DOE Bioenergy Technologies Office (BETO) 2019 Project Peer Review

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

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Technology Session Area Review

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Lumen Bioscience

Goal Statement

- *Arthrospira species ("Spirulina")*
  - Cyanobacteria with proven industrial outdoor 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 and cold-tolerance traits and increased lipid content.

- *Outcome:* Demonstrate successful and stable outdoor productivity of 6 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
  - Response to PEAK CHALLENGE FOA, MYP2019 for goals by 2021

PEAK CHALLENGE FOA, MYP2019 GOALS

"By 2021, develop strain improvement toolkits and technologies that enable algae biomass compositions in environmental simulation cultivation conditions that represent an energy content and convertibility of 80 GGE of advanced biofuel per ash-free dry weight ton of algae biomass."

Quad Chart Overview

Timeline

- Project start date: October 2017
- Project end date: September 2020
- Percent complete: 50%

	FY 18 Costs	Total Planned Funding (FY 19-Project End Date)
DOE Funded	385,396	1,465,980
Project Cost Share	159,337	440,206

•NREL (34%)

Barriers addressed

Aft-C. Biomass Genetics and Development

Aft-E. Algal Biomass Characterization, Quality, and Monitoring

Objective:

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

End of Project Goal:

Demonstrate successful and stable outdoor productivity of 6 *Arthrospira* strains with at least 1 achieving growth rates of at least 19 $g/m^2/day$ with at least 14% lipids and 16% carbohydrates.

Project Overview

Arthrospira species, "Spirulina"

PROS (Advantages of this platform)	**CONS (Addressed in this project)**
• Cultivated outdoors globally (food, feed, cosmetic industries) • Robust growers, resistant to predation • Easier to harvest compared to unicellular algae • Can be grown in saline or wastewater-based media	• Variable biomass composition with high protein content / low fatty acid content • Limited molecular biology tools were available • Decreased biomass accumulation under light and cold stresses

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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Approach (Management)

Lumen Bioscience	National Renewable Energy Laboratory (NREL)	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: PI	Lieve Laurens: Co-PI	John McGowen: Cultivation management

Approach (Technical)

Transform → Compete → Sequence → Build → Test → Stack → Grow

1. **Transform** libraries of traits with various coding sequences and promoter/terminator strengths (100+ variants in each library).
2. **Compete** transformed libraries against control cells (expressing YFP) or each other.
3. **Sequence** the genomes of the competed populations to identify constructs that imparted the best fitness or most wax esters without severe growth defects.
4. **Build** top constructs into *Arthrospira* to produce strains expressing traits at optimal levels.
5. **Test** newly made strains for improved growth rates or metabolic profiles relative to *wild type* control runs. End of Phase I, **GO/NO-GO 2 – (Sept 2019)**
6. **Stack** best performing traits to produce strains with combined improvements in both growth and wax ester accumulation.
7. **Grow** stacked strains in indoor and outdoor test beds alongside *wild type* controls. End of Phase II, **PEAK Challenge – (Sept 2020)**

Critical Success Factor	Challenge	Strategy
Expression of stress resistance and cold tolerance traits that improve growth.	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.
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. Monitor wax ester production with single-filament mass spectrometry population statistics.
Models that accurately determine biomass composition from high throughput data are needed.	Many representative samples must be used to train a robust model.	Generate a large number of samples from diverse growth conditions.
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.

Approach

	Year 1 - FY 18				Year 2 - FY 19				Year 3 - FY 20			
	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9	Q10	Q11	Q12
	Dec-19	Mar-19	Jun-19	Sep-19	Dec-19	Mar-19	Jun-19	Sep-19	Dec-19	Mar-19	Jun-19	Sep-19
Validation												
PHASE I: Strain development and phenotyping												
PHASE II: PEAK challenge												
Task 1: Validation												
1.1 Process and cultivation readiness demonstration	G/NG											
1.2 Interim performance validation								G/NG				
Task 2: Strain Development (LUMEN)												
2.1 Monitoring competition growth by fluorescence microscopy and Sequencing												
2.2 Stress tolerance												
2.3 Wax ester production												
2.4 Trait stacking												
Task 3: Metabolic Phenotyping of Improved Strains (NREL)												
3.1 Single filament phenotyping by high resolution mass spectrometry												
3.2 Whole cell biomass metabolic fingerprinting												
Task 4: Cultivation and Pond Operational Management (Lumen, NREL, ASU)												
4.1 Indoor sclae up and indoor / outdoor cultivation												
4.2 Test successful traits in ePBRs and indoor large cultivation												
4.3 PEAK challenge												

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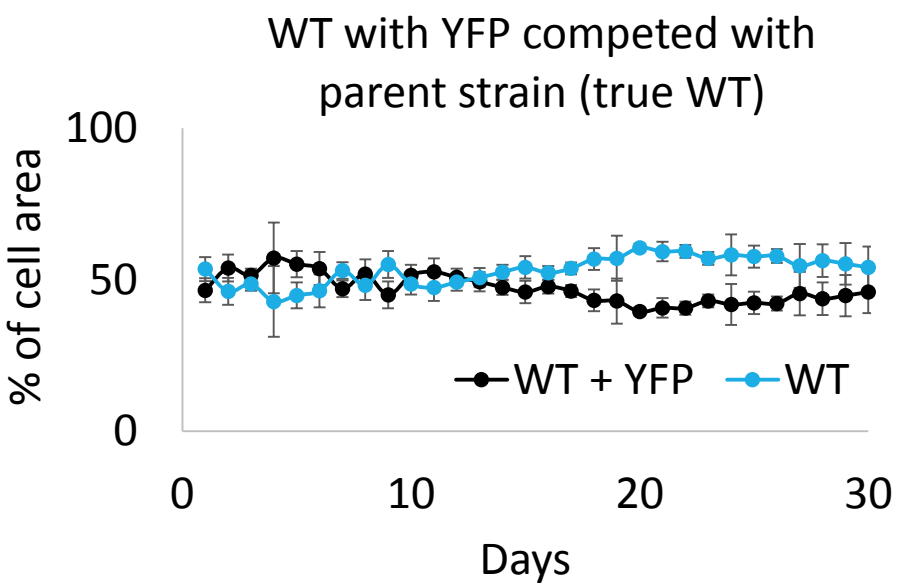
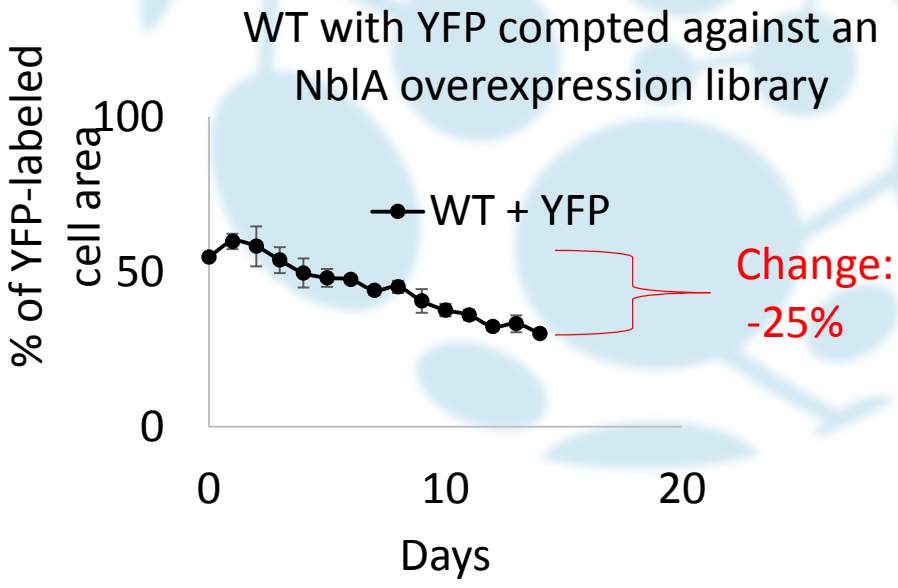
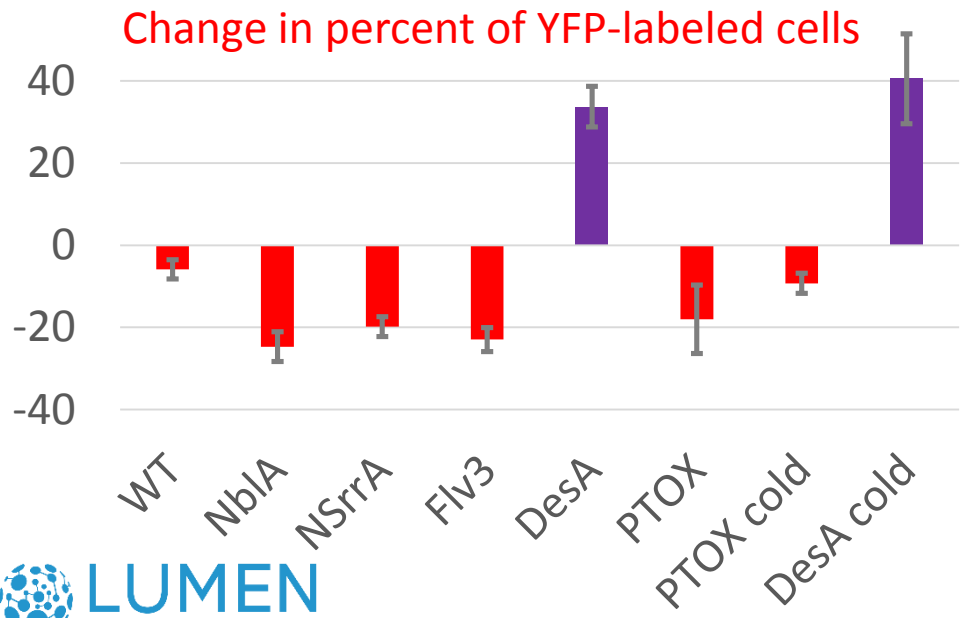
Progress, Traits: in situ competitions







Decline in % labeled cells → increase in % nblA cells

DesA was not a successful trait, but the "most tolerated" constructs will be identified by sequencing – similar future approach with wax ester traits

5 of 7 competitions expected to lead to constructs conferring stress resistance.

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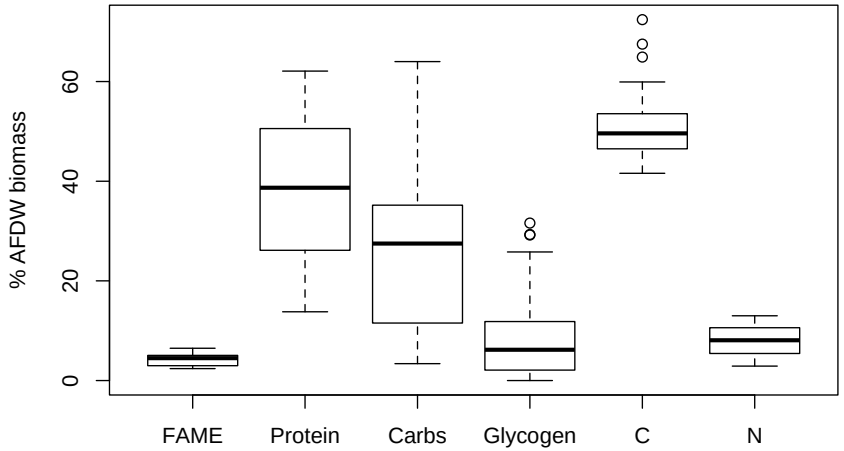
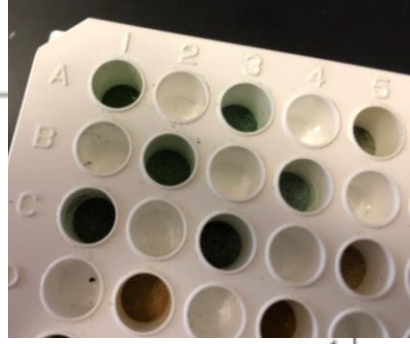
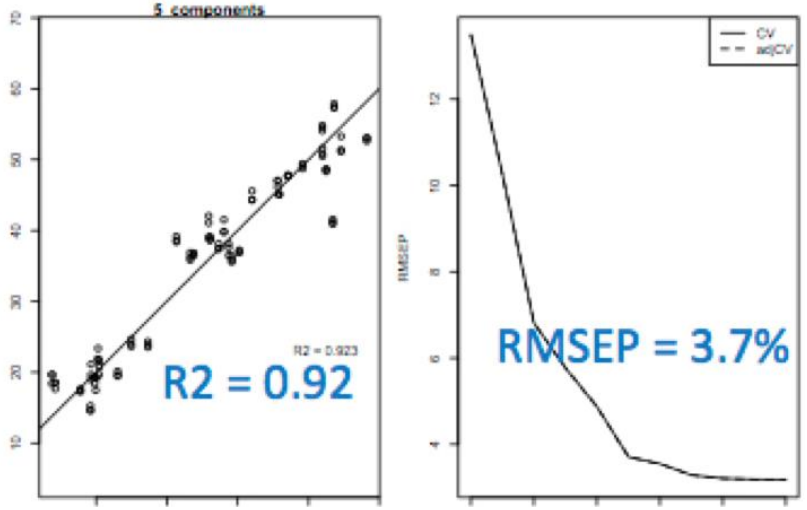
Progress, Metabolic Profiling

Compositional analysis of *Arthrospira* biomass (>35 samples) indicates a dynamic nature, responsive to media composition and cultivation conditions



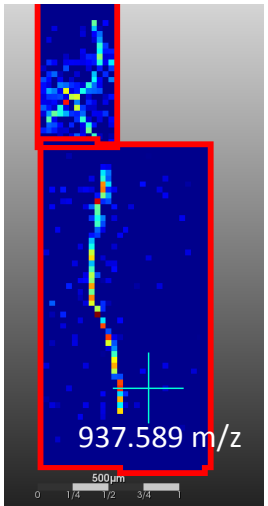


Protein



NIR spectroscopy used to build model that uses high throughput data to predict **protein content with ~4% accuracy**

Also: Ultrahigh-resolution mass spectrometry is allowing for rapid, **in situ lipidomic profiling** of <u>single filaments</u> of *Arthrospira* from wax ester strain competitions. (under method refinement)



NREL

Progress, Growth: Indoor

Parameter	Phenometrics ePBR	Raceway Pond (indoor)
Light cycle	16/8-hour light/dark cycle	16/8-hour light/dark cycle
Light intensity	1650 µE*m^{-2}*s^{-1} sinusoidal light	1000-1500 µE*m^{-2}*s^{-1} square wave light
Temperature	30°C constant	Low of 24°C (night) – high of 34°C (day)
CO_2 delivery	pH control with CO_2 (on/off)	pH control with CO_2 (on/off)
pH	Maintained below 10	Maintained below 10
Inoculation density	0.05 OD750	0.05 OD750
Culture volume	500 mL	100 L
Reactor illuminated area	37 cm^2 (0.0037 m^2)	0.5 m^2
Average growth rate	4.25 ± 0.18 g/m^2/day (AFDW)	11.84 ± 3.6 g/m^2/day washed ~ 9.5 g/m^2/day AFDW

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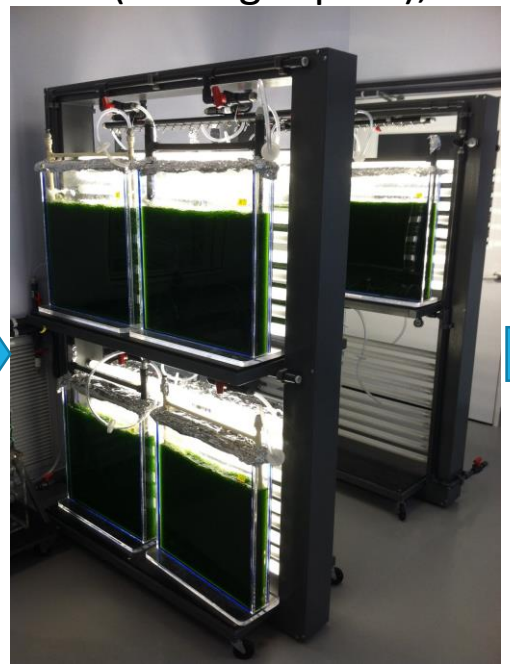
New experiments are planned for indoor, flat-panel reactors.

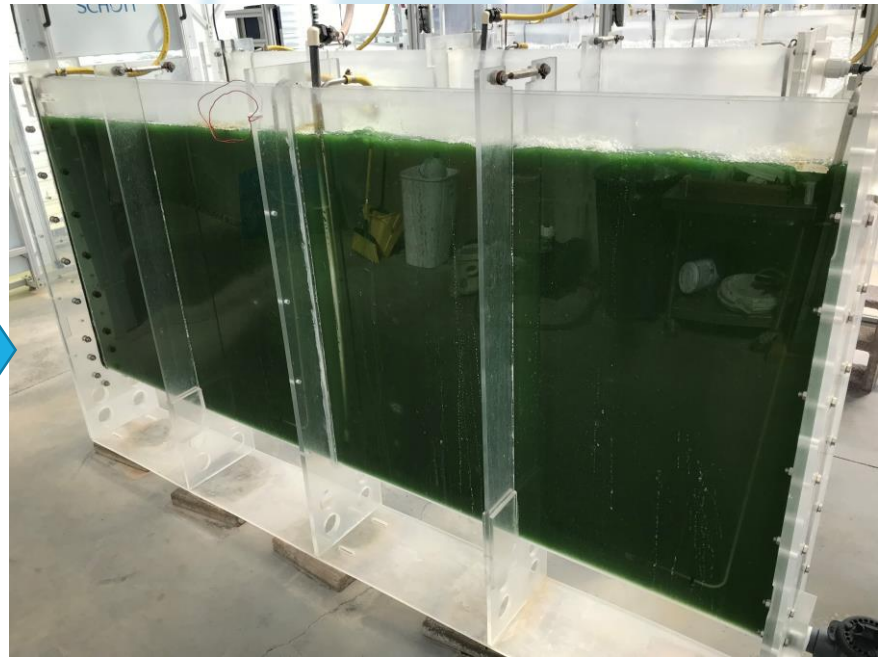
Progress, Growth: Outdoor

800 ml bubble columns

2’x2’ flat panels (1.5” light path), 15 L



4’x8’ flat panels (1.5” light path), 100 L, in greenhouse



More work needed to demonstrate baseline outdoor growth and relate with indoor culturing.

Setup	Conditions
Bubble columns and indoor flat panels	30°C Constant Light (150 µE*m^{-2}*s^{-1}) Air only sparging
Greenhouse panels	Seasonal light and temp 2% V/V CO_2 in air, day. Air only, night.

Relevance

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	Increases productivity and robustness to factors such as temperature, seasonality, and competition. **Addresses BETO Barrier Aft-C. Biomass Genetics and Development.**

Relevance

Project Feature	Relevance
Optimization of wax ester expression traits	Increases 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	Increases understanding and characterization of variability in novel feedstock characteristics. **Addresses BETO Barrier Aft-E. Algal Biomass Characterization, Quality, and Monitoring**
Cultivation targets	Demonstrates translation of laboratory improvements to industrially relevant (outdoor/solar) cultivation approaches, driving down the cost of biomass production. Produces strains that can be licensed with demonstrated enhanced ability to be grown outside for biofuels. Demonstrates traits and engineering tools that can be licensed for enhanced algal biofuel productivity in *Arthrospira* or other cyanobacteria.

Future Work

Key tasks to complete:

- Wax ester competitions. -- ON TRACK
  - Evaluate top 5 strains making wax esters, growth rates not more than 10% slower than wildtype control
- Trait building and validation -- ON TRACK Go/No-Go
  - Identify, build, and select a set of at least 4 specific improved strains (2 stress/cold, 2 wax ester). Target of 20% improved productivity in stress strains, >12% lipids in wax ester strains
- Trait stacking -- ON TRACK
- Demonstrate single filament phenotyping by mass spectrometry – ON TRACK
- Apply whole cell biomass metabolic fingerprinting to new strains – ON TRACK
  - Phenotyping analysis used to select top stacked strains for PEAK challenge
- Outdoor baseline cultivations -- DELAYED, changed to flat panels which will increase number of runs, expect to be back ON TRACK by Sept 2019
- Peak Challenge
  - Outdoor productivity of 6 improved *Arthrospira* strains, at least 1 achieving 19 g/m^2/day with simultaneous compositional improvements of >14% lipids and >16% carbohydrates.

Future Work: Highlighted Near-term Deliverables (FY2019)

Near-term Deliverables (FY 2019)	Deadline	Anticipated Delivery Date
Establish baseline growth (mean and variance) for wild-type *Arthrospira* indoor and outdoors and develop a predictive protocol to be used for translating growth improvements.	June 2018	September 2019
Rebuild at least **10 strains** from the best performers identified. (Towards demonstration of at least 20% productivity improvement over baseline (stress and cold tolerance), see below).	March 2019	March 2019
Identify and **rebuild** the top **5 strains** with the most promising constructs of genetically distinct wax ester expression traits.	March 2019	May 2019
Demonstration that complex libraries can be screened in growth competitions and metabolic phenotypes established by NREL technologies. Identify, **build, and select a set of at least 4 specific improved strains** (2 stress resistant strains and 2 wax ester producing strains). Targets are 20% improvement over baseline and 12% lipid for wax ester single trait strains, respectively.	September 2019	September 2019

Future Work: Highlighted Longer-term Deliverables (FY2020)

Longer-term Deliverables (FY 2020)	Deadline	Anticipated Delivery Date
Test 4 improved (single trait) strains in outdoor cultivation (Season 1: **Winter/Spring**) and demonstrate at least 50% productivity improvement in biomass or a lipid content of 16%.	March 2020	March 2020
Test 4 improved (single trait) strains in outdoor cultivation (Season 2: **Summer**) and demonstrate at least 50% productivity improvement in biomass or a lipid content of 16%.	August 2020	August 2020
Outdoor cultivation demonstration for stacked trait strains with demonstrated viability and report on growth data.	September 2020	September 2020
End of Project Goal: Outdoor productivity of 6 improved *Arthrospira* strains, at least 1 achieving 19 $g/m^2/day$ with simultaneous compositional improvements of >14% lipids and >16% carbohydrates.	September 2020	September 2020

Summary

The project aims 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. Transform → Compete → Sequence → Build → Test → Stack → Grow

Progress:

- ✓ In situ competitions
- ✓ Metabolic profiling
- ✓ Indoor growth baselines
- ❑ Outdoor growth baselines and indoor/outdoor comparisons

Relevance:

- *A robust strain, resistant to predation, with commercially demonstrated outdoor cultivation, will be 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.*

Future work:

BUILD new strains based on stress resistance and wax ester competitions.
TEST single traits against controls indoors and in outdoor testbeds.
STACK best traits.
GROW stacked strains with improved growth AND wax ester accumulation.

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

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

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Thank you

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Lumen	NREL	ASU
Jim Roberts	**Lieve Laurens**	**John McGowen**
Rachelle Lim Troy Paddock Lauren Goetsch Colin Brady	Peter V. Shanta Steven Rowland Nicholas Sweeney Kylie Smith	Jessica Forrester

DOE
Dan Fishman Elizabeth Burrows

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Supplemental: Stress Library Composition *as built*

Gene	Total Reads	Promoters Present	CDS present	Terminators present	Theoretical library diversity	Actual Library Diversity based on PacBio Sequencing
nblA	25400	21/22	3/3	3/3	198	173
Flv3	6817	21/22	2/2	3/3	132	121
NSrrA	26241	20/22	3/3	3/3	198	169
PTOX	35260	21/22	4/4	3/3	264	232
desA	19998	19/22	2/2	3/3	132	93