophy leads to increased intracellular polysacchride accumulation



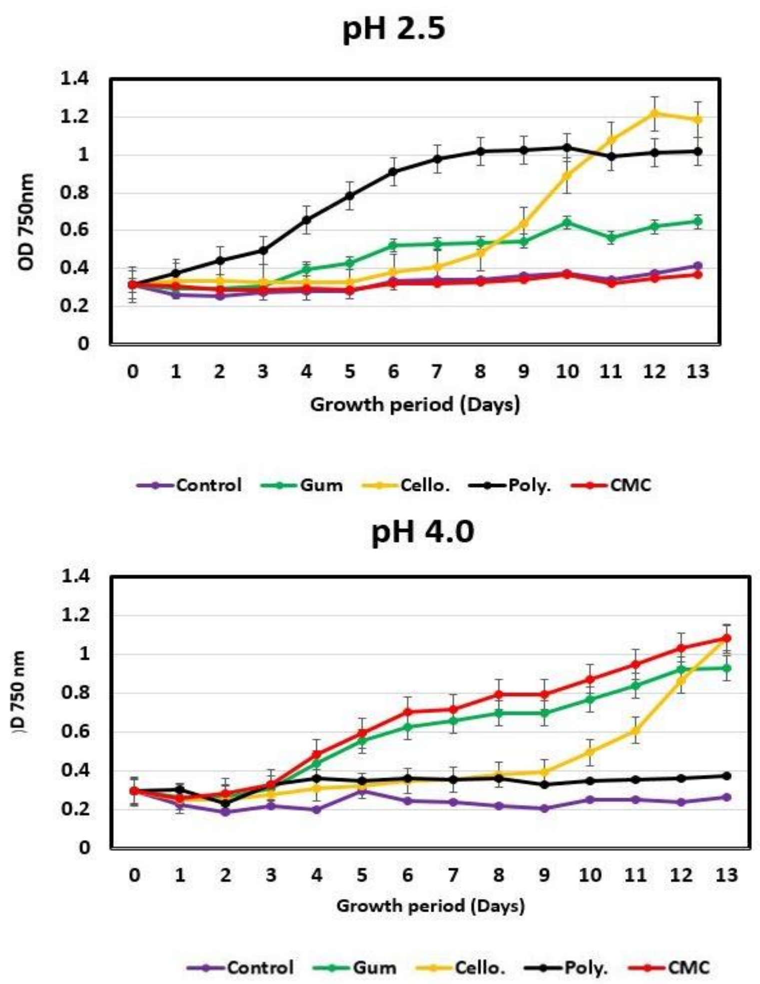
Heterotrophy leads to increased intracellular lipid accumulation

<u>TEM microscopy of *G. sulphuraria*</u> cells under autotrophic; exponential (A) and stationary cells (B), mixotrophic; exponential (C) and stationary cells (D) and heterotrophic; exponential (E) and stationary phase cells (F). Labels: CP – Chloroplast; N – Nucleus; FS – Floridean Starch; LB – Lipid Body; CW: Cell Wall.

Evidence of a β-Glycosidase in Secretome



Dinitrosalisylate Cellulase Activity Assay Secretomes from 5587.1 cultures grown on CO_2 (cont), corn syrup α-linked polysaccharices (poly), β-linked carboxymethyl cellulose (CMC), galactomannan (gum), or cellobiose (cello) substrates and at two pH conditions. "A" indicates preadapted cultures vs "N" indicates naïve cultures. Commercially available cellulase from Aspergillus was run as positive control. Cellobiose-grown cultures at both pH values yielded the greatest activity response.



pH dependency of cellulolytic secretome

Technical Accomplishments/Progress/Results

- Initial (high level) TEA and LCA were developed in tandem with outdoor experiments to determine if any PBR designs considered were "non-starters"

Horizontal Tubular

TEA - ✓
LCA - ✓
Operations - ✗

Clumping and Settling Issues

Hanging Bags

TEA - ✓
LCA - ✗
Operations - ✗

High Gas Sparge Rate Needed

Clumping and Settling Issues

Glass Helix System

TEA - ~
LCA - ✓
Operations - ✓

TEA – would need a high value added coproduct

Rigid Flat Panels

TEA - ✗
LCA - ✗
Operations - ✓

Too capital intensive

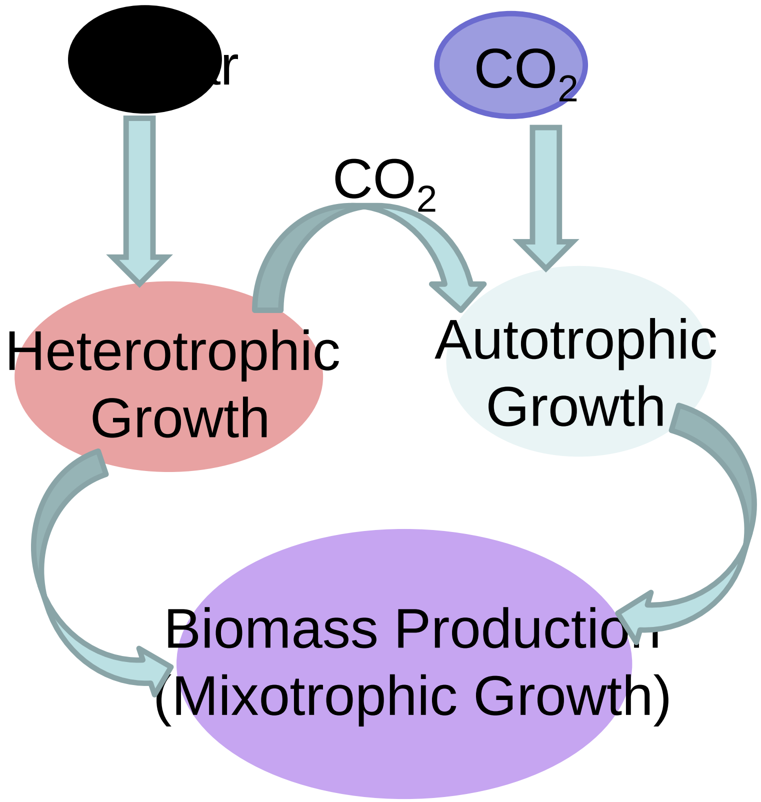
High Gas Sparge Rate Needed

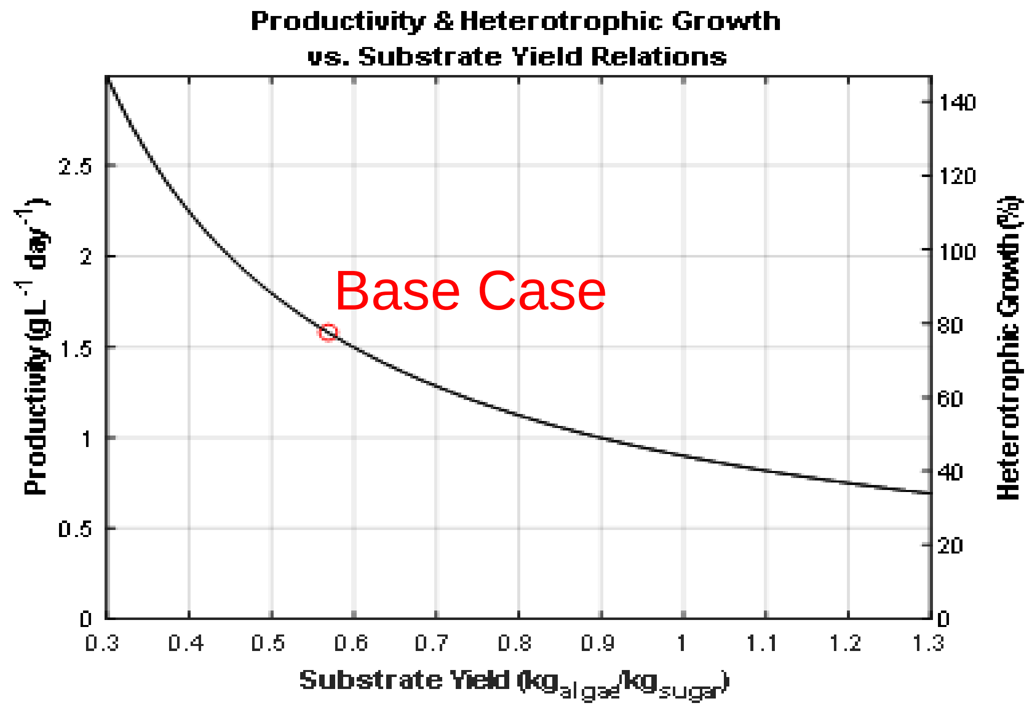
Open Ponds

TEA - ~
LCA - ✓
Operations - ✓

Depending on Productivity

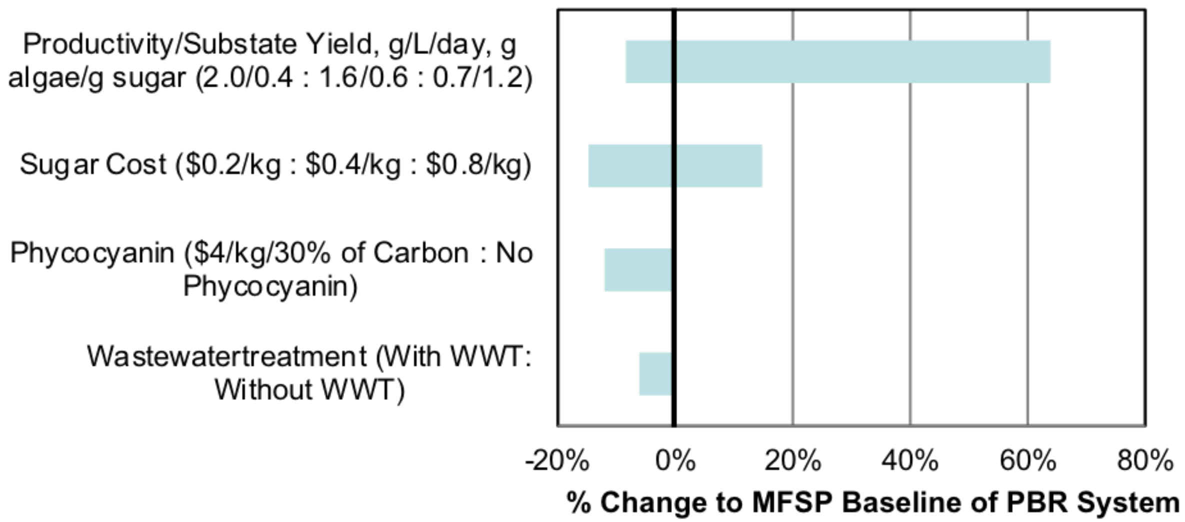
Substrate Yield to Productivity Modeled Relationship





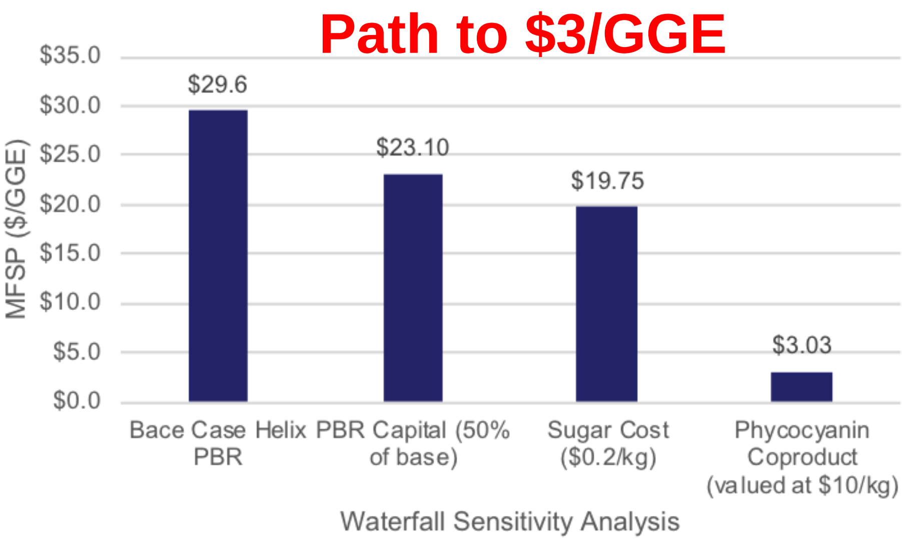
- Productivity relates to the relationship between heterotrophic growth, and autotrophic growth that are inherent in a mixotrophic system. Based on modeling relationships (as shown by the black curve in the above figure), the greater the substrate yield the more autotrophic the system is and less productivity is likely.
- The modeling aims to find optimized productivity/substrate yield for research to target.

TEA Results



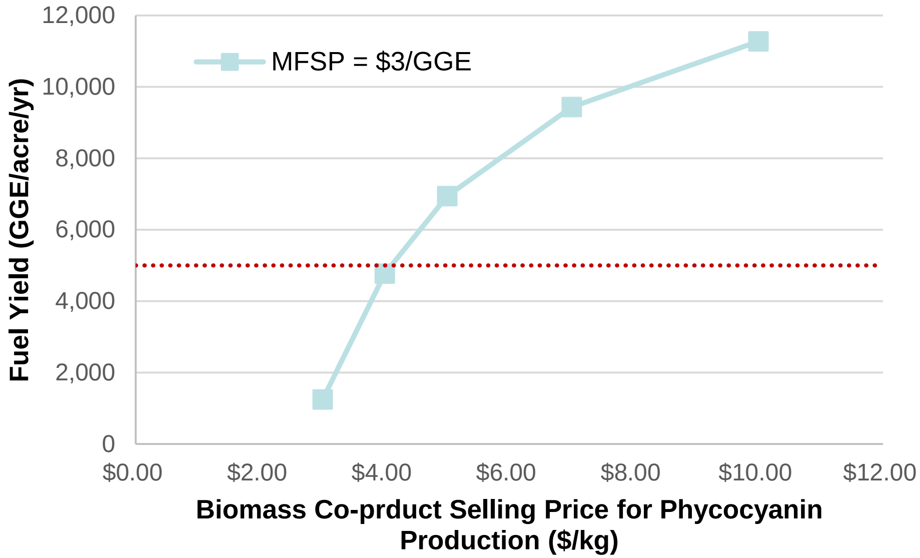
- Productivity combined with the resulting substrate yield is a main driver for the TEA causing over a 50% increase in costs when productivity reduced from 2.0 g/L/day to 0.7 g/L/day.

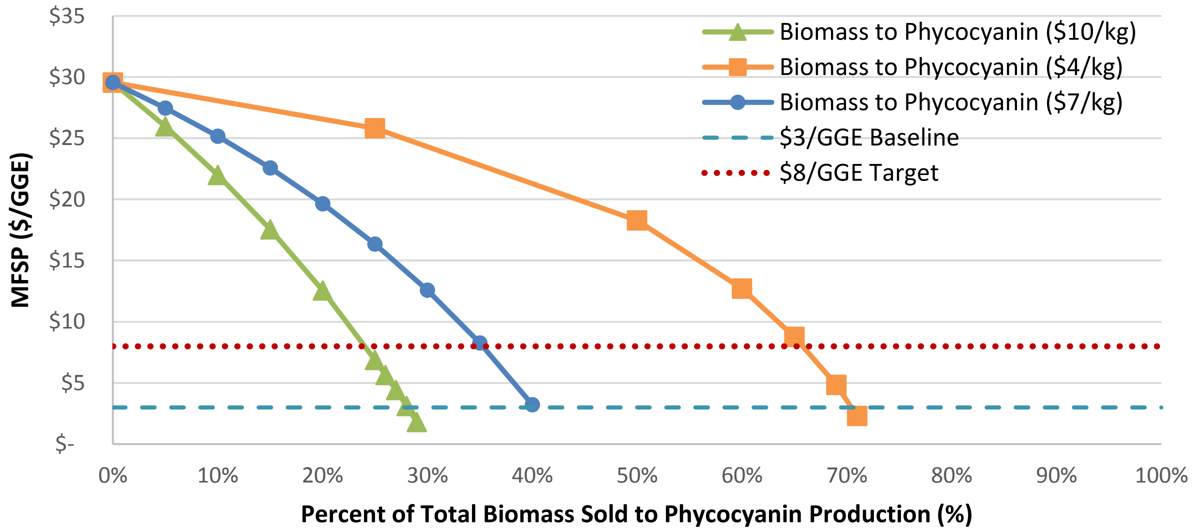
- Without additional improvements to productivity, the path to $3/GGE is dependent on reduced PBR capital, reduced sugar cost, and high value added coproducts such a phycocyanin.



Phycocyanin Sensitivity Analysis

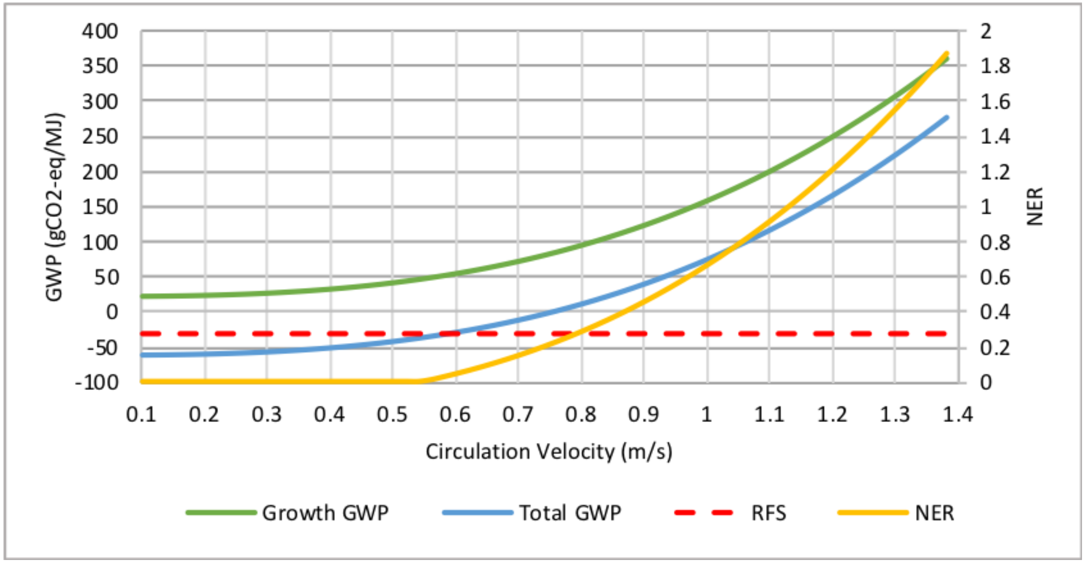
- Several Phycocyanin sensitivity cases were run to understand changing markets and the minimum phycocyanin coproduct credit needed while still maintaining 5,000 GGE/acre/year fuel potential for the *Helix Base Case* process





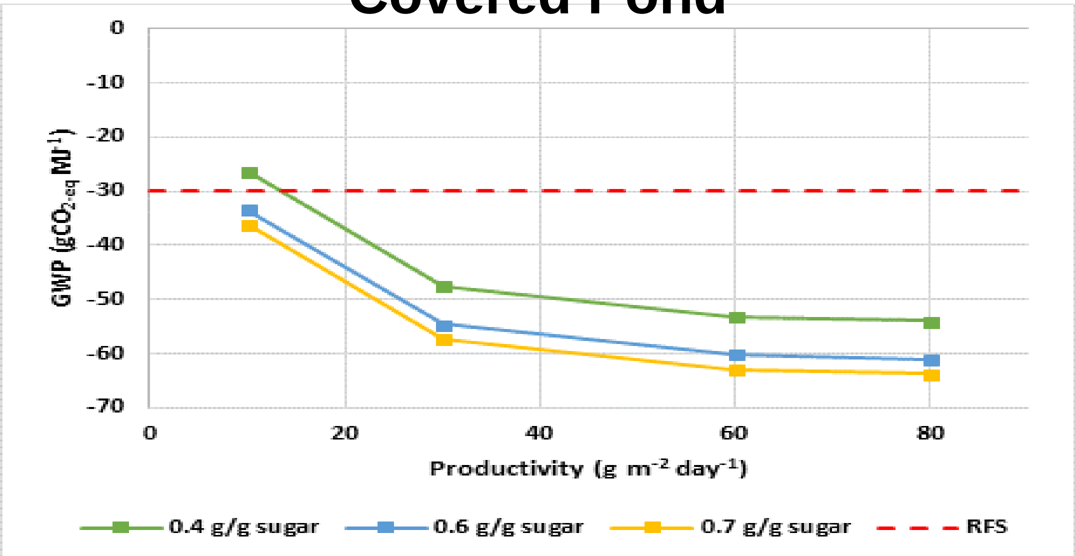
LCA Results

Helix Photobioreactor



- A main driver to the LCA for the Helix system is the circulation velocity and power requirement of mixing within the system.
- Circulation velocity should remain below 0.6 m/s based on the pump efficiencies modeled.

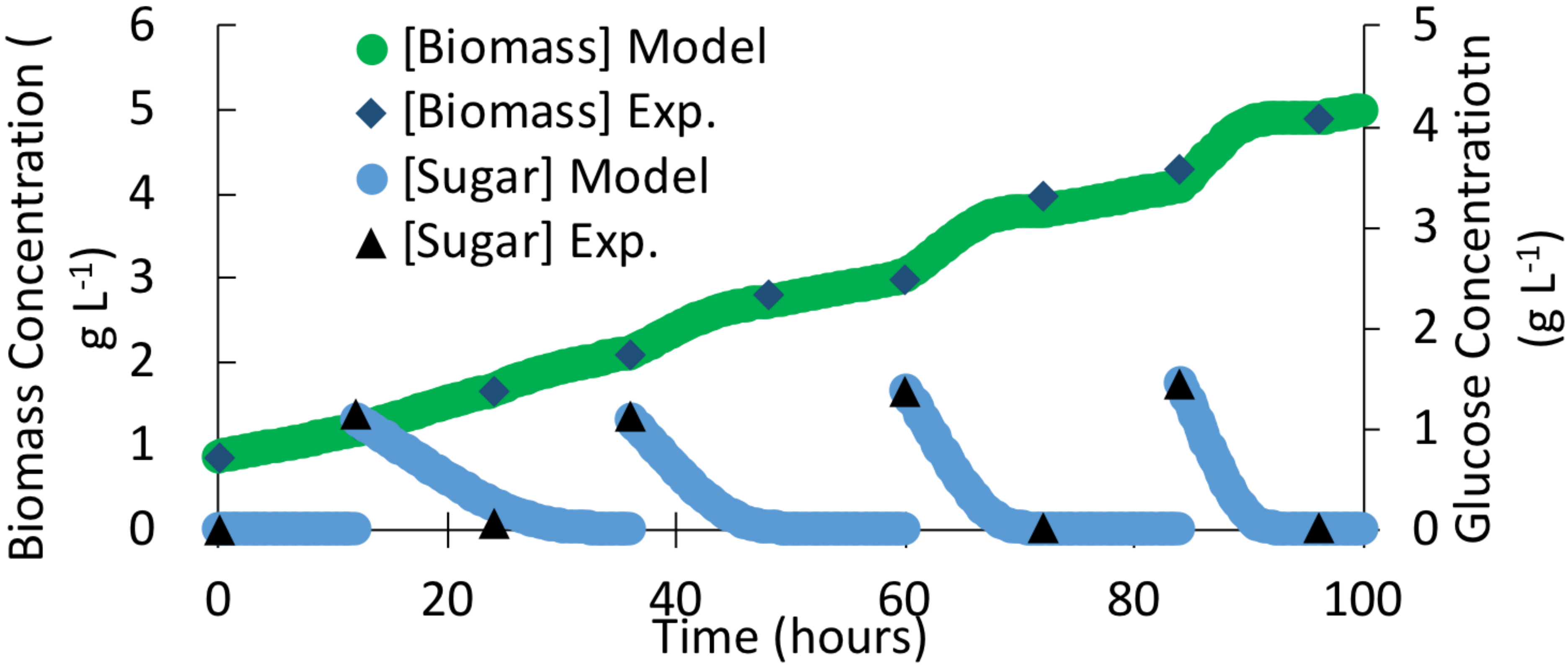
Covered Pond



- A system using covered ponds also has circulation power to consider in the LCA, but productivity and substrate yield can play a large role as well.
- Between the two (productivity or substrate yield), productivity affects the LCA to a greater degree.

Mixotrophic Growth Modeling

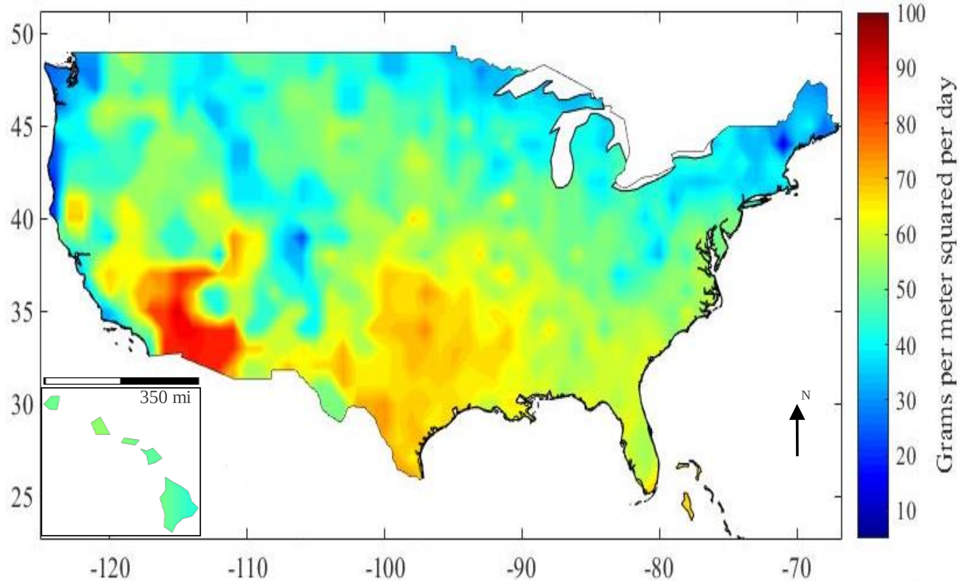
Model Validation



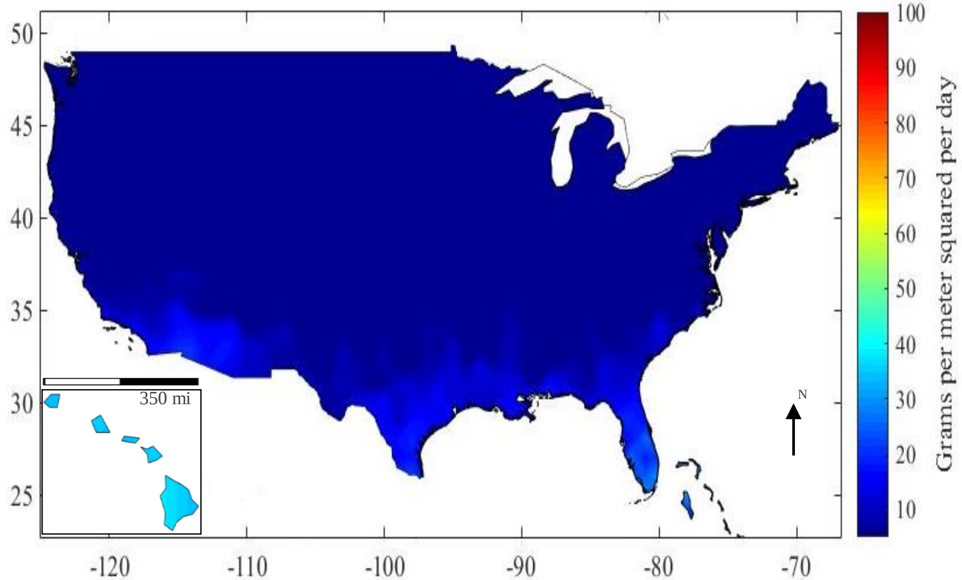
Mycotrophic growth in indoor tubular reactors at growing *Galdieria s.* at AzCATI. Modeling work coupled thermal, phototrophic and heterotrophic growth modeling. Minimal strain characterization parameters combined with experimentally derived heterotrophic growth were required.

Mixotrophic Growth Modeling

July



January



4 – Relevance

• Abundant flat land in arid regions of the U.S. could support biofuel production in evaporation-resistant reactors coupled with thermotolerant, high-productivity mixotrophic algae

• Heat, low pH and low O_2 in microaerobic mixotrophic conditions combine to severaly limit growth of heterotrophic contaminants

• Project outcomes show photobioreactor cost reductions are needed to reach cost targets

• Lower cost PBR re-design enabled by balanced, microaerobic mixotrophy (patent submitted)

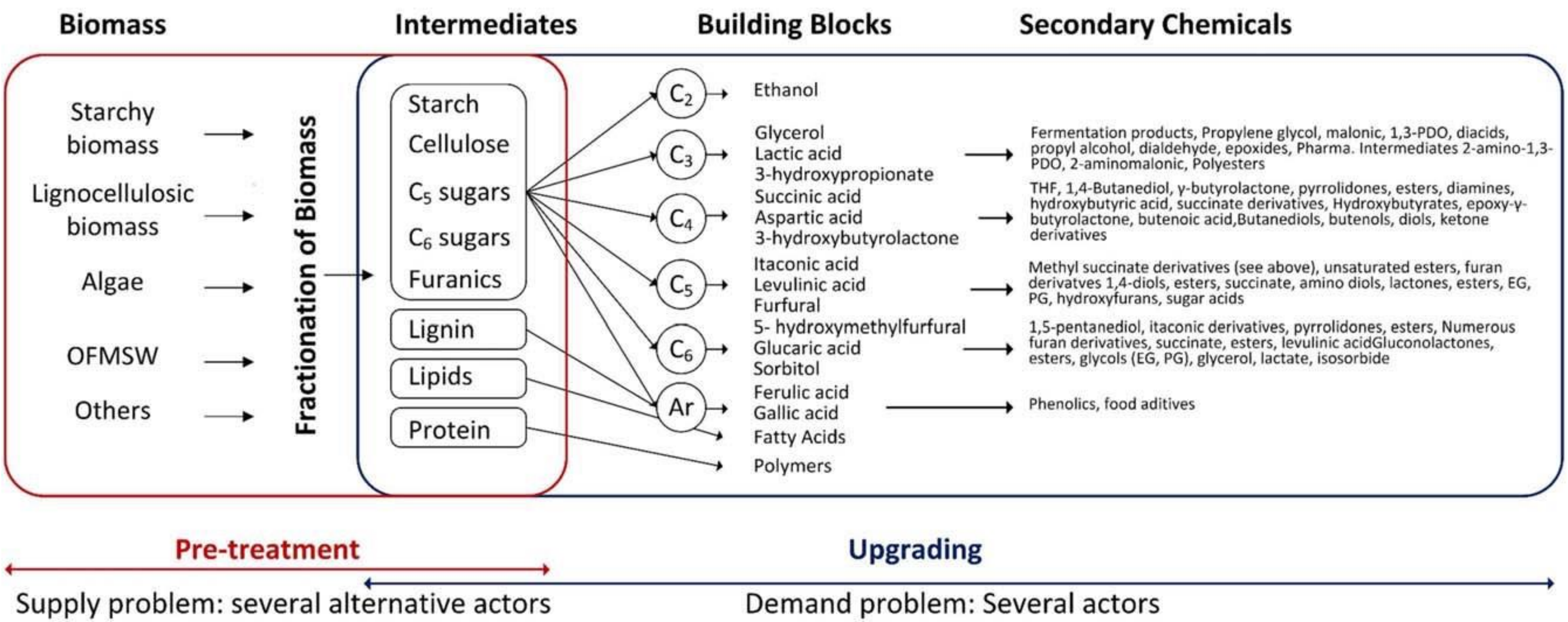
Summary

1. Red microalgae can use PSII to provide O_2 for respiration of cellulosic glucose and xylose which afford options for intensification of algal biomass production
2. Unique thermotolerant phycocyanin co-product, β-1,3 glucan and others waiting to be discovered
3. Modeling and experimentation used to downselect tubular PBR option with covered raceway systems identified as potential option
4. Nine manuscripts published, in review or in preparation, 25 meeting presentation
5. Relevance to MYPP goals for 30% increase in biomass value over 2015 value; route toward 2020 - 3,700 gal/ac/yr target based on high mixotrophic growth modeling results
6. Future work to address biorefinery model using wastewater and lignocellulosic sugars to produce high-value algal biomass

The Future: Product/Co-Product Design Options [a]

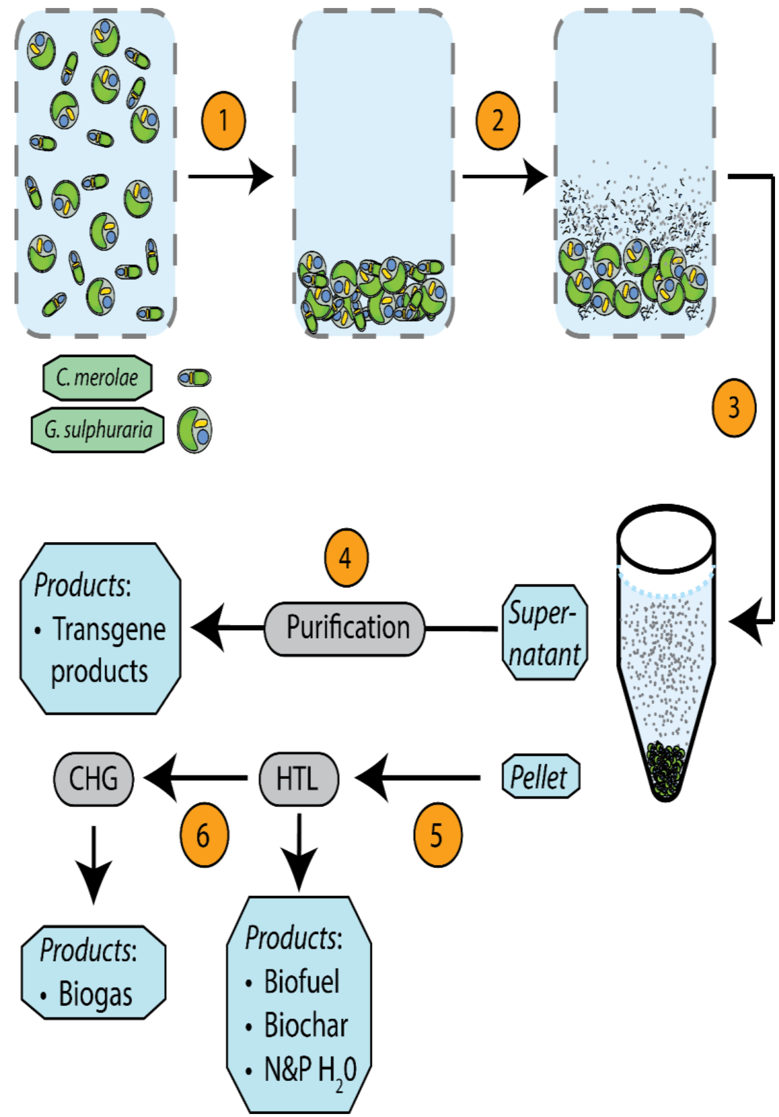
A) Further development of biorefinery options using wastewater nutrients and lignocellulosic sugars as feedstocks for intensified *Galdieria* cultivation, with multiple downstream products.
B) Utilize modeling and biochemical specifications to negotiate with other “actors” wrt costs, outputs and intermediate product streams
C) Explore different designs for covered bioreactors to reduce PBR costs relative to glass tubular systems.
D) Explore co-culture/genetic engineering options for building blocks & secondary chemical markets

[a] A.I. Torres, G. Stephanopoulos, Design of Multi-Actor Distributed Processing Systems: A Game-Theoretical Approach, AICHE Journal, 62 (2016) 3369-3391.



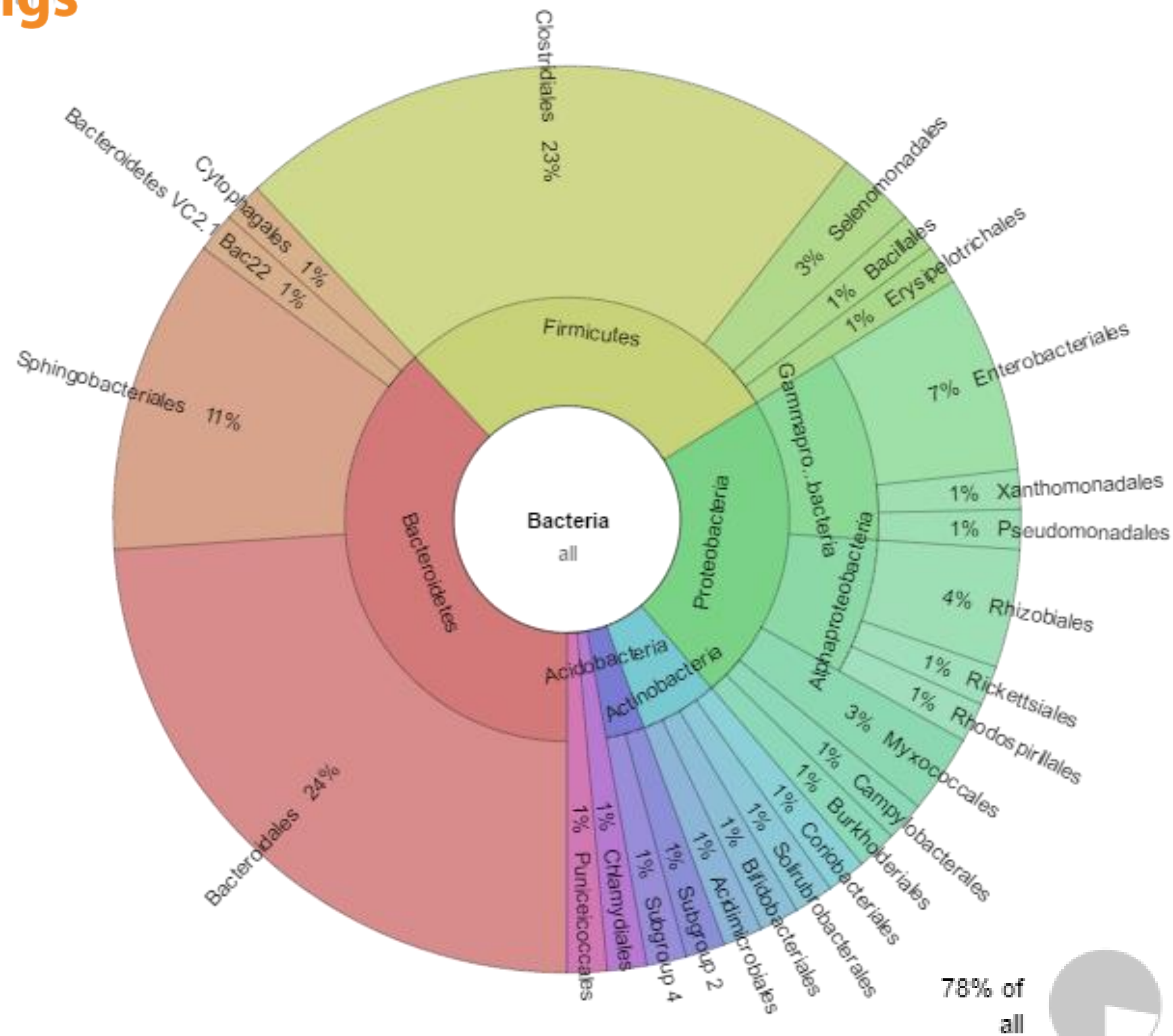
Co-Culturing with *C. merolae* 10D strain:

- transformable w/ multiple auxotrophic selectable markers, CRISPR compatible
- *Cyanidioschyzon* strains are highly compatible with *G. sulphuraria* with metabolic gas exchange
- 2nd method for overcoming catabolic repression of photosynthesis
- Co-culture of *C. merolae* with *Galdieria* strains that bleach with sugars to provide for green and non-green biomass
- no cell wall enabling differential lysis via osmotic shock for product separation



Additional Slides

silva ngs



VL2-*Galdieria sulphuraria* outdoor 1,000-L culture, Flat Panel

Biomass Yield and Exogenous Carbon Utilization

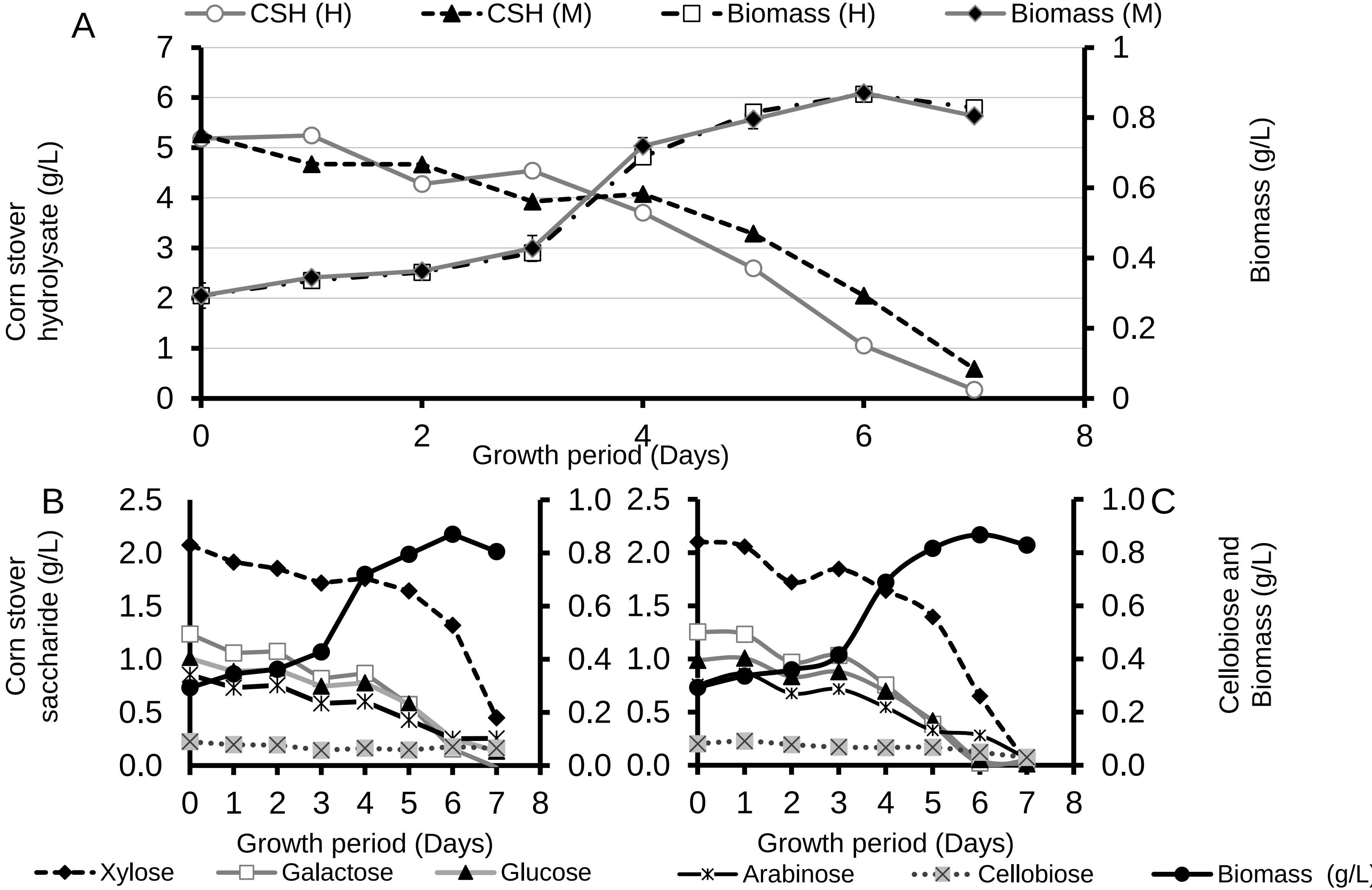


Figure X. A. Biomass yield in both growth conditions **B.** Cornstover uptake rate in heterotrophic and **C.** mixotrophic cultures. Error bars indicate SD (n=3).

Proteomic Investigation of Mixotrophy vs Autotrophy

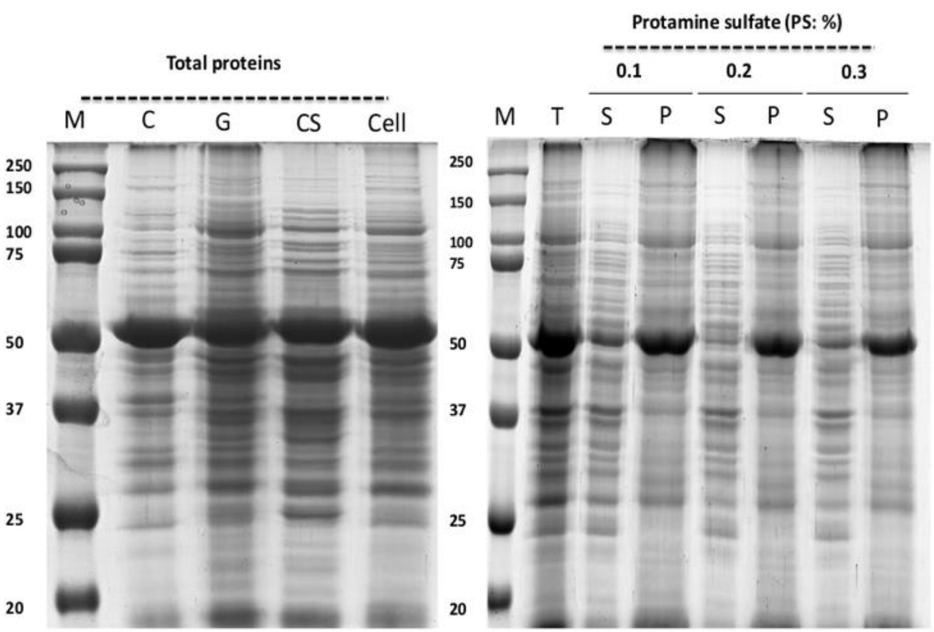


Figure X. SDS-PAGE analysis of proteins in *G. sulphuraria before and* after the RuBisCO precipitation. Total proteins (T) were subjected to different concentrations of PS (0.1%-0.3%). Both supernatant (S) and PS-treated (P) resolved on SDS-PAGE. Arrow shows the large subunit of rubisco. 15ug of each protein was loaded. M: Marker, T: Total protein, S: PS-treated cell P: Pellet C:Control G: Glucose CS: Cornstover Cell: Cellobiose. These findings showed that the 0.2% PS is more effective in RuBisCO depletion from the supernatant.

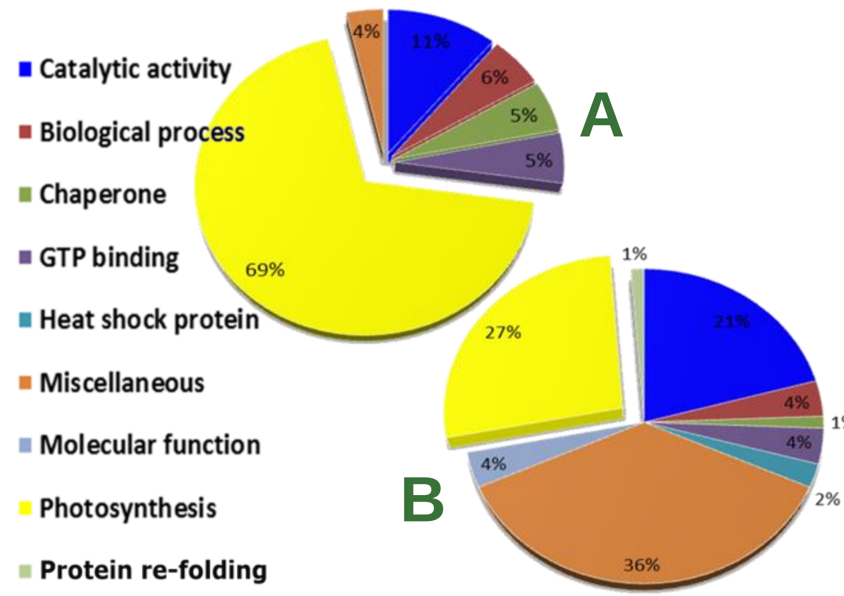
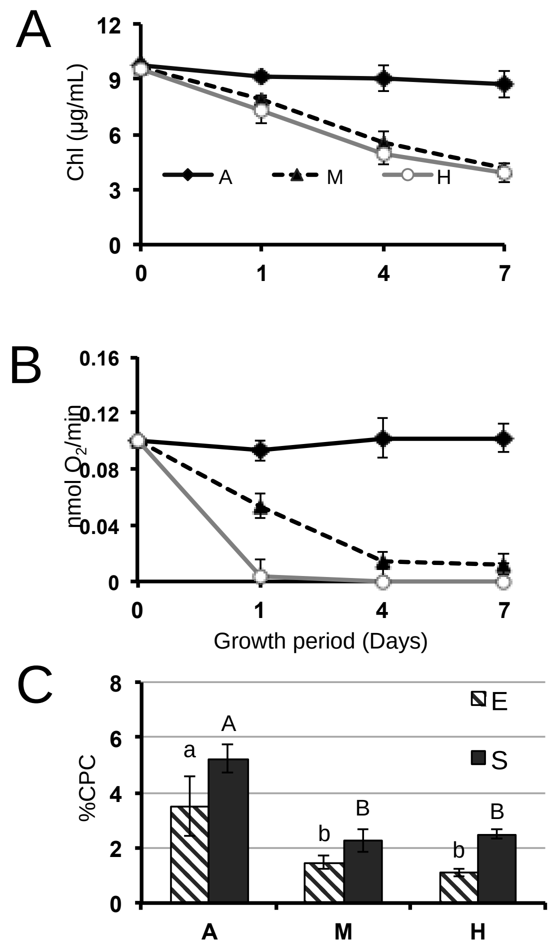


Figure X. Functional analysis of identified proteins from *G. sulphuraria* under photoautotrophic and mixotrophic growth. Distribution of proteins involved in different cellular, biological and molecular processes based on their GO annotations is shown. Note the higher percentage of the proteins involved in photosynthesis are highly abundant proteins in control cultures (A) compared to Cornstover (B). Low amounts of proteins were identified due to the large abundance of photosynthetic proteins.

Cornstover Culture Photosynthetic Measurements

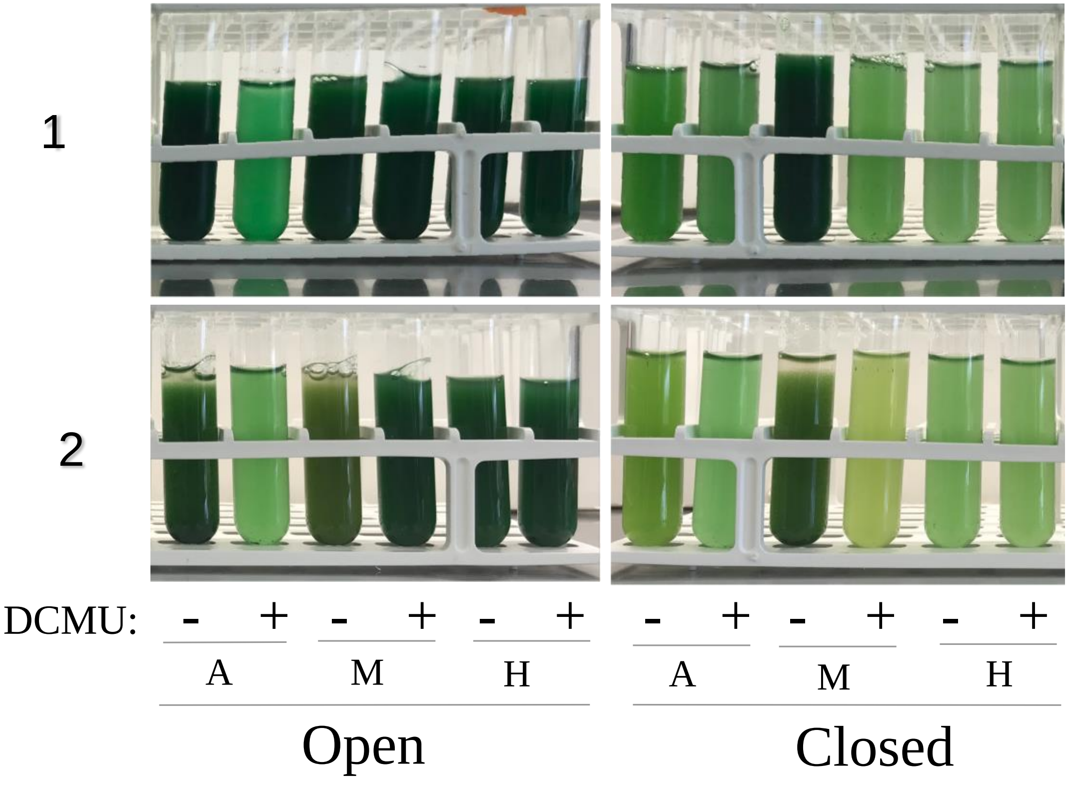


Gradual repression of photosynthesis in mixotrophic cultures

Phycoyanin decreases during Cornstover supplementation during exponential and stationary growth

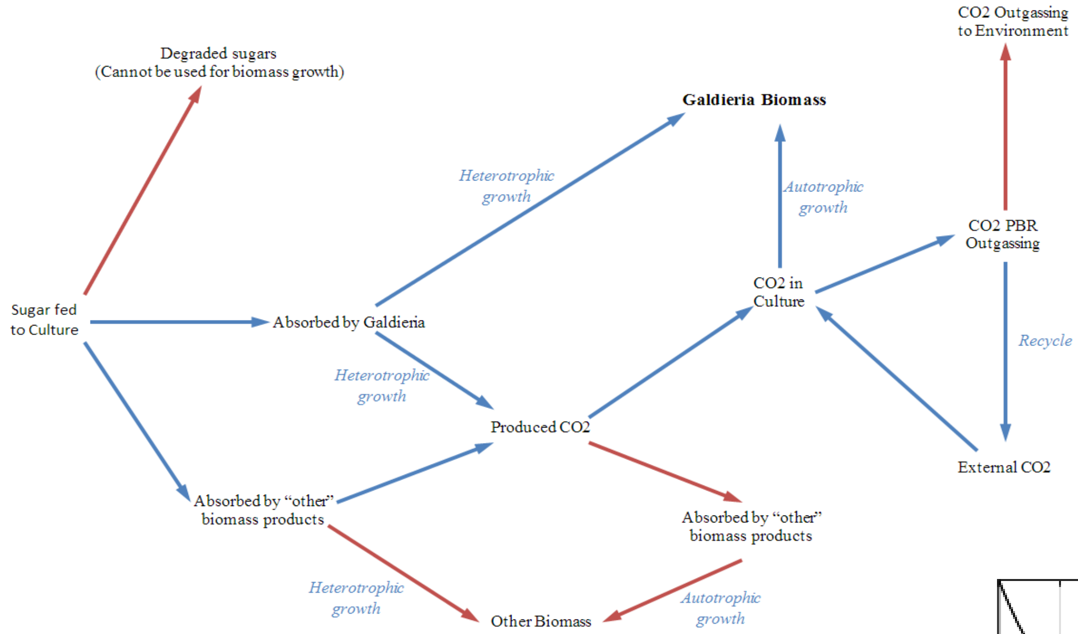
Photosynthetic measurement and pigment analysis A. Micrograms of Chlorophyll A per culture volume of autotrophic (A) mixotrophic (M) and heterotrophic (H) cultures in *G. sulphuraria*. **B**. C-phycocyanin production under autotrophic (A), mixotrophic (M) and heterotrophic (H) growth conditions. **C.** Oxygen evolution of A, M and H cultures. Cells were harvested in the exponential (Day4) with diagonal lines (E), and stationary (Day7) phases with solid lines (S). Error bars indicate SD (n=3).

Metabolic gas exchange in mixotrophic systems
Closed test-tube growth on sugars requires photosynthesis (no DCMU)
A = autotrophic, M = mixotrophic, H = heterotrophic



1: *G. sulphuraria* strain 074G
2: *G. sulphuraria* strain 5587.1

Substrate Yield to Productivity Modeled Relationship



The yield on sugar for the mixotrophic system is referred to here as substrate yield and is dependent on the productivity and carbon balance for *Galdieria*.