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DOE Bioenergy Technologies Office (BETO) 2021 Project Peer Review

SOFAST: Streamlined Optimization of Filamentous *Arthrospira*/*Spirulina* Traits

March 22, 2021

Advanced Algal Systems

Rachelle Lim

Lumen Bioscience

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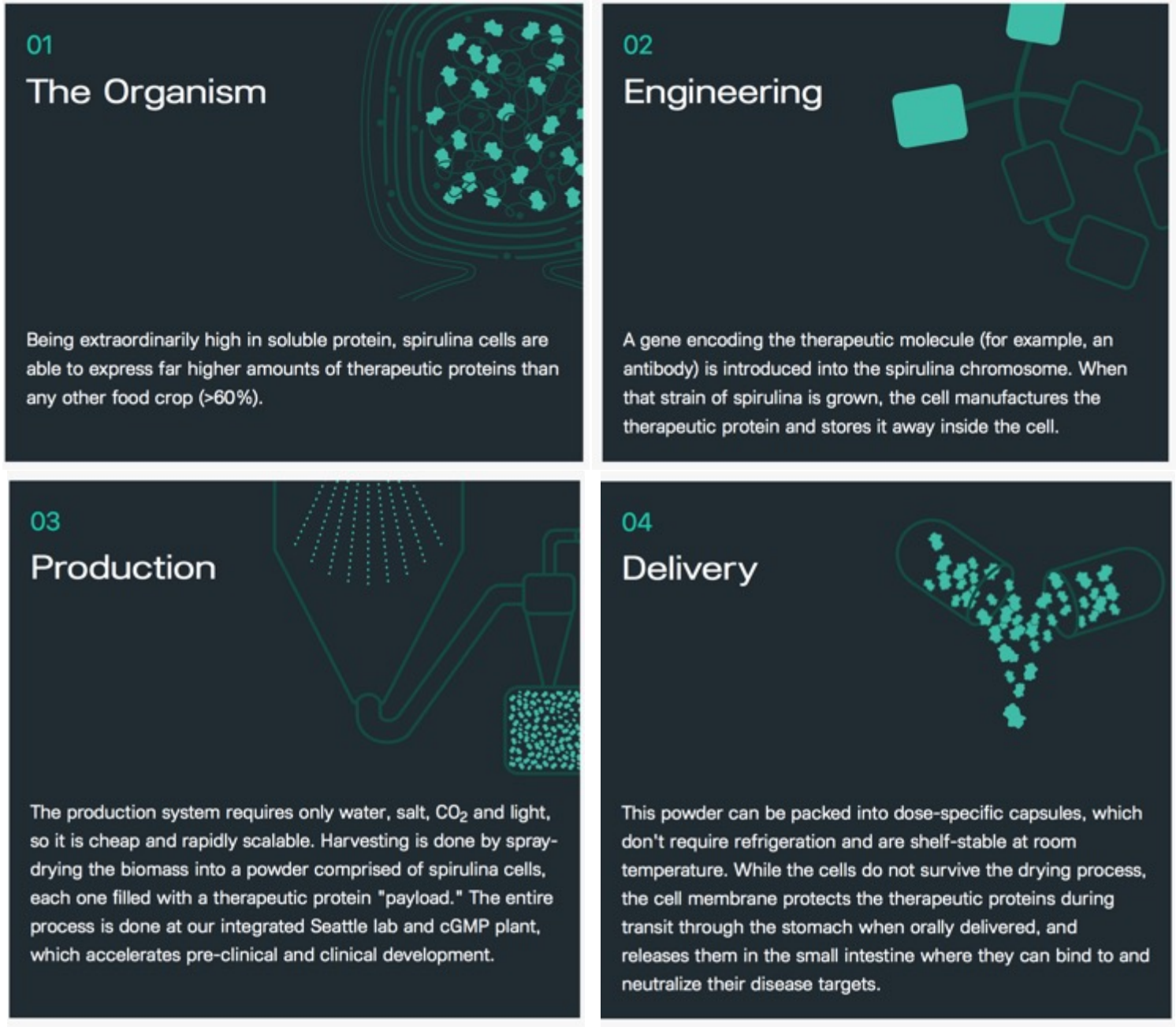
Lumen Bioscience, Inc.

Lumen's interests center around using spirulina to tackle problems in the development of oral therapeutics:

- Commercially viable
- Reduction in costs and risks
- Accelerated time-to-market
- Shortened development process



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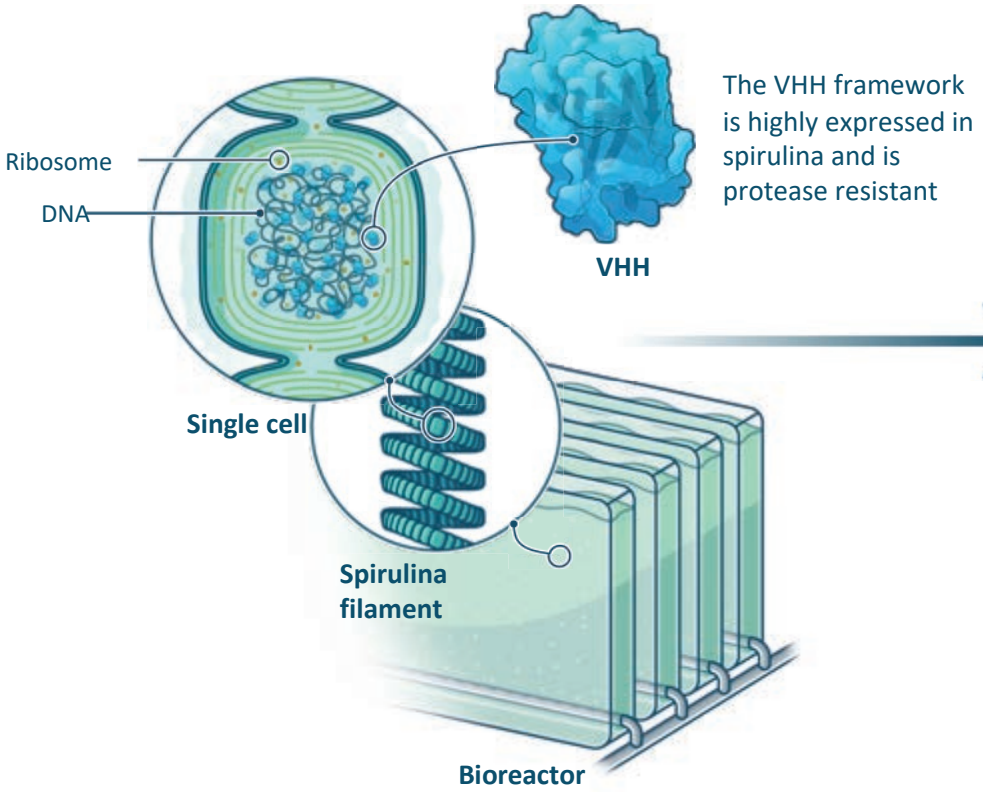
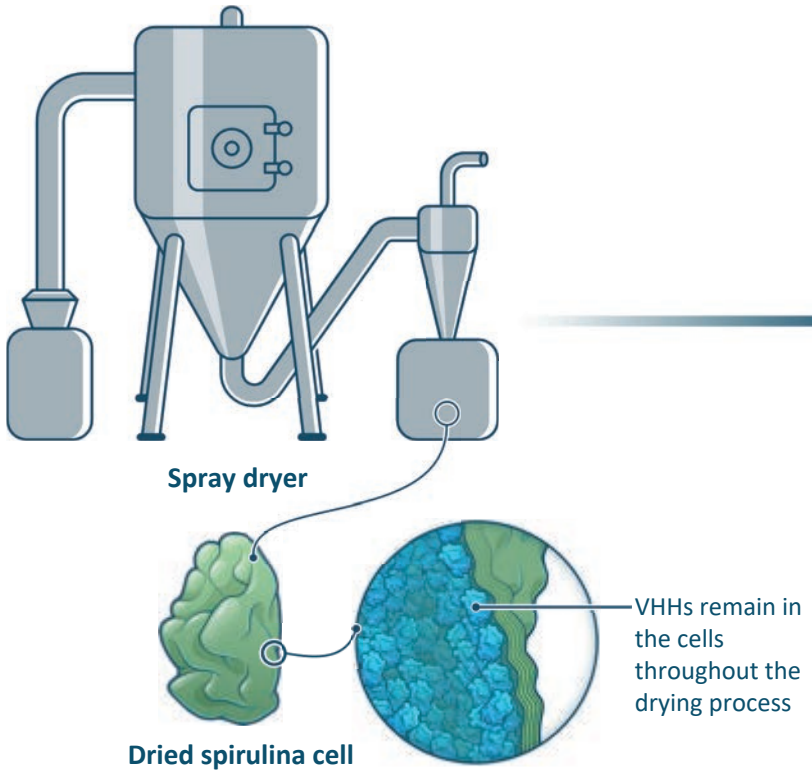
Ultra simple manufacturing enables patient safety at *de minimis* cost

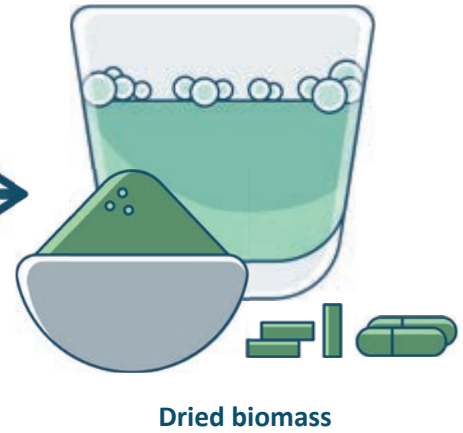
1 Harvest spirulina semi-continuously; only minerals, light, water, and air are needed



2 Spray-dry the biomass



3 Resuspend in water and administer orally (optionally formulate or encapsulate)



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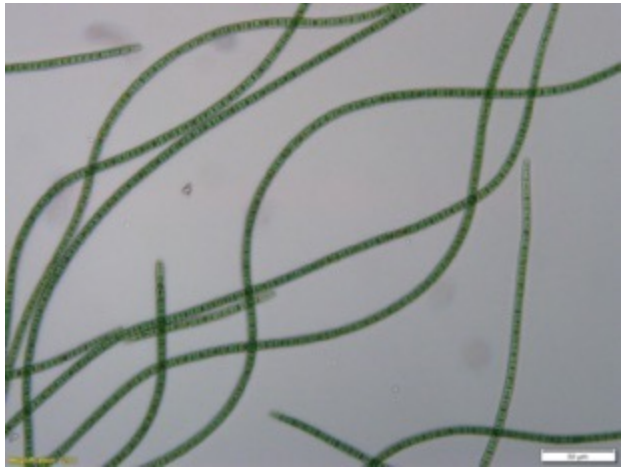
Patent pending.

Spirulina (*Arthrospira platensis*): a new biotechnology platform



Lumen developed genetic engineering for Spirulina	
Advantages	**Challenges**
• Robust grower, resistant to predation • Easier to harvest compared to unicellular algae • Can be grown in saline or wastewater-based media	• Its protein rich biomass is naturally low in fatty acids and it therefore has a lower energy density than oleaginous algae. • **It is a new chassis. More molecular tools must be developed and demonstrated.** • **Its photosynthetic performance is still limited, especially at high culture densities and under stress.**

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Project Overview – Goal Statement

- *Arthrospira species ("Spirulina")*
  - Cyanobacteria with proven industrial cultivation capabilities.
  - Lumen has unique, patented technology to genetically enhance *Arthrospira* for improved growth and biomass composition for biofuels and bioproducts.

- *Goal:* To produce engineered *Arthrospira platensis* strains with improved photosynthetic traits and increased lipid content.

- *Outcome:* Demonstrate successful and stable outdoor productivity of 4 engineered *Arthrospira* strains with at least 1 achieving growth rates of at least 19 $g/m^2/day$ (AFDW) and at least 14% lipids and 16% carbohydrates.
  - Develop strain improvement toolkits and methods (SOFAST)
  - Increased areal productivity AND projected biofuel yield using these toolkits and methods

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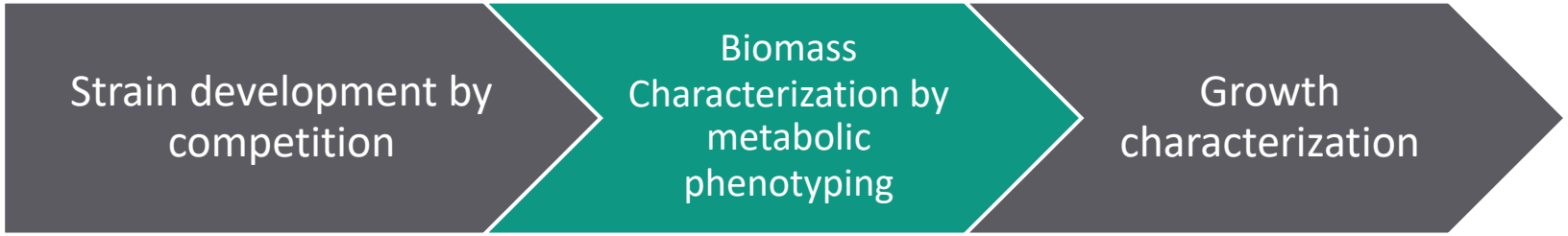
Project Overview

- *Goal:* To produce engineered *Arthrospira platensis* strains with improved photosynthetic traits and increased lipid content.

National Renewable Energy Laboratory	Lumen Bioscience
- High throughput methods for biomass characterization	- Increases lipid production in cyanobacteria (wax esters) - Can genetically engineer *Arthrospira* and select for optimal expression - Demonstrated traits that alleviate light and cold stresses in cyanobacteria

This project aims to optimize expression of traits in *Arthrospira* that increase outdoor biomass productivity and enhance biomass composition for production of biofuel intermediates



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1 – Management: Collaboration overview

Project split into three tasks to fulfill project objectives

TASK 2: STRAIN DEVELOPMENT **Lumen Bioscience**	TASK 3: METABOLIC PHENOTYPING **National Renewable Energy Laboratory (NREL)**	TASK 4: OUTDOOR GROWTH **Arizona Center for Algae Technology and Innovation (ASU)**
<u>Molecular biology and traits</u> • Strain and library construction • Streamlined selection of traits • Strain engineering, sequencing, and characterization • Artificial light (indoor) culturing and validation	<u>Analytical</u> • Biomass characterization by traditional and higher throughput methods • Lipidomics characterization in WT and engineered strains • Development of single-filament phenotyping and NIR high-throughput methods for *Arthrospira*	<u>Outdoor cultivation</u> • Outdoor (sunlight) culturing • Media optimization for outdoor culturing
Jim Roberts – Lumen PI Team: Damian Carrieri, Rachelle Lim, Troy Paddock, Lauren Goetsch	Lieve Laurens – NREL co-PI, Algae Platform Team Lead Team: Peter Shanta, Hannah Alt, Kaitlin Lesco	John McGowen - Director of Operations Team: Jessica Forrester

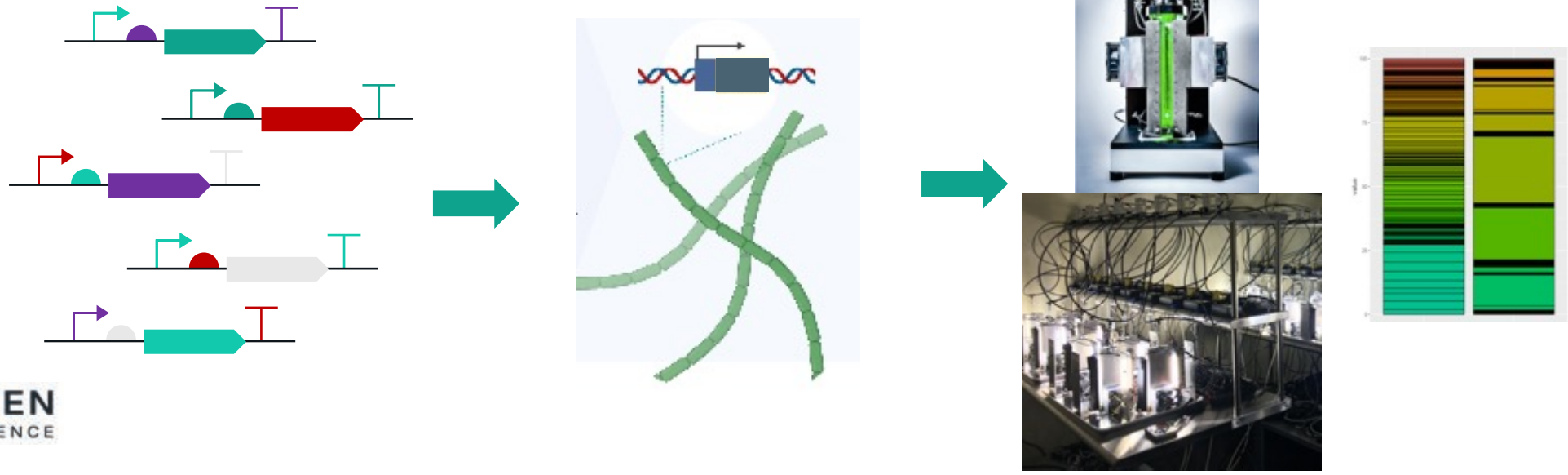
Communication plan:

- Biweekly meetings between NREL and Lumen
- Biweekly calls with ASU during project work
- SharePoint site
- Benchling LIMS platform for sample tracking and data management
- Monthly meetings with BETO project team

2 – Approach Overview

Construct libraries → Transform → Compete → Build → Characterize → Grow

1. **Construct** libraries of traits with various coding sequences and promoter/terminator strengths (100+ variants in each library).
2. **Transform** libraries of traits into *A. platensis* using Lumen's patented genetic engineering technology
3. **Compete** transformed libraries against control cells (expressing YFP) or each other and sequence to identify constructs that imparted the best fitness or most wax esters without severe growth defects.



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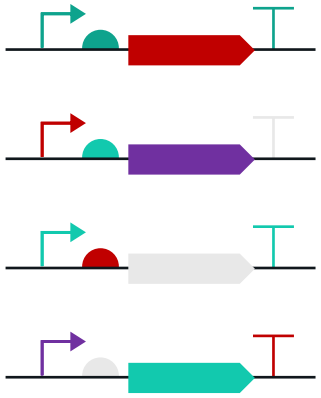
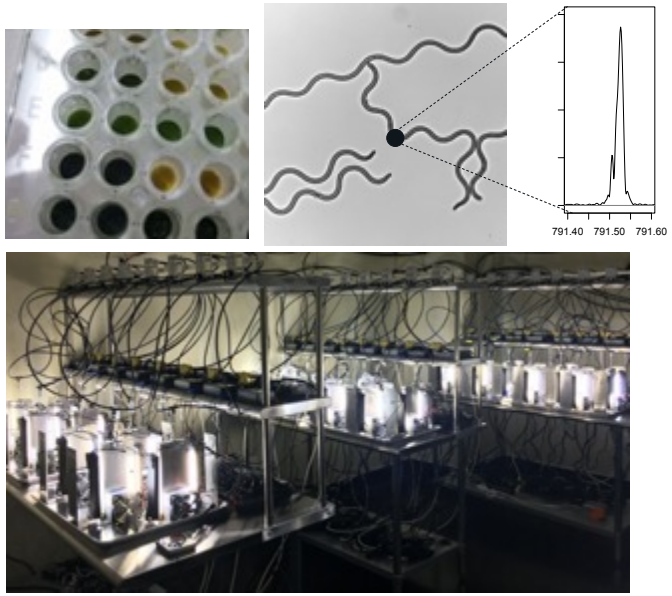
2 – Approach Overview

Construct libraries → Transform → Compete → **Build** → Characterize → Grow

4. **Build** top constructs into *Arthrospira* to produce strains expressing traits at optimal levels.
5. **Characterize** newly made strains for improved growth rates or metabolic profiles relative to *wild type* control runs.
6. **Grow** improved strains in indoor and outdoor test beds alongside *wild type* controls.





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2 – Approach: Challenges

Critical Success Factor	Challenge	Strategy	Go/No Go evaluation
Positive effect of stress resistance over-expression resulting in improved growth performance	Stress resistance and cold tolerance traits can adversely affect growth if expressed too strongly or be ineffective if expressed too weakly.	Use traits already demonstrated to improve growth in cyanobacteria. Compete diverse libraries with a wide range of expression levels. Frequently monitor competitions over time and with replicates.	• Successful library construction • Rebuild 4 strains • Increase in productivity >20% over baseline
Production of wax esters without severe growth defects.	Expression levels of enzymes in the wax ester synthesis pathway must be well-proportioned to avoid accumulation of damaging intermediates.	Same as above and: Use two-gene cassettes, minimizing the number of enzymatic steps.	• Successful library construction • Rebuild 4 strains • 12% extractable lipids and detectable levels of wax esters
Improvements to strains in the laboratory must translate to improve outdoor performance.	They may not.	Compete and test strains under conditions that closely resemble outdoor cultivations.	PEAK challenge: strain with >50% growth improvement over WT, 14% extractable lipids

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3 – Impact

Goals of project: To enhance outdoor growth and lipid content in *Arthrospira platensis* strains through streamlined optimization of genetic traits resulting in at least one achieving growth rates of at least 19 g/m^2/day and at least 14% lipids and 16% carbohydrates.

Project Feature	Relevance
Working with *Arthrospira platensis*	A robust strain, novel to the BETO AAS program, resistant to predation, with commercially demonstrated outdoor production. More easily harvested than unicellular algae.
Optimization of stress-resistance traits	Increased productivity and robustness to factors such as temperature, seasonality, and competition. **Addressed BETO Barrier Aft-C. Biomass Genetics and Development.** **Lumen impact: exploration of strain for use as parental background**

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3 – Impact

Project Feature	Relevance
Optimization of wax ester expression traits	Increased fraction of compounds important for biofuel / bioenergy production thereby enabling algae biomass compositions in environmental simulation cultivation conditions with increased energy content and convertibility.
Development of high-throughput metabolic profiling methods	Increased understanding and characterization of variability in novel feedstock characteristics. **Addresses BETO Barrier Aft-E. Algal Biomass Characterization, Quality, and Monitoring** **Lumen impact: improved TEA model inputs, improved understanding of growth parameter impacts on metabolic profiles**
Cultivation targets	Demonstrated translation of laboratory improvements to industrially relevant (outdoor/solar) cultivation approaches, driving down the cost of biomass production. Produced strains that can be licensed with demonstrated enhanced ability to be grown outside for biofuels. Demonstrated traits and engineering tools that can be licensed for enhanced algal biofuel productivity in *Arthrospira* or other cyanobacteria.

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3 – Impacts: Lumen future plans

Lumen Bioscience impacts and future plans:

1. **exploration of strain for use as parental background**
2. **improved TEA model inputs, improved understanding of growth parameter impacts on metabolic profiles**
3. **Toolkit applications to ACCESS CARBON award**

Data sharing methods:

- **Publication in scientific journals**
- **Invention disclosures**
- **Patent applications**

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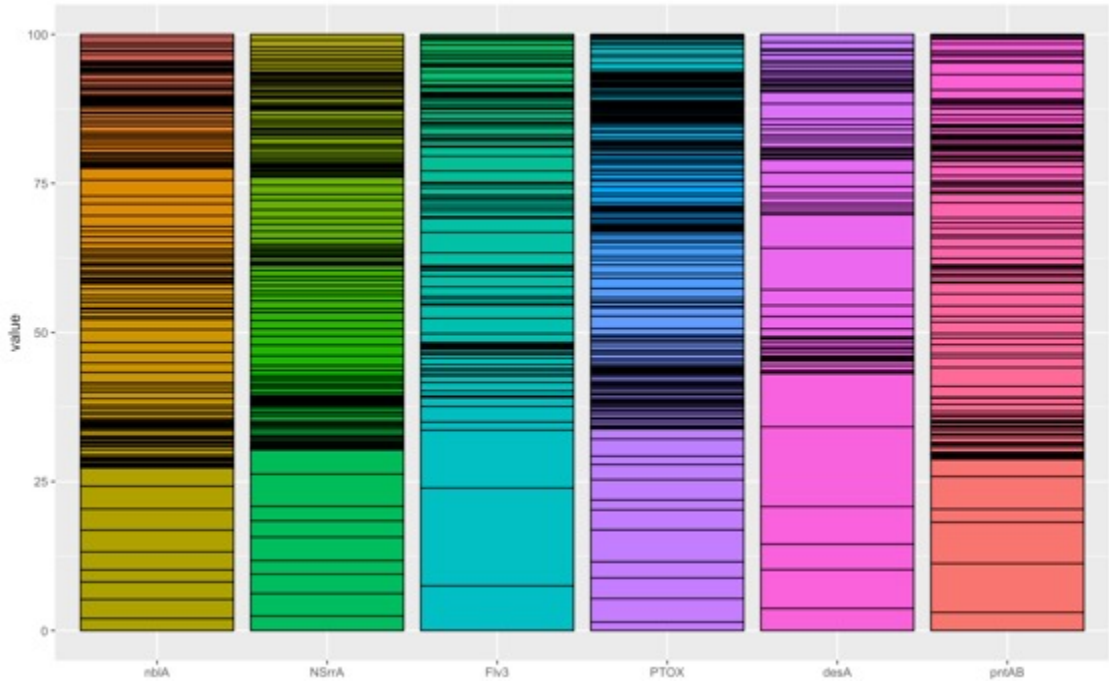
4 – Progress and Outcomes

Construct libraries → Transform → Compete → Build → Characterize → Grow

Deliverable 2.2.1 Successfully create transformation of 4 libraries (theoretical diversity >100) of stress resistance traits (Lumen) [M9]

Gene library	Unique Promoters	Unique Coding Sequence Versions	Terminators present	Theoretical library diversity	Actual library diversity (NGS)
nblA	22	3	3	**198**	173
flv3	22	2	3	**132**	121
NSrrA	22	3	3	**198**	169
PTOX	22	4	3	**264**	232
desA	22	2	3	**132**	93
pntAB	22	3	3	**198**	138

plasmid library distribution used for transformation



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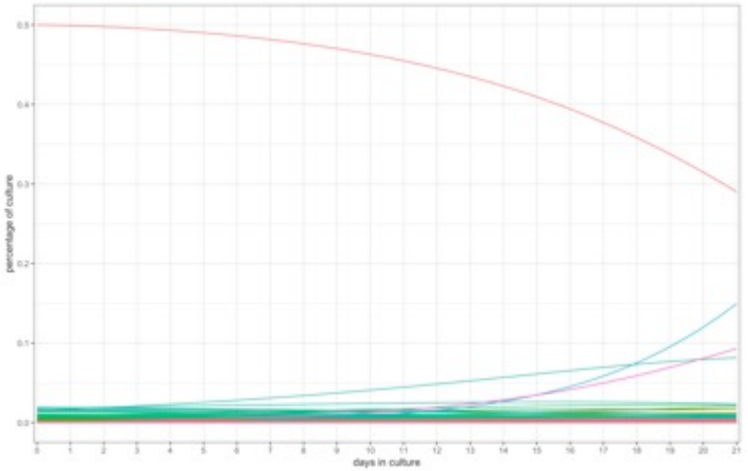
4 – Progress and Outcomes

Construct libraries → Transform → **Compete** → Build → Characterize → Grow

Competition model for growth at Wdt = 13.86 hours: 21 days of growth sufficient to identify winners at 0.1% of the starting population



Turbidostat mode:
OD = 0.1

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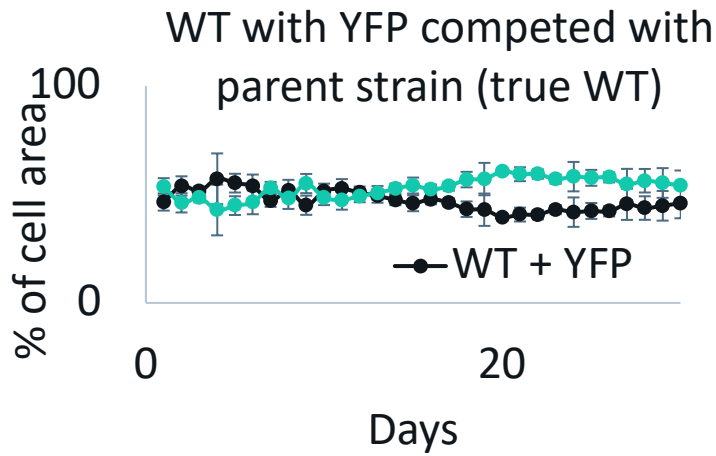
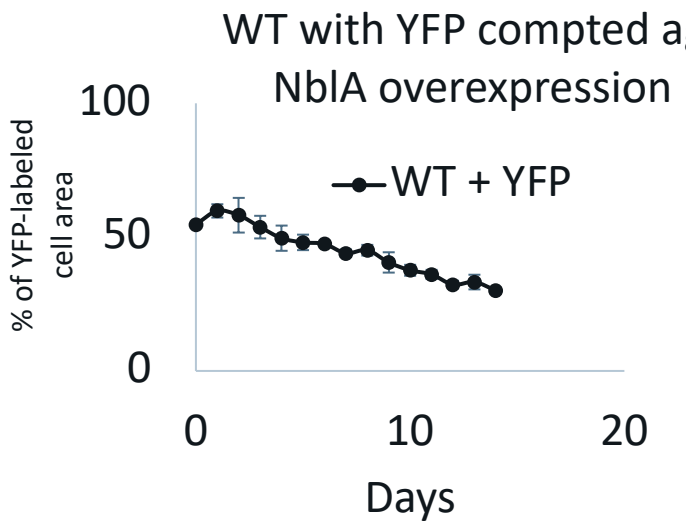
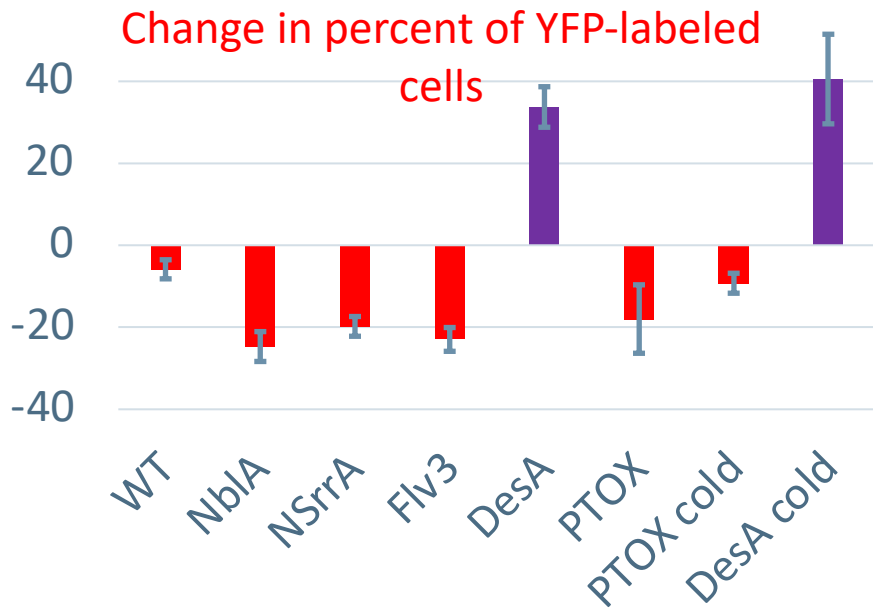




5 of 7 competitions led to decrease in share of WT-YFP population relative to stress tolerant libraries

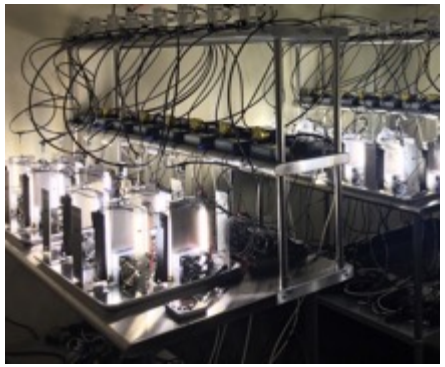


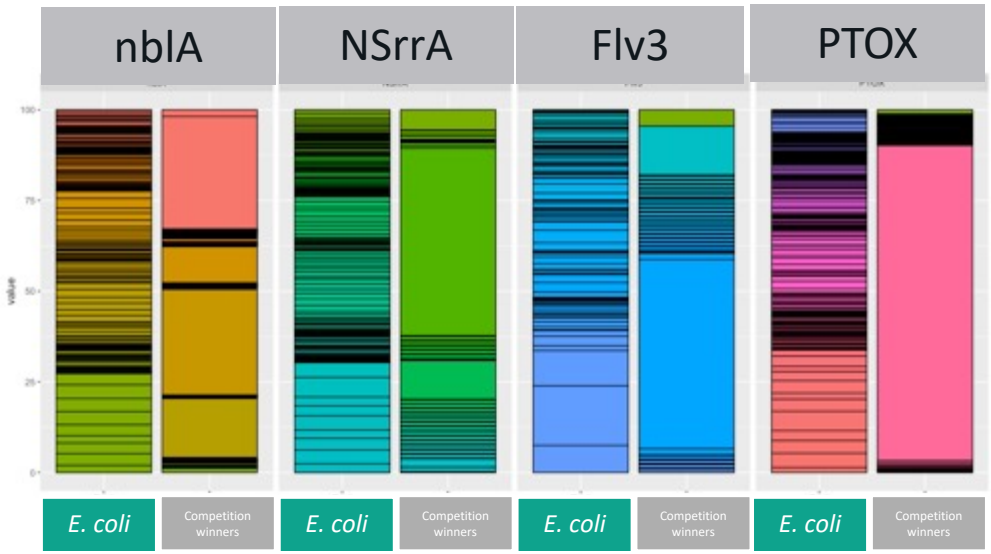
4 – Progress and Outcomes

Construct libraries | Transform | Compete | **Build** | Characterize | Grow

Milestone 2.2.2 Demonstrate productivity improvement over baseline growth for improved strains, by evaluating top strains by indoor, artificial light testing of productivity improvements with continuous monitoring of compositional improvements (Lumen) **[M24]**

- Rebuilt 20 strains from stress-tolerance libraries
- Characterized growth in flask and flat panel reactors





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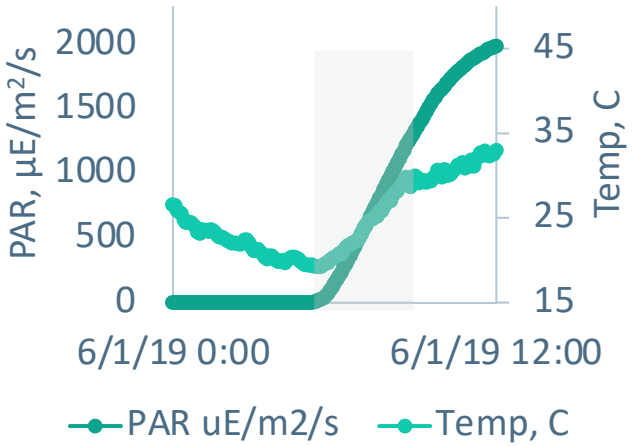
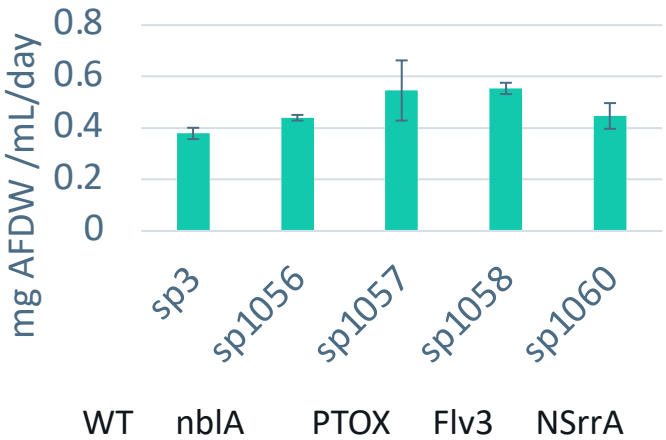
Outdoor growth in Arizona (6/1/2019)



4-day growth rates of rebuilt strains in flask : continuous light, 23C

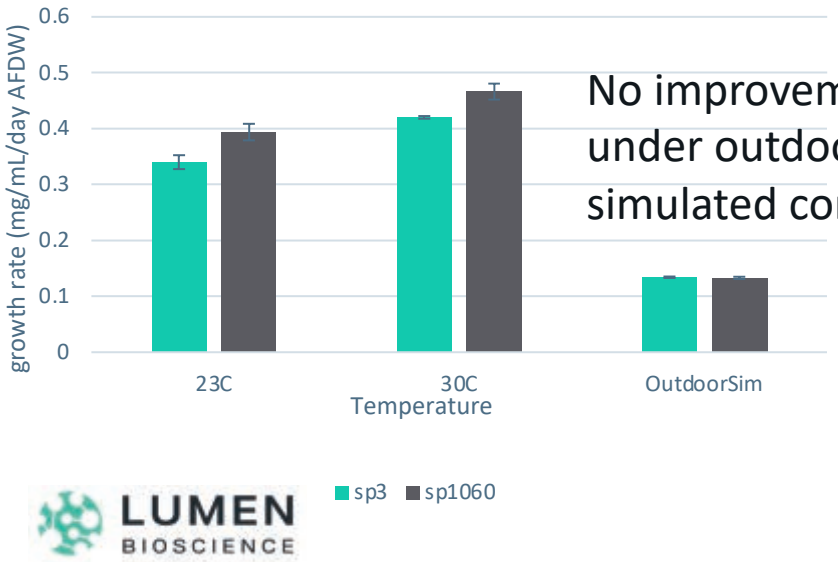


4 – Progress and Outcomes

Construct libraries → Transform → Compete → Build → Characterize → Grow

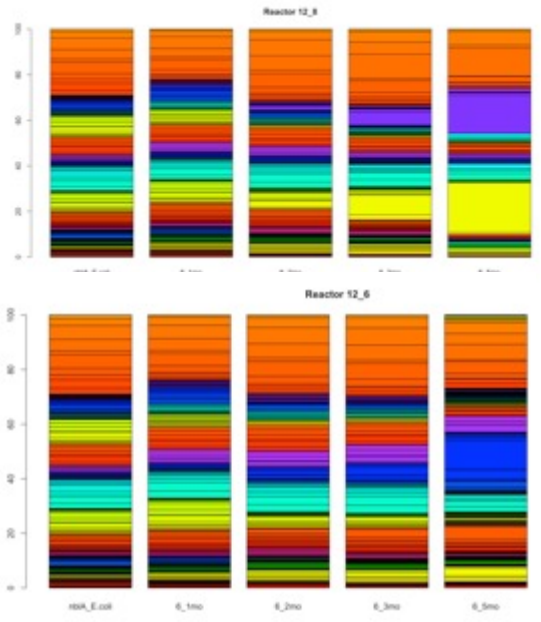
Milestone 2.2.2 Demonstrate productivity improvement over baseline growth for improved strains, by evaluating top strains by indoor, artificial light testing of productivity improvements with continuous monitoring of compositional improvements (Lumen) **[M24]**

Growth performance at constant and outdoor simulated light and temperature regimes



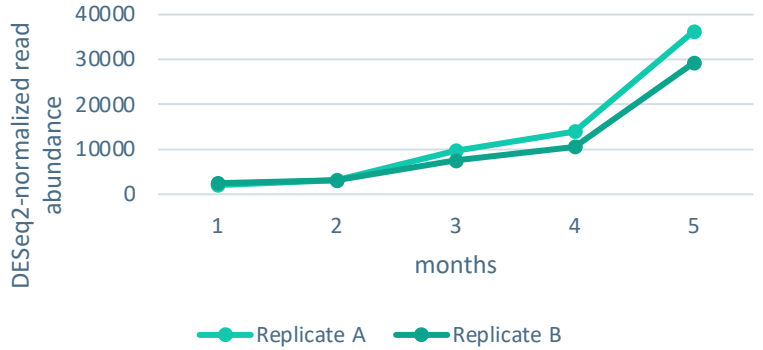
No improvement under outdoor simulated conditions

Repeated transformation of SOFAST libraries in flat-panel reactors under outdoor simulated conditions

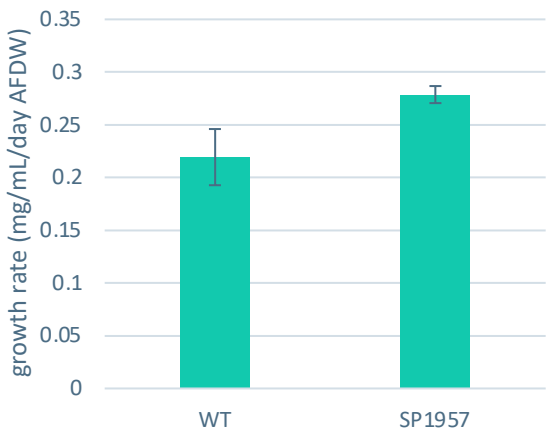


	base Mean	Log_2 FoldChange	lfcSE	padj
Patp1_nsrra sp87_5v1	11802.6	3.86	0.24	9.56E-69
PdnaK_nsrra synPCC coop_4v1	16272.2	3.7	0.17	2.08E-120

Patp1_nsrra-sp87_5v1 - clone abundance



Growth rate of improved SOFAST strain



4 – Progress and Outcomes

Construct libraries → Transform → Compete → Build → Characterize → Grow

Deliverable 2.3.1 Successfully create transformation libraries (theoretical diversity >100) of a two-gene wax-ester cassette.
Deliverable 2.3.2 Demonstrate continuous strain selection in ePBR of wax ester libraries for at least 14 days
Milestone 2.3.1 Evaluate top 5 strains by indoor, artificial light testing of productivity. Demonstrate growth of wax ester strains at rates not more than 10% slower than the wild type baseline.

Library	*E. coli* Sanger-sequencing estimated diversity (Chao1)	MinION-determined Library diversity of *A. platensis* library at end of competition
FAR12-AtfA45	128	35
FAR14-AtfA45	135	19
FAR16-AtfA45	135	37
FAR18-AtfA45	135	19
FAR12-AtfA67	128	51
FAR14-AtfA67	135	27
FAR16-AtfA67	135	37
FAR18-AtfA67	135	55

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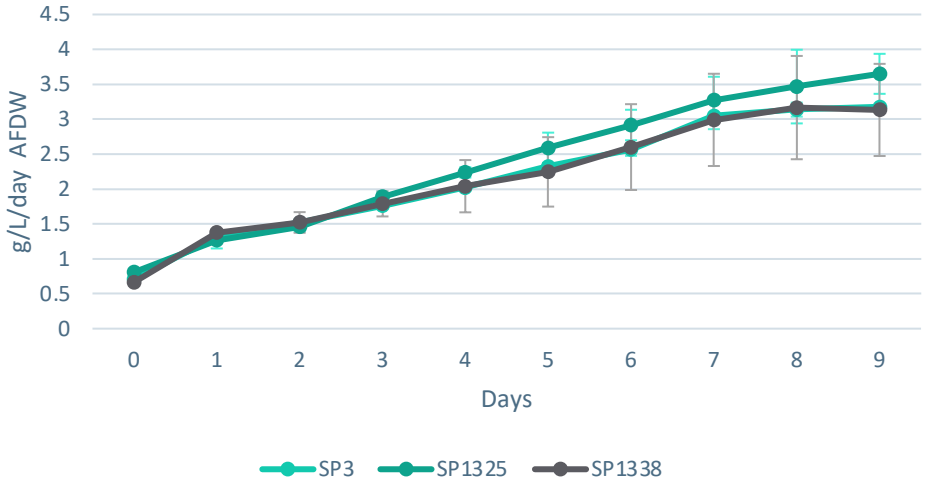
Characterization of wax ester-producing strains in 9L reactors – constant light



NREL GC and ESI data informed clones to rebuild potential wax ester-producing strains

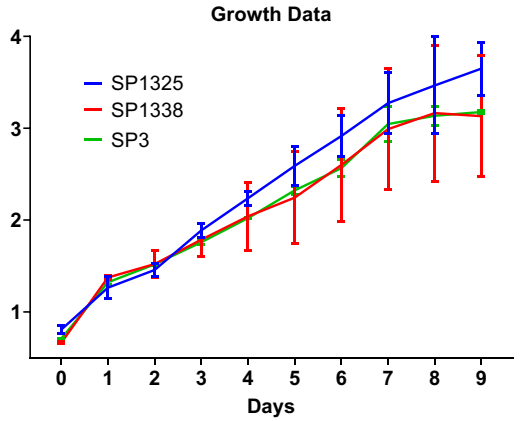
No impact to growth for wax-ester producing strain SP1325 under constant light or outdoor simulated conditions

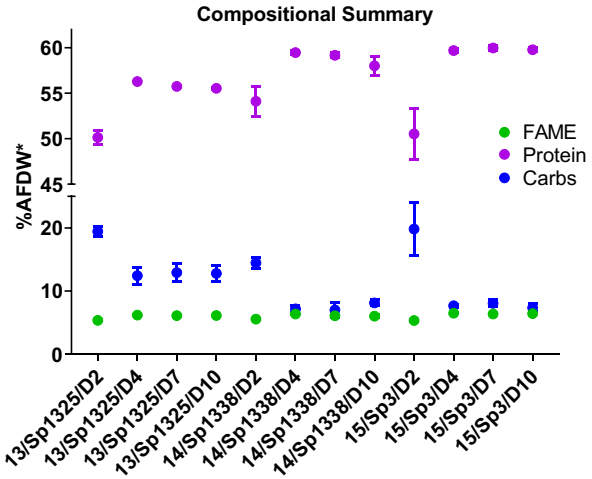
Novel Wax Ester strains	ID	Wax ester content as % lipid	Growth rate: Constant light/temp in miniarray (mg/mL/day) [%WT]	Growth rate: Outdoor simulation in miniarray (mg/mL/day) [%WT]	Growth rate: Constant light/temp in 9L reactor (mg/mL/day) [%WT]
PpsaF-FAR14coop_Pgap205-AtfA45	SP1325	1.14	0.35 [103.2]	0.159 ± 0.021 [83]	0.324 ± 0.042 [118]
PpetB-FAR16coop_PccmK-AtfA67	SP1338	0.74	0.148 [43.6]	0.145 ± 0.023 [76]	0.271 ± 0.09 [98]

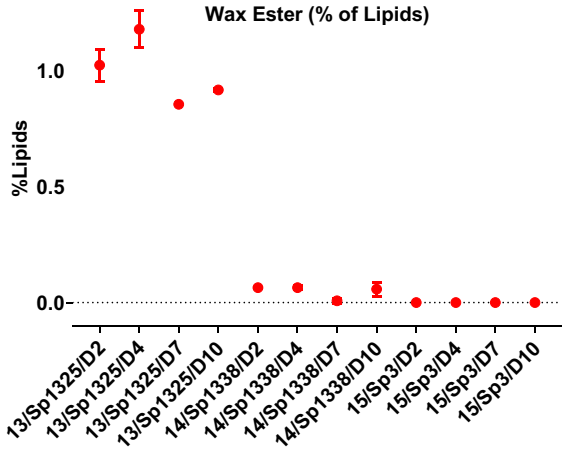
4 – Progress and Outcomes

Construct libraries | Transform | Compete | Build | Characterize | Grow

Milestone 2.3.1 Evaluate top 5 strains by indoor, artificial light testing of productivity. Demonstrate growth of wax ester strains at rates not more than 10% slower than the wild type baseline.







Sample	Fame	Protein	Carbs
17/Sp1325/D4	6.09	56.16	**11.46**
17/Sp1325/D7	6.01	55.83	**13.88**
17/Sp1325/D10	6.09	55.46	**13.71**
14/Sp1338/D4	6.4	59.35	7.6
14/Sp1338/D7	6.13	58.98	7.88
14/Sp1338/D10	6.16	57.27	8.46
18/Sp3/D4	6.54	59.82	**7.84**
18/Sp3/D7	6.36	60.18	**7.68**
18/Sp3/D10	6.41	59.63	**7.78**

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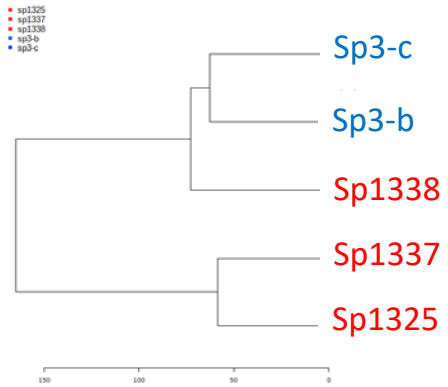
4. Progress and Outcome

- Wax ester producing strains shift biomass composition over time
- SP1325 exhibits significantly increased carbohydrate content at no reduction (minor increase) in growth rate
- Lipidomics profile shows distinct clustering of SP1325 mutant dominated by polar lipid and WE physiological changes
- Detailed metabolomics analysis is ongoing to narrow down carbon sequestration pathways in SP1325



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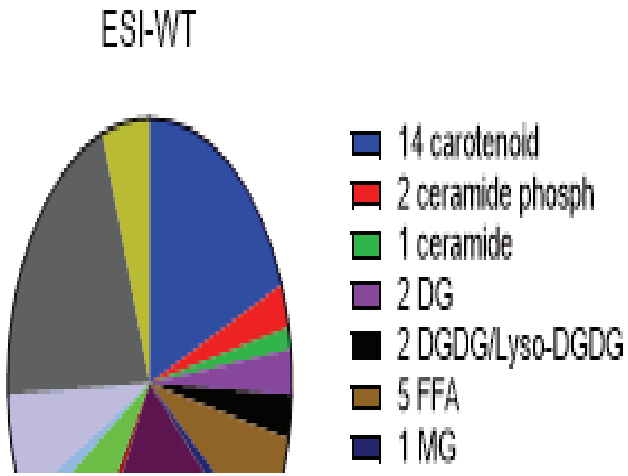
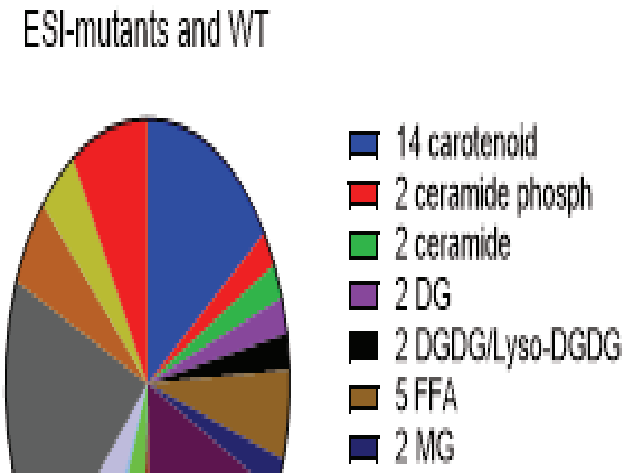
14/Sp1338/D7	6.13	58.98	7.88
14/Sp1338/D10	6.16	57.27	8.46
18/Sp3/D4	6.54	59.82	7.84
18/Sp3/D7	6.36	60.18	7.68
18/Sp3/D10	6.41	59.63	7.78





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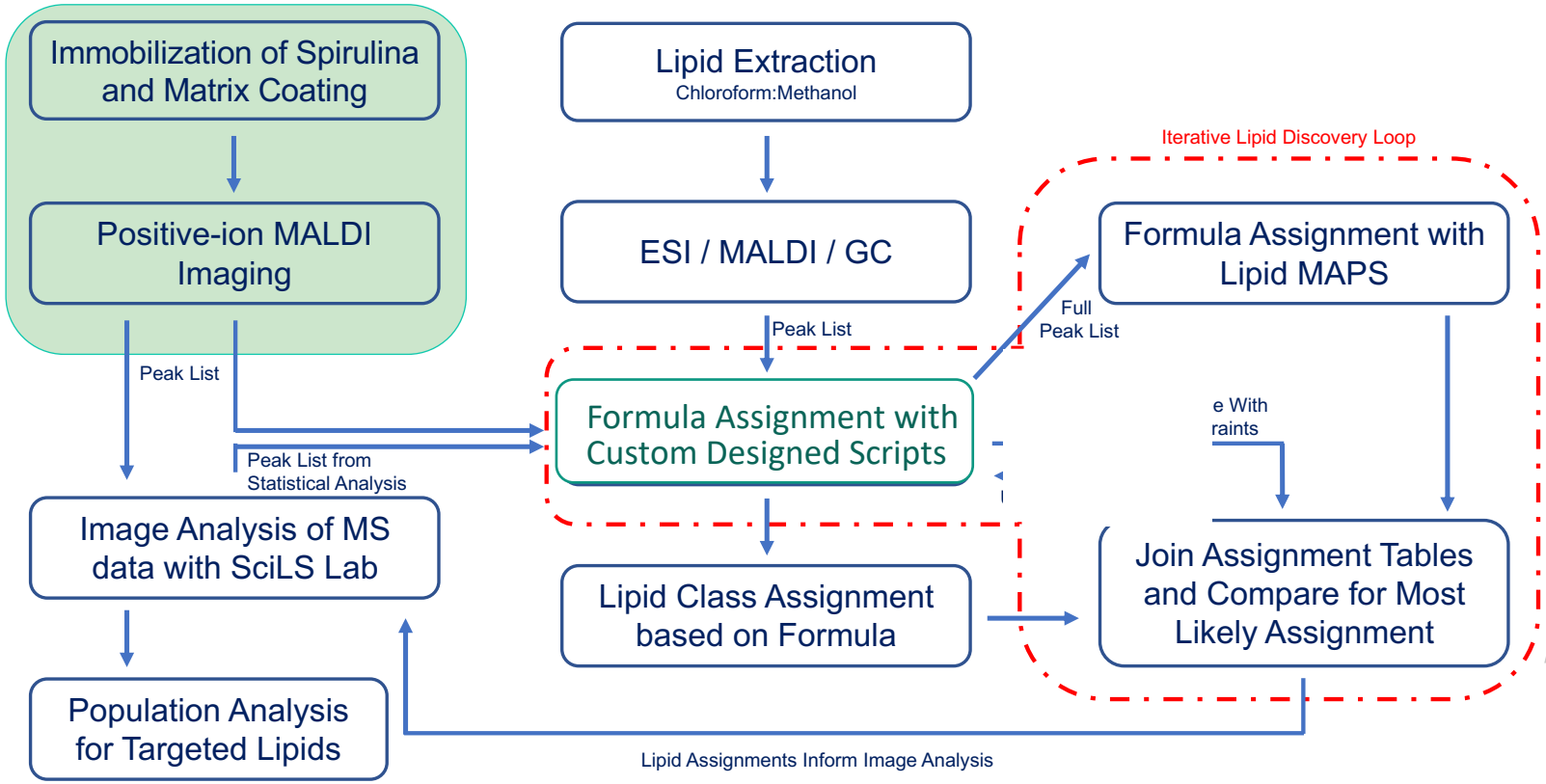
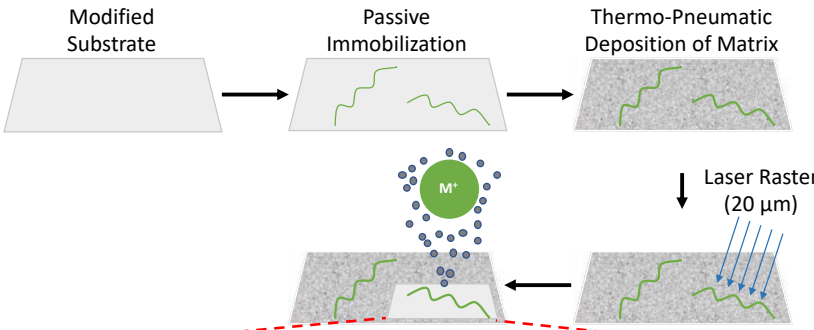
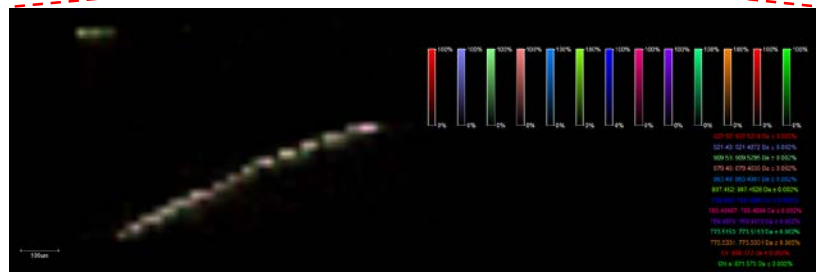
NREL Transforming ENERGY

4. Progress and Outcome



- Novel characterization pipeline developed and tailored for spirulina
- Ultra-high resolution mass spectrometry (MS), and imaging MS used to map lipids in engineered combinatorial arrays of strains
- Imaging MS Analysis of population heterogeneity and intrafilament heterogeneity





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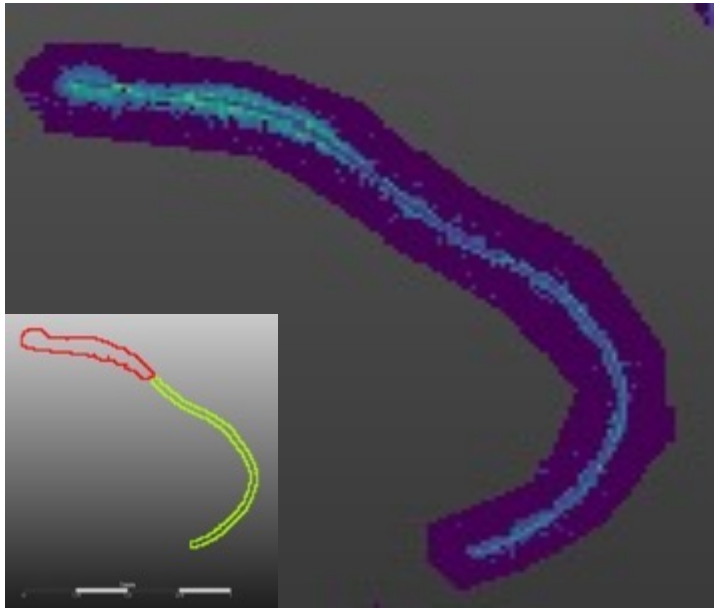
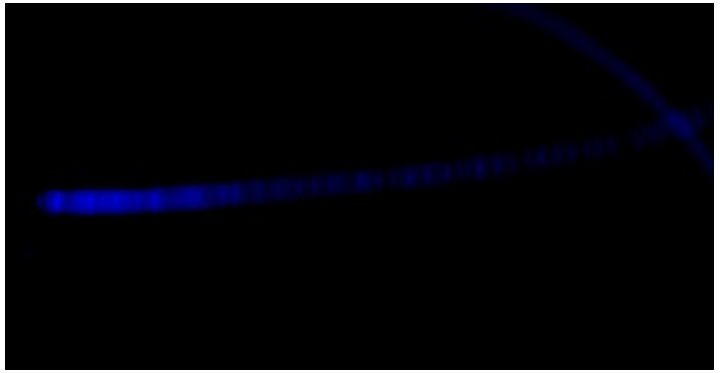
4. Progress and Outcome: Toolkit development

- First demonstration of MALDI-imaging MS shows population heterogeneity
- Intrafilament imaging lipidomics shows differential concentration of distinct physiology within spirulina filament
- MS multivariate analysis narrows down lipidome changes associated with filament ends, a total of 26 lipids can be differentially expressed



Imaging MS profile of MGDG (775.5331 m/z) across a filament



Fluorescent D-amino acid dye (7-hydroxycoumarin 3-carboxylic acid (HCC-OH) attached to a D-amino acid backbone, 3-amino-D-alanine, HADA) image, showing peptidoglycan intercellular boundaries on one filament end

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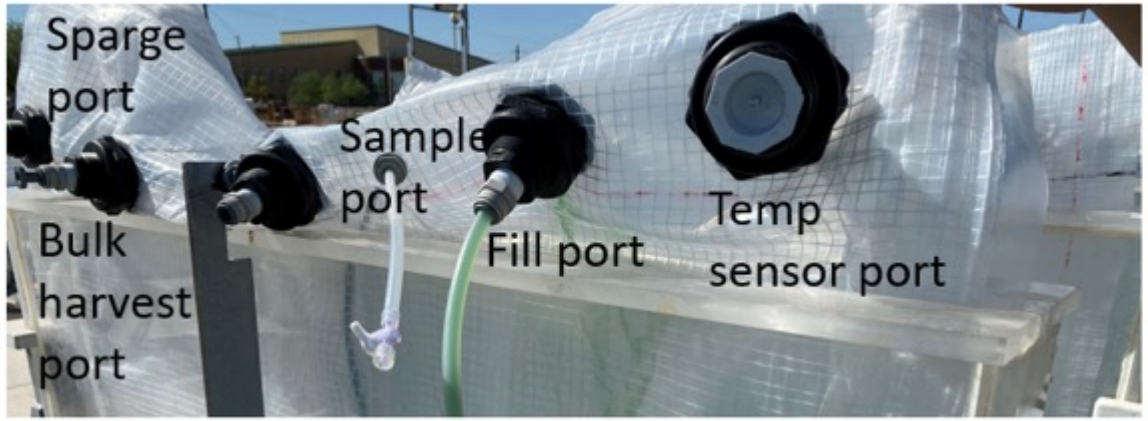
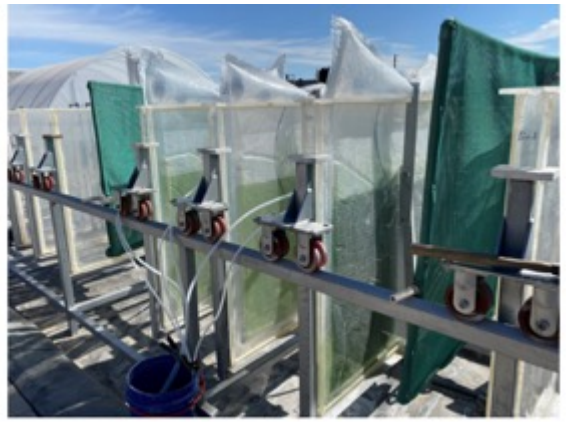
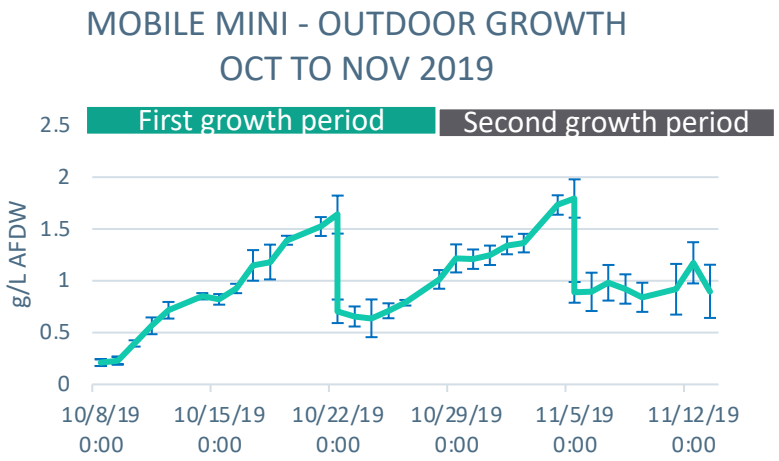
Shanta, P., Intrafilament Imaging Mass Spectrometry-based Lipidomics, *manuscript in preparation*

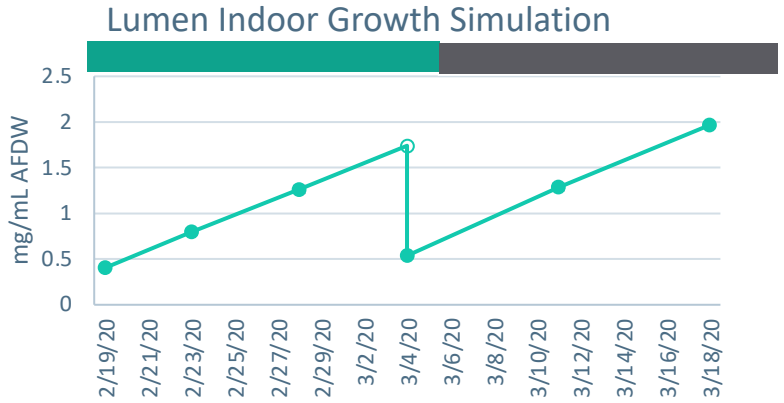
4 – Progress and Outcomes

Construct libraries → Transform → Compete → Build → Characterize → **Grow**









Milestone 4.3.1 Test two stress resistant strains in outdoor cultivation (season 1, winter/spring), demonstrating at least 50% productivity improvement in biomass growth relative to WT baseline. (ASU) **[M33]**

growth	g/L/day	error
First	0.102	0.004
Second	0.0904	0.005

Lumen Indoor Growth performance

growth	g/L/day	error
First	0.0953	0.0015
Second	0.102	0.003

Best performing stress tolerance and wax ester strains for outdoor testing at AZCATI

- SP1060 and SP1325

Summary

The project aimed to enhance growth and lipid content in an industrially relevant cyanobacterium, *Arthrospira platensis.*

Approach: combine genetic engineering and selection approaches with high-throughput biomass characterization to select for winning genetic constructs that we transform into *Arthrospira* stains.

Progress:

- ✓ In situ competitions
- ✓ Metabolic profiling
- ✓ Indoor growth characterization for wax esters
- ❑ Outdoor growth seasons

Target: 20% enhancement in growth / >12% lipids

19 g/m^2/day, > 14% lipid, >16% Carbohydrate.

Relevance:

- ➢ *A robust strain, resistant to predation, with commercially demonstrated outdoor cultivation, that shows improved for outdoor growth AND biomass composition, driving down the cost of biomass production.*
- ➢ *Potential for licensing strains, traits, and genetic tools for algal biofuel production.*

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Quad Chart Overview (Competitive Project)

Timeline

- October 2017
- June 2021

	FY20 Costed	Total Award
DOE Funding	(10/01/2019 – 9/30/2020) $377,730	(negotiated total federal share) $1,193,682
Project Cost Share	$182,040	$556,331

Project Partners*

- NREL (34%)

Project Goal

To produce engineered *Arthrospira* platensis strains with improved photosynthetic and cold-tolerance traits and increased lipid content.

End of Project Milestone

End of Project Goal: Demonstrate at least 1 improved platform spirulina strain achieves at least a 15 percent improvement in biomass productivity relative to wild type baseline, displaces 25 percent of delivered CO2 with DAC, and has a biomass composition containing at least 2% therapeutic protein and achieves a 100% relative increase in components of biomass that can be used for bioenergy production relative to baseline strain and conditions

Funding Mechanism

DOE-FOA-0001628: Productivity Enhanced Algae and Tool-Kits (PEAK) 1 Topic Area 1: Strain Improvement

*Only fill out if applicable.

Additional Slides

Responses to Previous Reviewers' Comments

Reviewer Comments	Response to Comments
Demonstrated progress on most milestones, unfortunately the project pivoted away from outdoor raceway trials to indoor simulated trials instead.	The project pivoted from outdoor raceway trials to outdoor flat-panel trials. Indoor simulations were always planned and remain in the project goals.
The SOFAST project addresses both the development of tools for strain improvement and characterization as well as the use of those tools to improve an industrially relevant strain. Focusing on Spirulina as a target is logical and relevant given the long history of outdoor cultivation at scale. Key steps have been made towards the project goals around tool development. The use of competition experiments to identify top performing strains under selective pressure is clever and likely to result in a highly efficient methodology. It is unfortunate that the validation of the strain performance will not be conducted in outdoor trials.	Outdoor validation trials will indeed be completed.

Publications, Patents, Presentations, Awards, and Commercialization

LIST ANY PUBLICATIONS, PATENTS, AWARDS, AND PRESENTATIONS THAT HAVE RESULTED FROM WORK ON THIS PROJECT

Patents:
None

Publications / Presentations:

Presentation: Damian Carrieri, Rachelle Lim, Troy Paddock, Jim Roberts, John McGowen, Peter Shanta, and Lieve Laurens. "SOFAST: Streamlined Optimization of Filamentous *Arthrospira*/*Spirulina* Traits". The Rules of Life: Complexity in Algal Systems; Summer Science Symposium. July 2020.

Manuscript in preparation: "Engineering of Bioenergy Pathways in Cyanobacteria has a Global Metabolic Impact on Photophysiology and Lipid Metabolism" Shanta P., et al.

Manuscript submitted: "Overexpression of NblA decreases phycobilisome content and enhances photosynthetic growth of the cyanobacterium Synechococcus elongatus PCC 7942" Algal Research. (submission 2020_833). Damian Carrieri, Tracey Jurista, Nina Yazvenko, Adan Schafer Medina, Devin Strickland, and James M. Roberts.

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