DOE Bioenergy Technologies Office (BETO) 2023 Project Peer Review

Enhanced production of algae lipids and carbohydrates for fuel and polyurethane precursors

EE0009671 – BETO WBS 1.3.1.672

4/4/2023
Advanced Algal Systems
Algae Productivity Exceeding Expectations - APEX

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Project Overview

Topic Area 2: Algae Productivity Exceeding Expectations (APEX)

Subtopic 2a: Improvements in productivity with traditional CO2 supply

Topic Area 2 should target a 20% productivity increase over their baseline productivity using strain and/or cultivation improvement approaches under both environmentally simulated and outdoor conditions

For Strain Improvement:

- ✓ Directed evolution experiments that improve stress tolerance of industrially relevant strains
- ✓ Strain improvement approaches such as genetic engineering to achieve target biochemical composition while maintaining high productivity to reduce overall costs of downstream processing.
- ✓ Breeding strategies to increase productivity of algae.

For Cultivation Improvement:

- ✓ Alteration of cultivation operations, like culturing at high salinity, to reduce contamination from pests and competition from non-production algae strains.

❖ **Extremophile algae** are grown worldwide – have been for centuries

❖ **Breeding** and selection is the foundation of Agriculture

❖ **Co-products** and by-products can drive economic viability

Participants:

Michael Burkart, PI, UCSD, Chem/Biochem

Alissa Kendall, UC Davis, Civ Engineering

Ryan Simovsky, Algenesis Materials

Project Overview

Generate high quality biomass for the production of fuels and high value polyurethane precursor (PUP) as co-products

From the FOA - Improvements in production systems must address:

- Using non-potable water sources, such as brackish or salt water reservoirs
- Limiting contamination and frequency of crop protection interventions
- Producing co-products that are recoverable and of high value

Our solutions in this project:

- ➢ Select and develop strains that can grow under high pH (> pH 10.5; this has worked well for Spirulina); bonus: alkaline media enhances direct air capture (DAC)
- ➢ Select and develop strains that can grow productively under high salt conditions
- ➢ Develop advanced genetic AND breeding technologies for extremophile strains
- ➢ Select and develop strains that can over produce high value co-products
- ➢ Develop methods of purifying as many components of value (lipids, carbohydrates, diacids, etc.) as possible from biomass and culture media

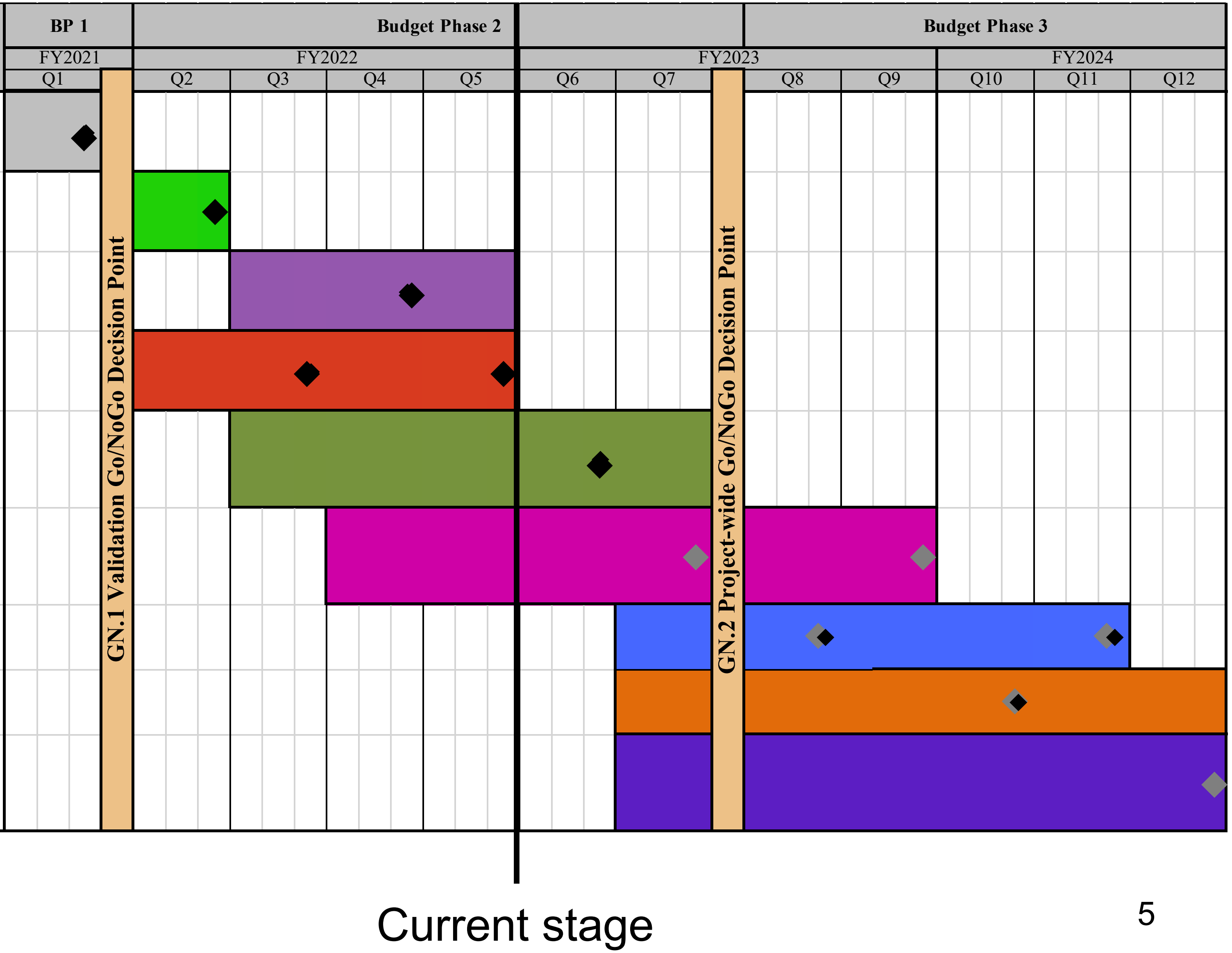
1 – Approach

Generate high quality biomass for the production of fuels and high value polyurethane precursor (PUP) as co-products

- Develop commercially relevant extremophile algae and cyanobacteria
  - Adapt/evolve to grow at ≥ pH 10.5, and 18 g/L NaCl (1/2 sea salt)
  - Adapt/evolve to high light (≥ 800 µE) and high temperatures (≥ 40°C)
  - Develop advanced genetic tools AND breeding technologies for extremophile
  - Make all tools and strains available to the algae community
- Increase algae biomass productivity and PUP yields utilizing:
  - genetic engineering, Breeding, *in vitro* evolution, high-throughput screening, process & cultivation optimization
- Develop chemical methods to convert biomass & PUPs into fuels and PU monomers
- Validate system at pilot scale: ***Generate PU products*** from cultivated algal biomass and/or PUPs
- Conduct a Techno-Economic and Life Cycle Assessment of the entire process

2 – Progress and Outcomes

Task	Title or Description	Responsible Group(s)
0	Validation	All
1	Characterize baseline composition of naturally occurring PUPs in production strains	Mayfield, Simkovsky
2	Evolve strains of algae for improved growth in high salt alkaline medium at lab-scale	Mayfield, Simkovsky
3	Develop advanced genetic tools and improve algae biomass quality using synthetic biology	Simkovsky, Mayfield, Pomeroy
4	Develop purification strategies for succinic acid, lipids, and carbohydrates from algae and cyanobacteria	Pomeroy
5	Improve algae biomass quality using the UCSD-PEAK Process	Mayfield, Simkovsky, Pomeroy
6	Chemically convert algal PUPs to polyurethanes and residual lipids to fuels	Burkart & Algenesis
7	Use polyurethane monomers to create commercially viable polyurethane products	Algenesis, BASF
8	Conduct a life cycle assessment (LCA) and techno-economic assessment (TEA) on data from pilot-scale cultivation process	Kendall



◆ Achieved milestones
◆ Next milestones

2 – Progress and Outcomes

- Validation of algal genetic tools, pilot-scale cultivation systems, and chemical processing
- Baseline PUP levels evaluated for production strains
- >20% increase in biomass productivity in high salt alkaline medium of production strains
- Generate 1+ functional vector for each production strain to be used for heterologous expression/metabolic engineering
- Generate 1+ production strain with >20% improved production of 1+ PUP over baseline

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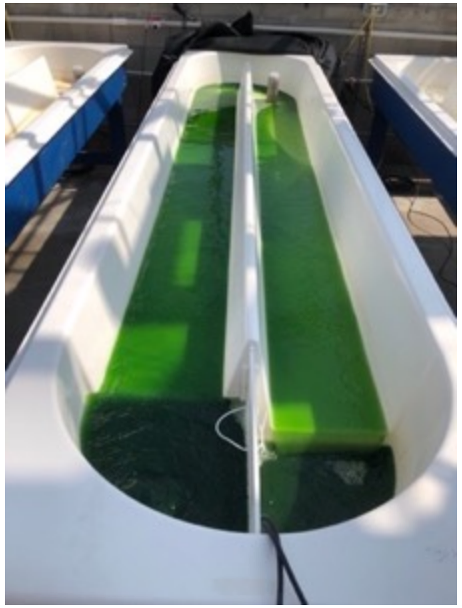
Current stage

- Demonstrated capacity to purify 1+ PUP from a biological sample to >90% purity
- Demonstrate >50% increase in biomass and/or PUP productivity after 2+ rounds of PEAK process
- Evaluated growth and yields of at least one improved strain at pilot scale in an outdoor greenhouse
- Demonstrate conversion of algal-derived PUPs to PUs at gram scale
- Demonstrate conversion of algal-derived PUPs to PUs and residual lipids to fuel precursors
- Generate a >50% algae-based PU product using PUPs produced from Task 7
- Economic assessment performed to evaluate the costs and environmental effects of a pilot-scale PUP bioproduction facility generating both PUPs and biomass for biofuels and PU monomer

2 – Progress and Outcomes

Extremophile Cyanobacterial (11901) Cultivation

Recovery after adaptation
to open ponds in greenhouse

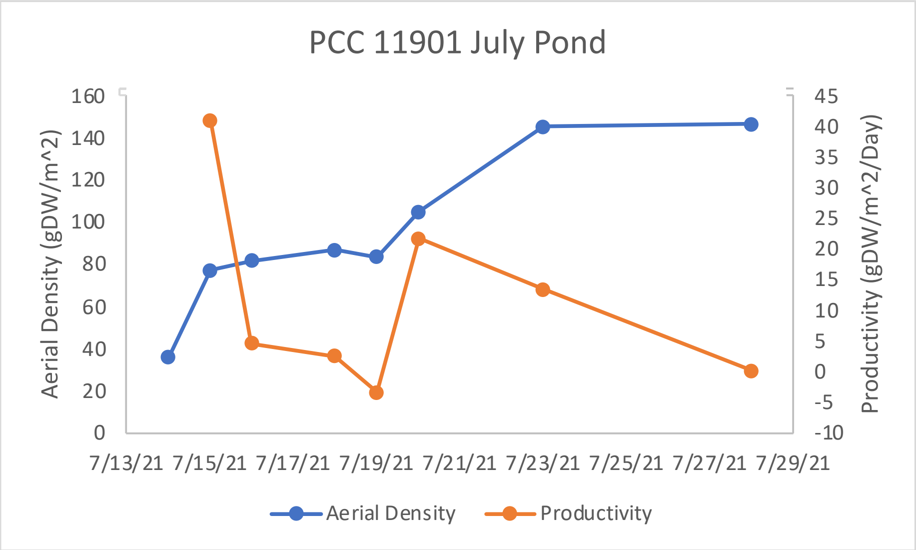


Volume 80 L
High pH & High salt

Sustained growth in ponds under extremophile conditions for > 4 months

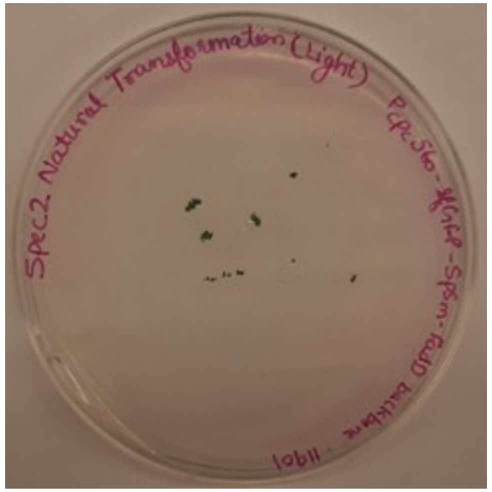
Biomass Saturation > 4.5 g/L (DAC)

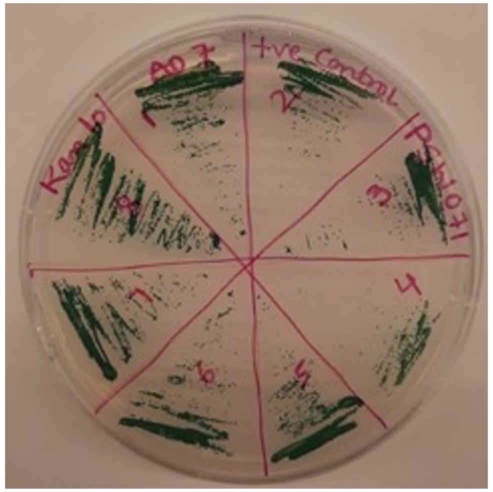
Average Productivity: 11.4 $gDW/m^2/day$
Maximal Productivity: > 40 $gDW/m^2/day$

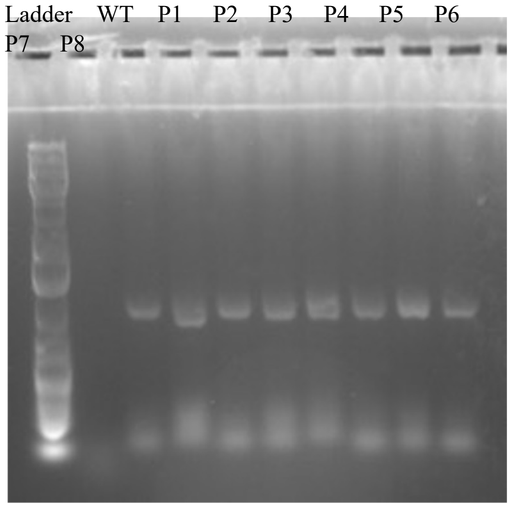


2 – Progress and Outcomes

Extremophile Cyanobacteria tool development





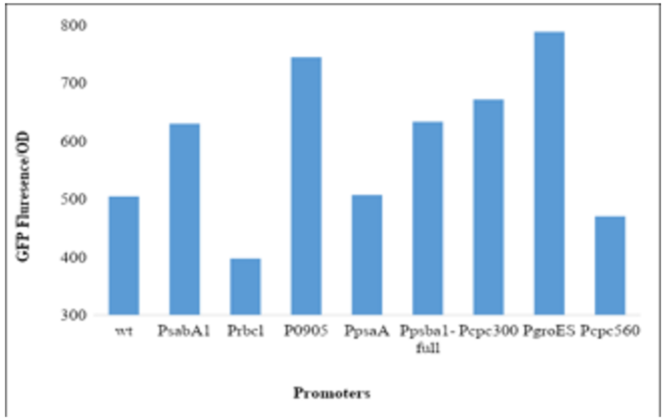


Colonies appeared on antibiotic selection

Restreaked 3-times on antibiotic plate

Confirmation of transformants through PCR

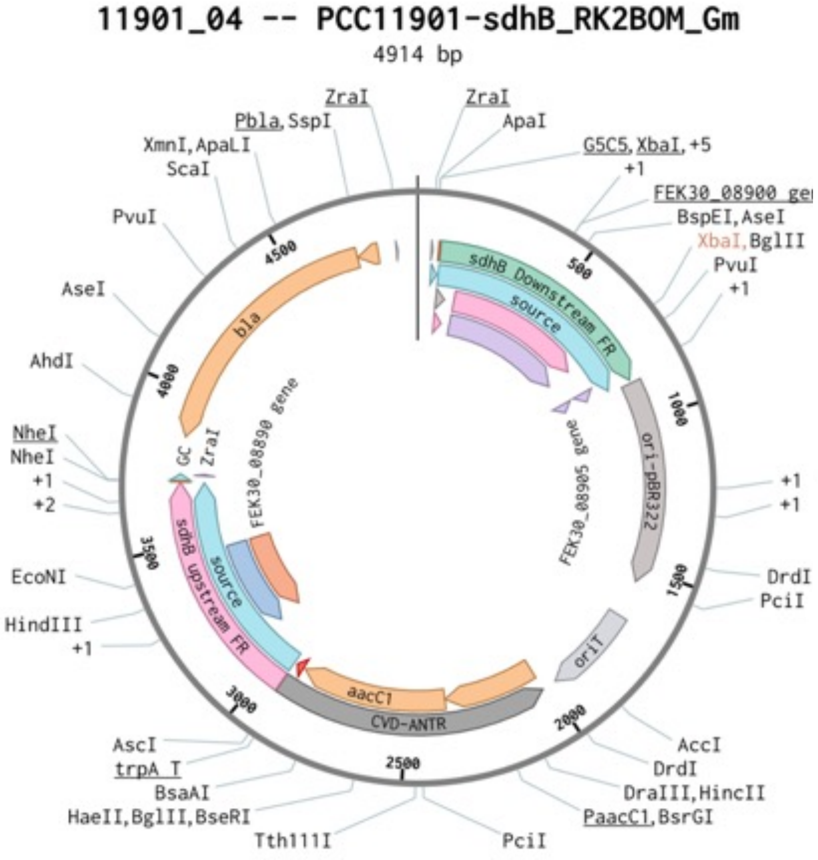
Transformations are efficient and easily confirmed using freshwater cyanobacteria vector

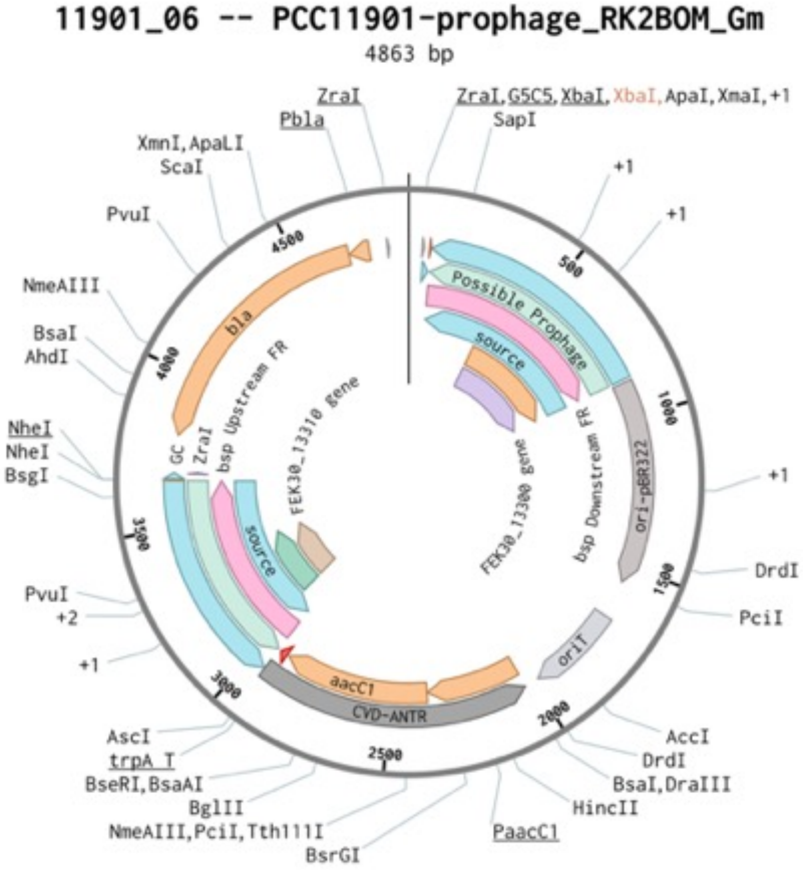


Promoters derived from freshwater strains show poor GFP expression in marine extremophile. Currently developing promoters from marine strains and cyanophage.

2 – Progress and Outcomes

Extremophile Cyanobacteria Genetic Tool Development





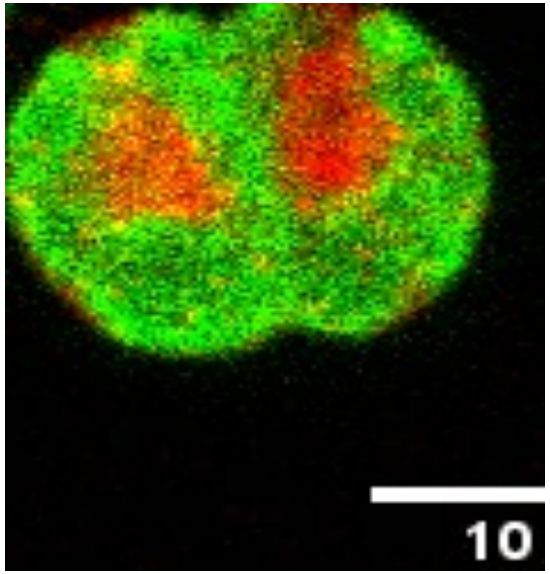
11901 KO Integration Backbones Developed

- fadD (fatty acid recycling)
- acsA (acetate -> acetyl-CoA)
- psbA2 (Photosystem II protein D)
- sdhB (succinate -> fumarate) (2 versions made)
- Prophage baseplate (possible neutral site)
- idi (terpenoid pathway)
- g6pd (OPP)

2 – Progress and Outcomes

Extremophile Green Algae – *Chlamydomonas sp.* 402

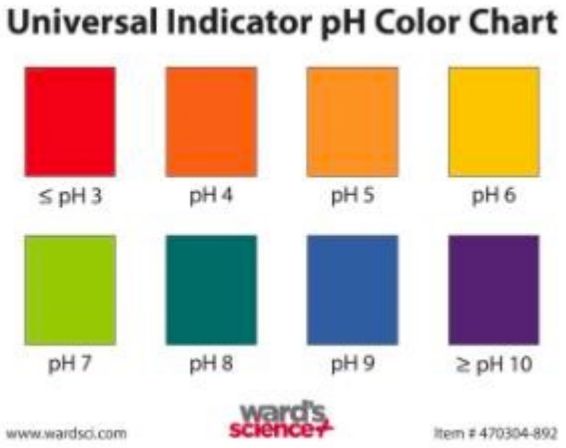
- Complete genome sequenced and annotated
  - 121 mbp, ~17000 genes,
- Complete metabolic profile
  - 30% starch from DCW, without optimization
  - **Secreted PUPs – 3 Hydroxy proprionic acid (3HP) Bioplastic**
- Capacity to grow in brackish water and high pH (>10.5)
- Complete suite of working vectors
- ***Both mating types identified and functional 402/403***

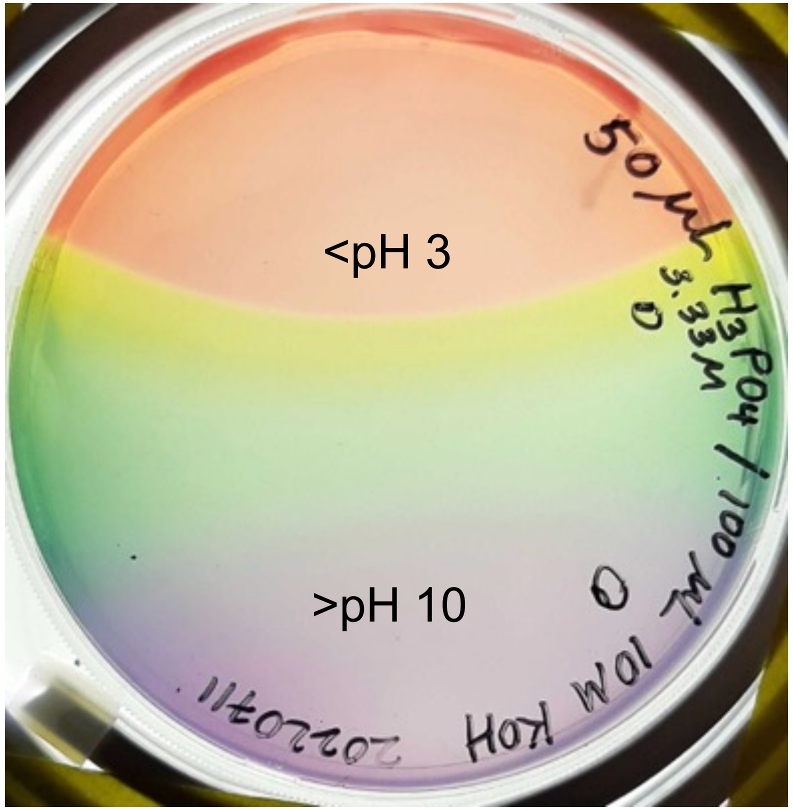


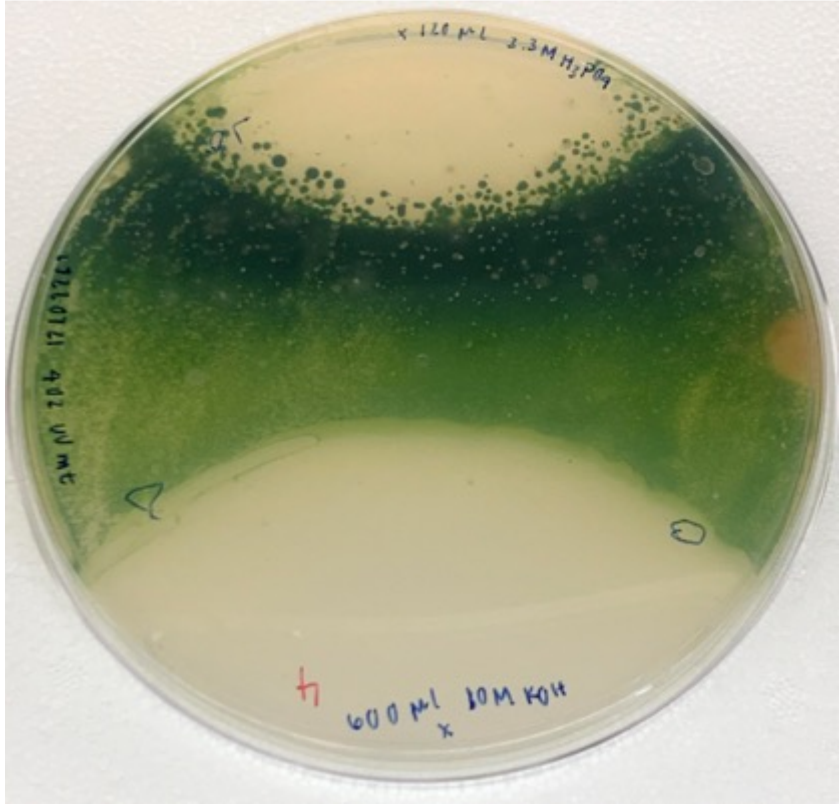
2 – Progress and Outcomes

In vitro evolution utilizing both Breeding and Mutagenesis

- Screening plate
  - Gradient pH

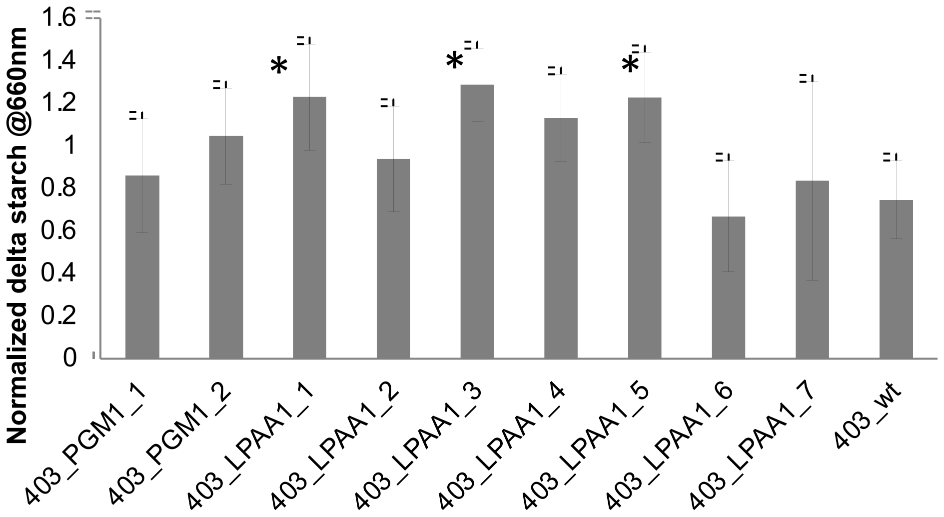




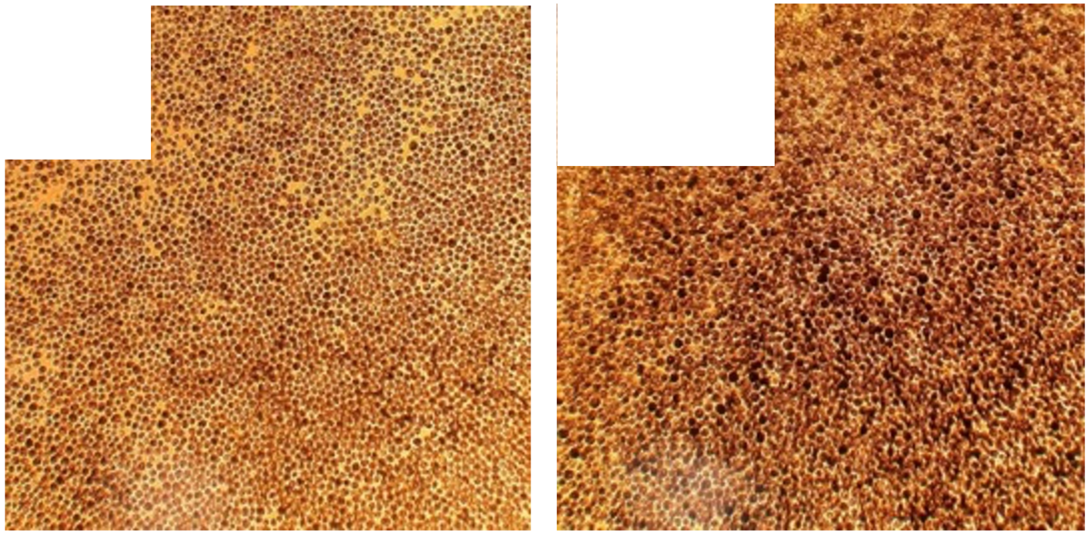


2 – Progress and Outcomes

Starch overaccumulation in 403



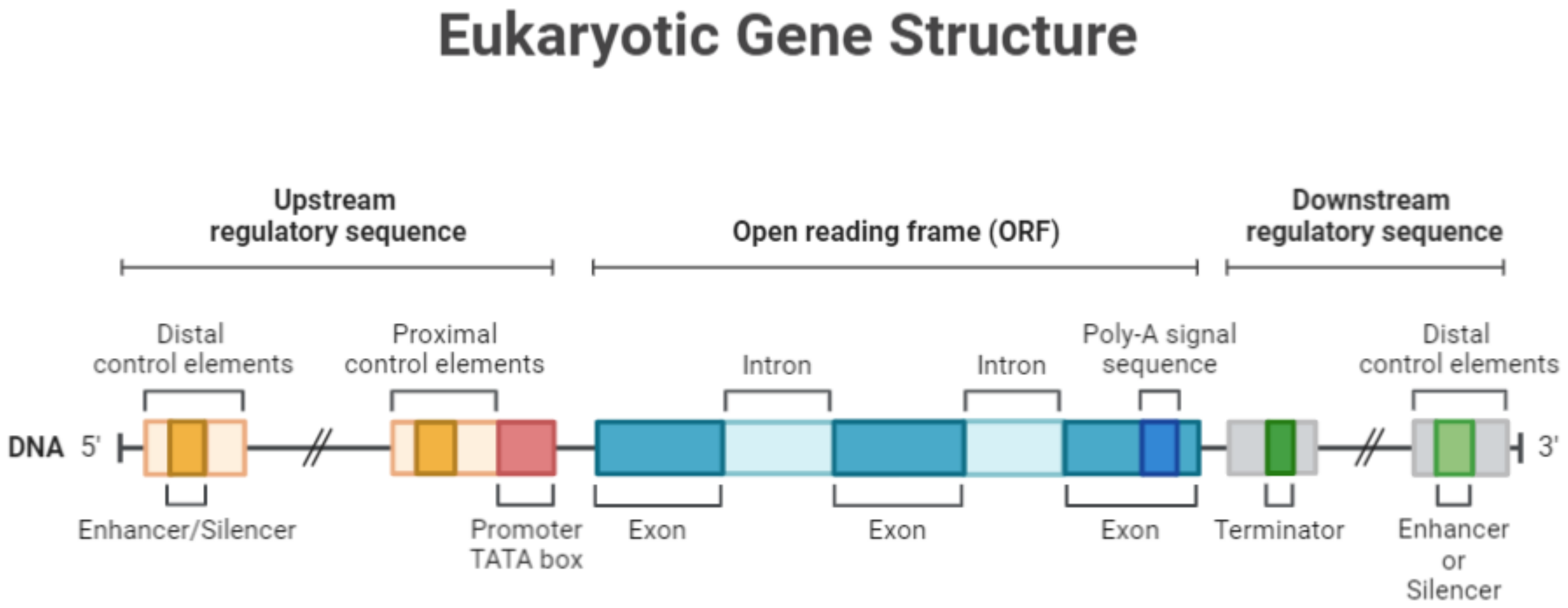
Cells starved for 24h in HSM-S
* Statistically significant, alpha 0.05



Cells starved for 24h in HSM-S

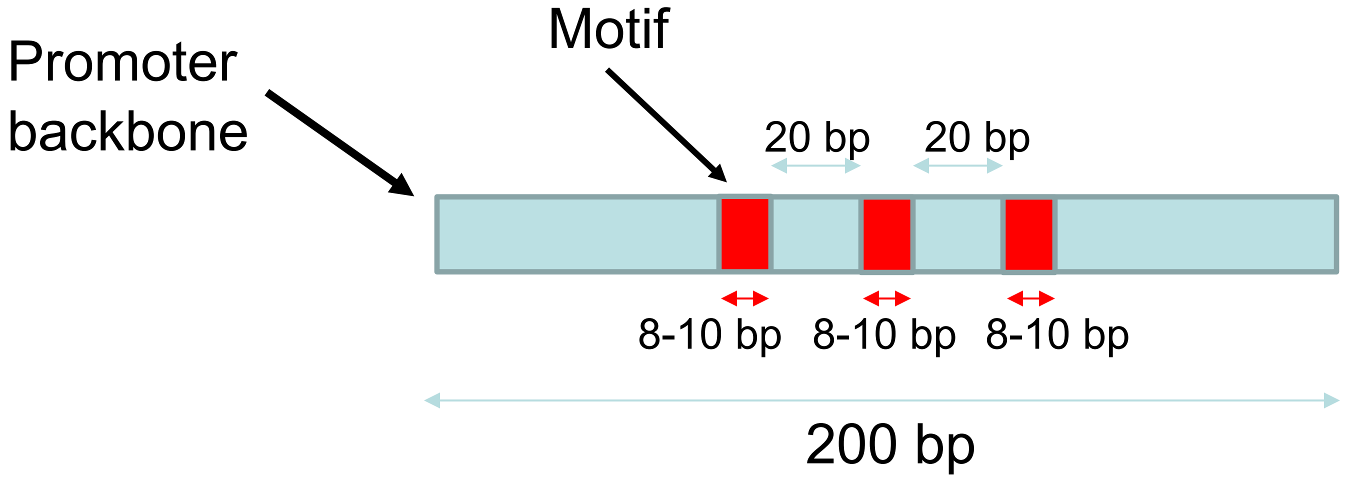
2 – Progress and Outcomes

Extremophile green algae lack strong algal promoters



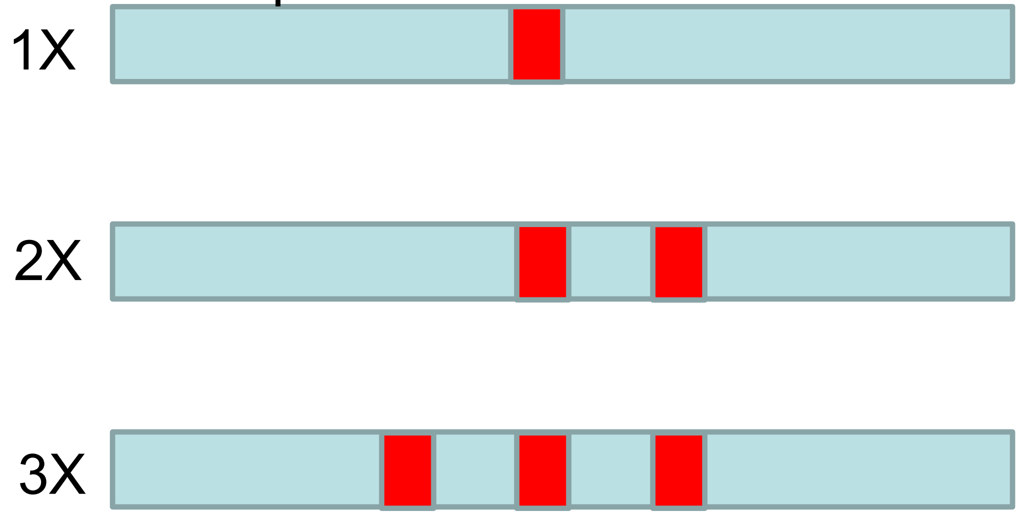
2 – Progress and Outcomes

402 synthetic promoter design



Two pools generated:
- **Pool 1: ~ 700 different motifs, ~ 2100 different promoters**
- **Pool 2: ~ 800 different motifs, ~ 2400 different promoters**

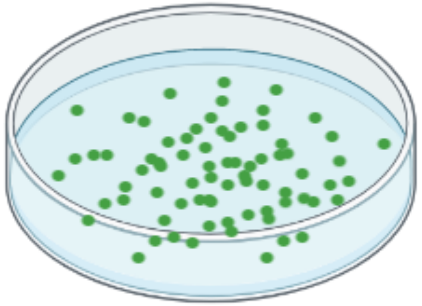
Promoter variations, same motif with varying number of copies



2 – Progress and Outcomes

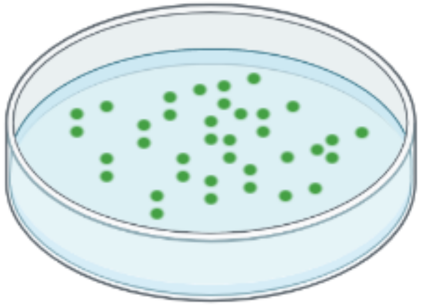
SynPro selection design

Synthetic Promoter



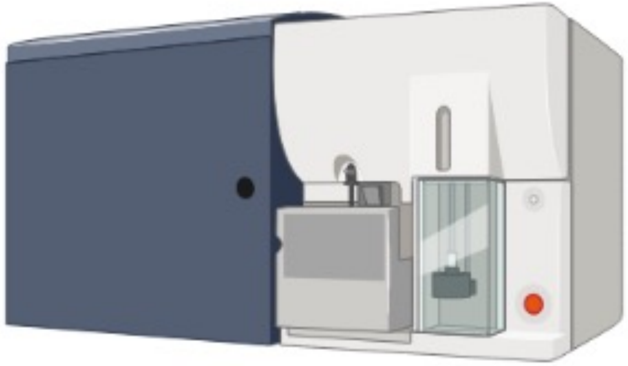
Hygromicin agar plate

Control for promoter distribution within the DNA library



Hygromicin and Zeocin agar plate

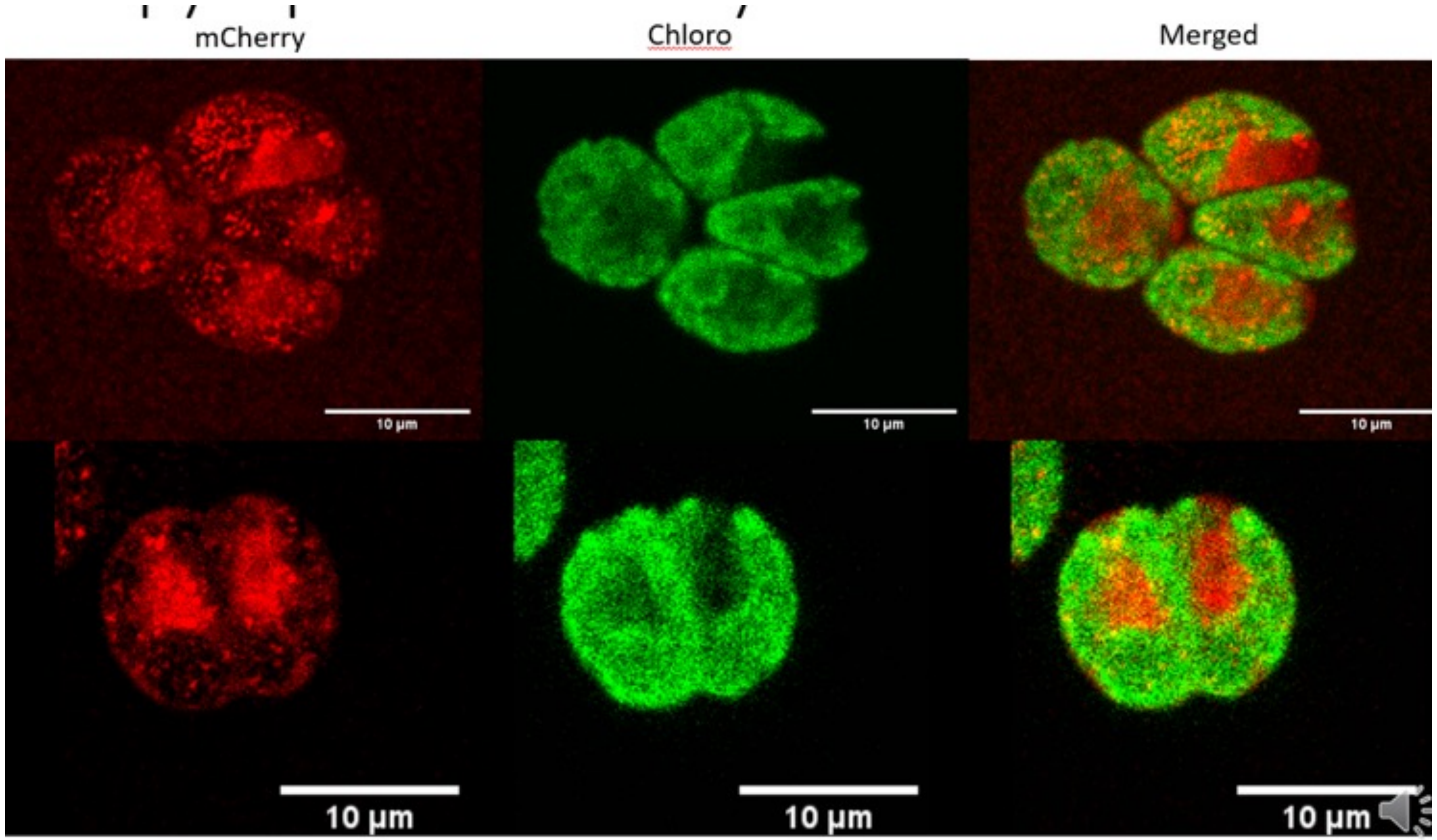
Synthetic promoter activity test



Synthetic promoter strength measurement

2 – Progress and Outcomes

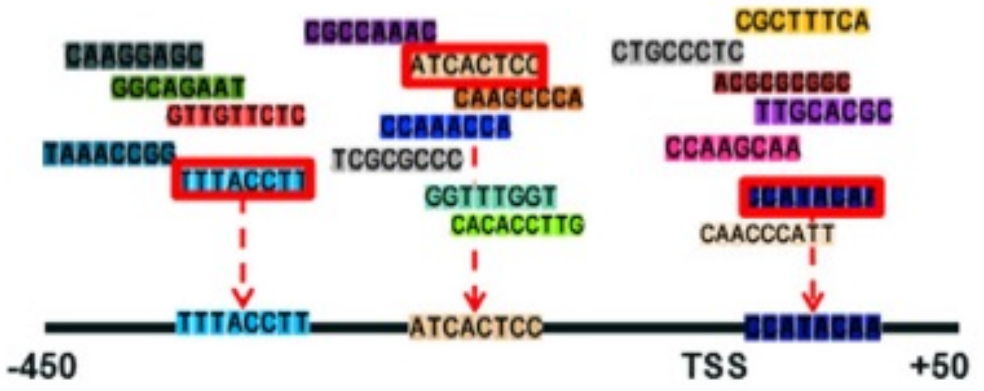
Expression of mCherry using SynPro in *Chlamydomonas* 402

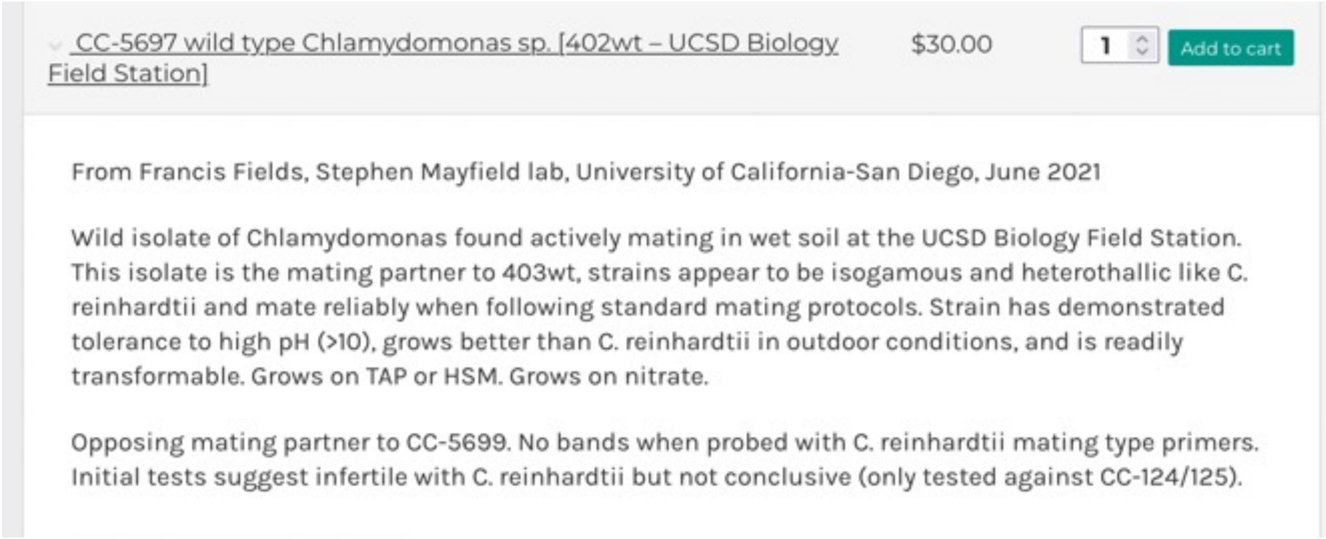


2 – Progress and Outcomes

Developing a Promoter Element Catalog for 402

- Catalog of promoter elements with varying transcriptional activities under different environmental conditions
- Allow for the creation of synthetic promoters that can respond to different conditions
- Stronger recombinant product yields, and tunable metabolic engineering
- Made available to the entire community along with 402/403




CC-5697 wild type Chlamydomonas sp. [402wt – UCSD Biology Field Station] $30.00 1 Add to cart

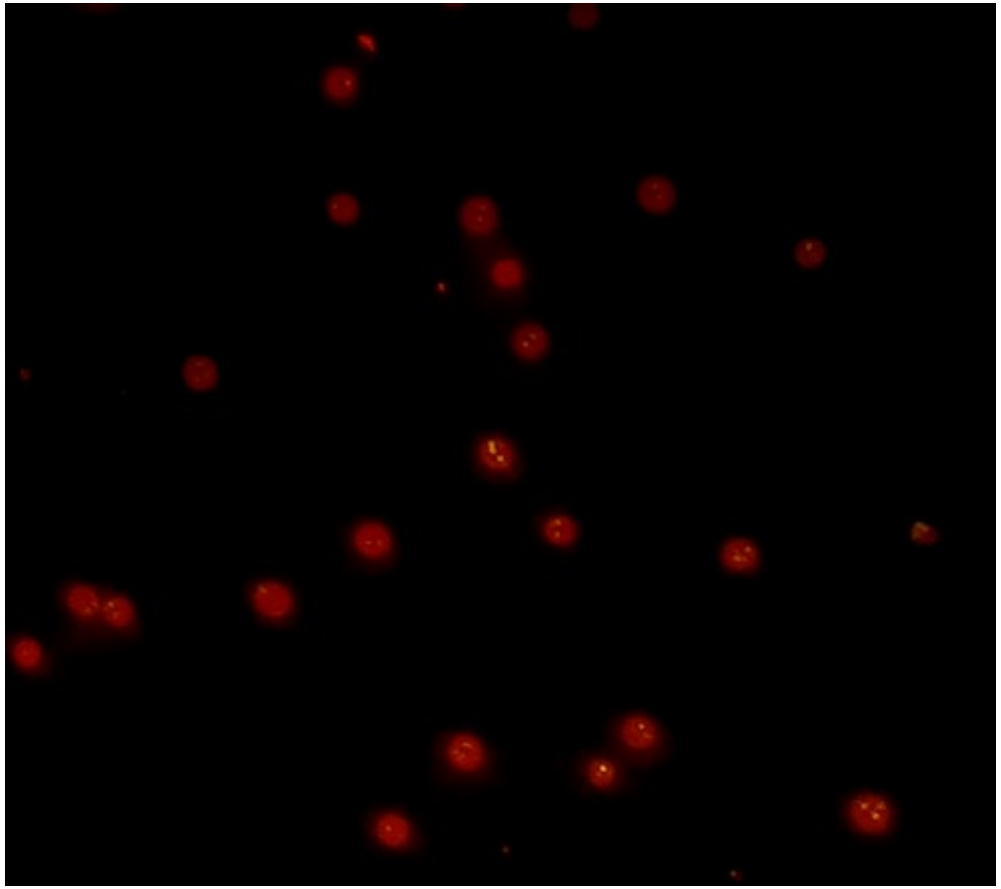
From Francis Fields, Stephen Mayfield lab, University of California-San Diego, June 2021

Wild isolate of Chlamydomonas found actively mating in wet soil at the UCSD Biology Field Station. This isolate is the mating partner to 403wt, strains appear to be isogamous and heterothallic like C. reinhardtii and mate reliably when following standard mating protocols. Strain has demonstrated tolerance to high pH (>10), grows better than C. reinhardtii in outdoor conditions, and is readily transformable. Grows on TAP or HSM. Grows on nitrate.

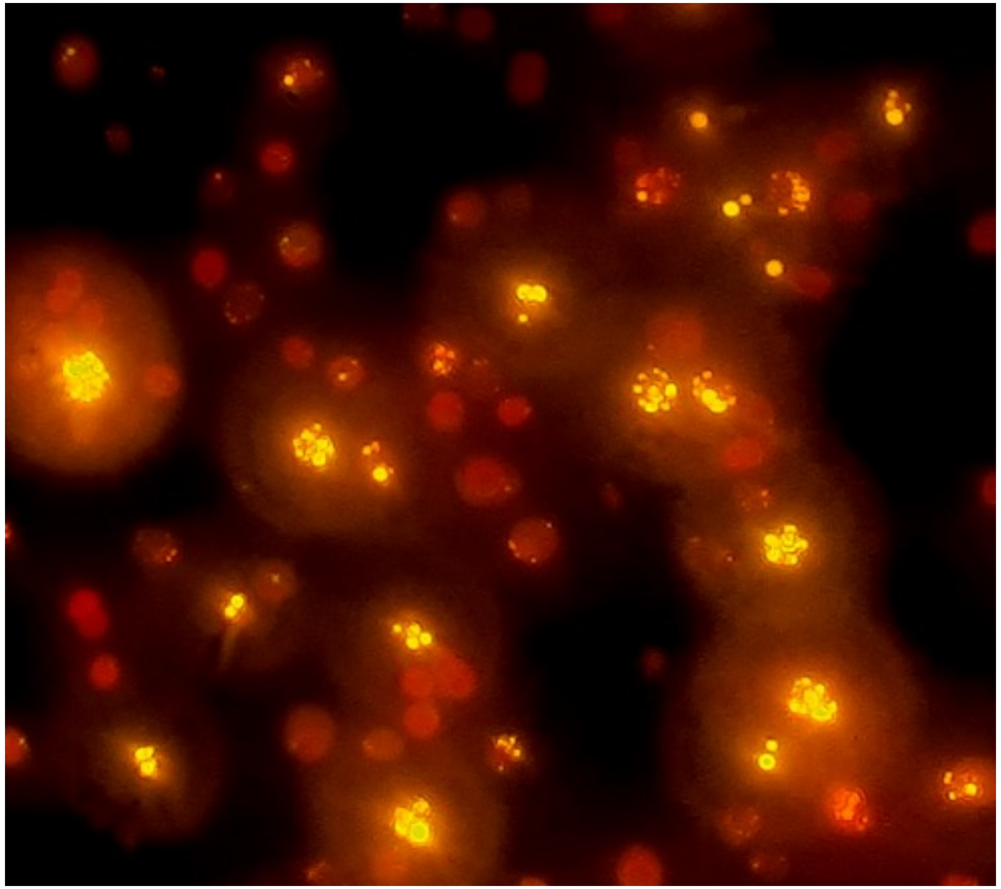
Opposing mating partner to CC-5699. No bands when probed with C. reinhardtii mating type primers. Initial tests suggest infertile with C. reinhardtii but not conclusive (only tested against CC-124/125).

2 – Progress and Outcomes

Using Synthetic TFs to drive lipid accumulation in extremophile algae



403 - Wildtype
HSM – N, 24h

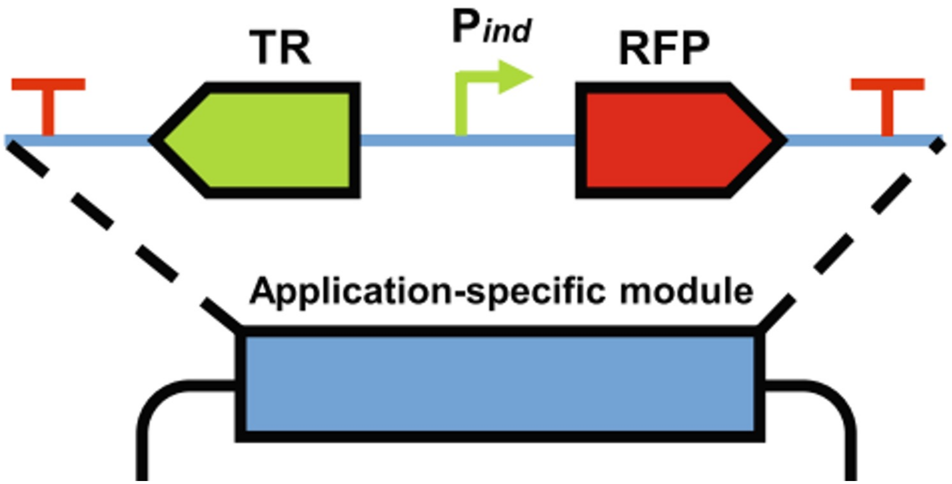


403 - DOF
HSM – N, 24h

2 – Progress and Outcomes

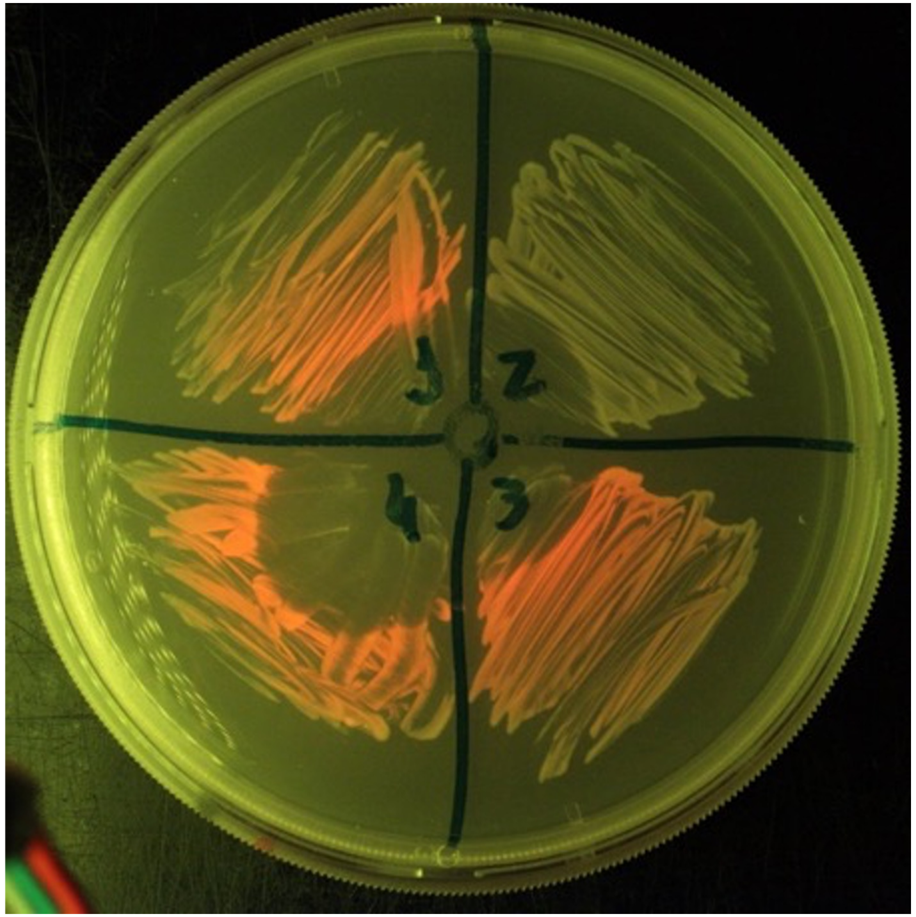
3HP accumulation - Biosensor

- Biosensor to 3 Hydroxy Propionic Acid from *Pseudomonas*



Hanko, E. K. R., Minton, N. P., & Malys, N. (2017). Characterisation of a 3-hydroxypropionic acid-inducible system from Pseudomonas putida for orthogonal gene expression control in Escherichia coli and Cupriavidus necator. *Scientific Reports*, *7*(1), 1–13. https://doi.org/10.1038/s41598-017-01850-w

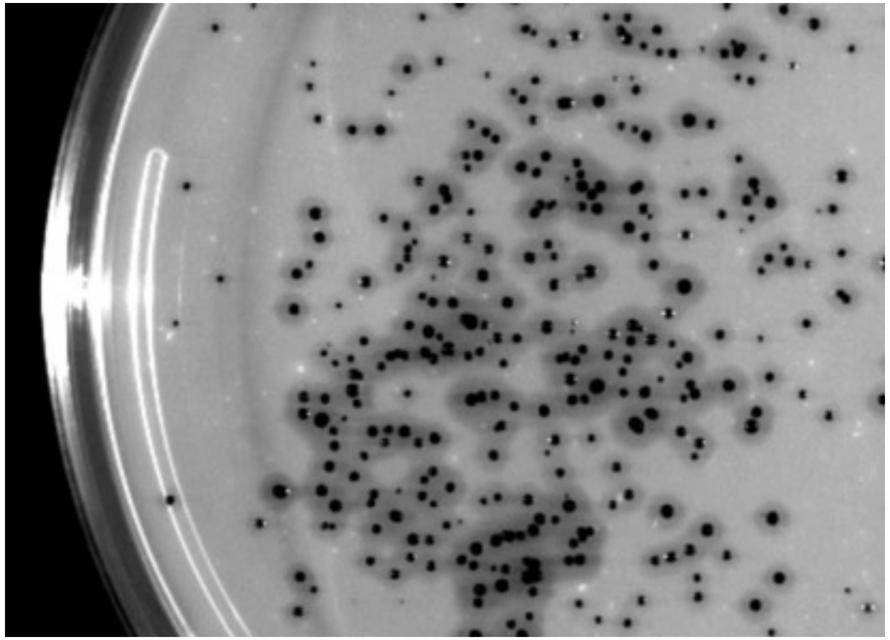
Not one of our proposed tasks but potentially very valuable



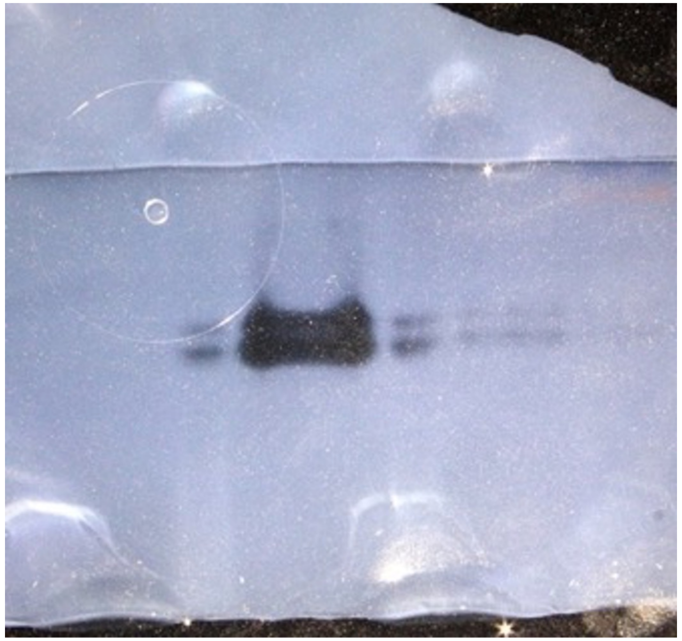
4 assembled vectors – 3 responding to 3HP placed in the middle of the plate. (#2 had a missing part)

2 – Progress and Outcomes

PHL7 – PET Degrading Enzyme expressed in green algae



Transformed algae secreting PHL7
on plate containing polyurethane



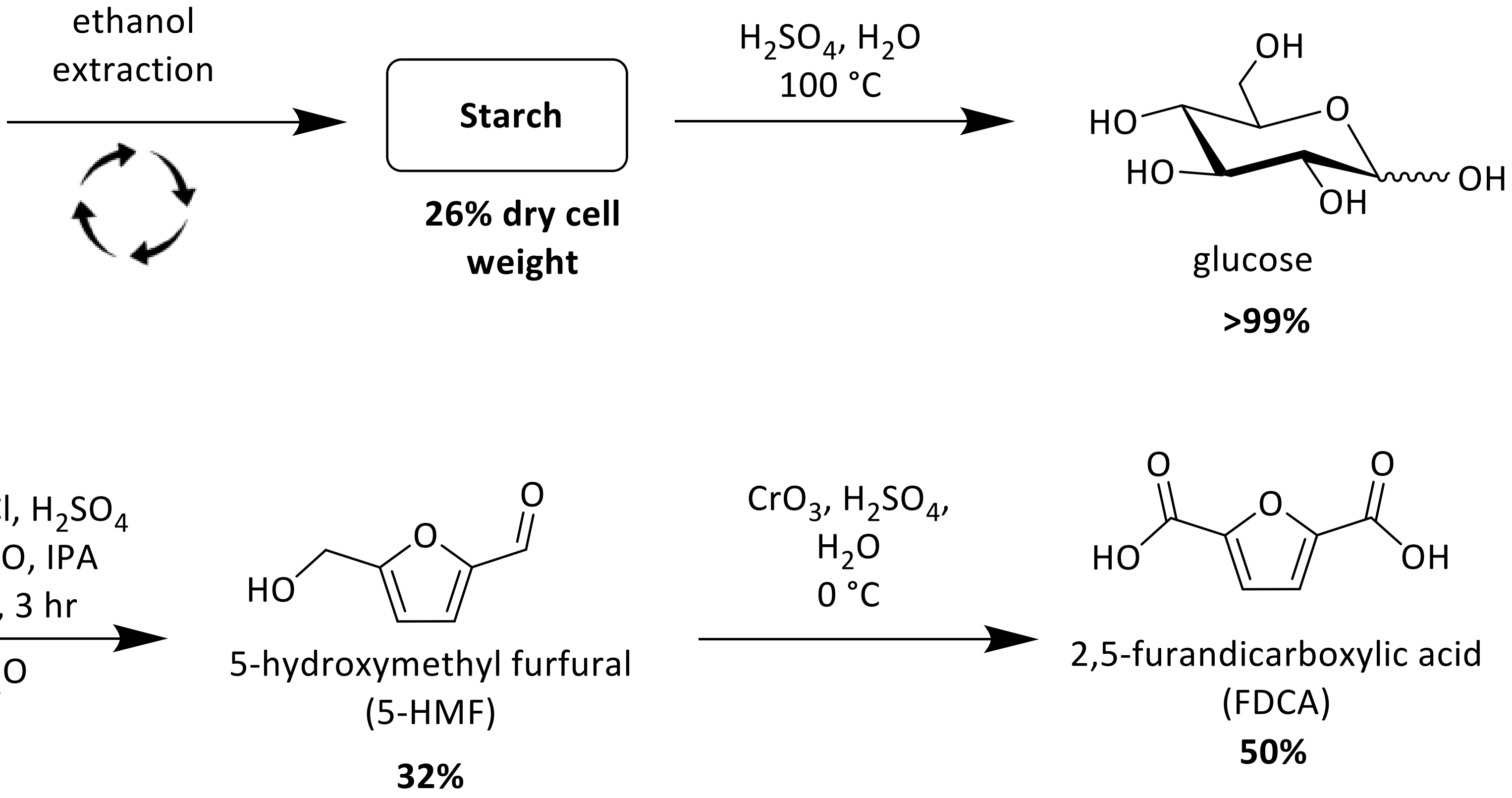
Purified PHL7 degrading PU
In a PAGE plate

Not one of our proposed tasks
but potentially very valuable

2 – Progress and Outcomes

Downstream process of algae biomass to plastic precursors – Algenesis collaboration

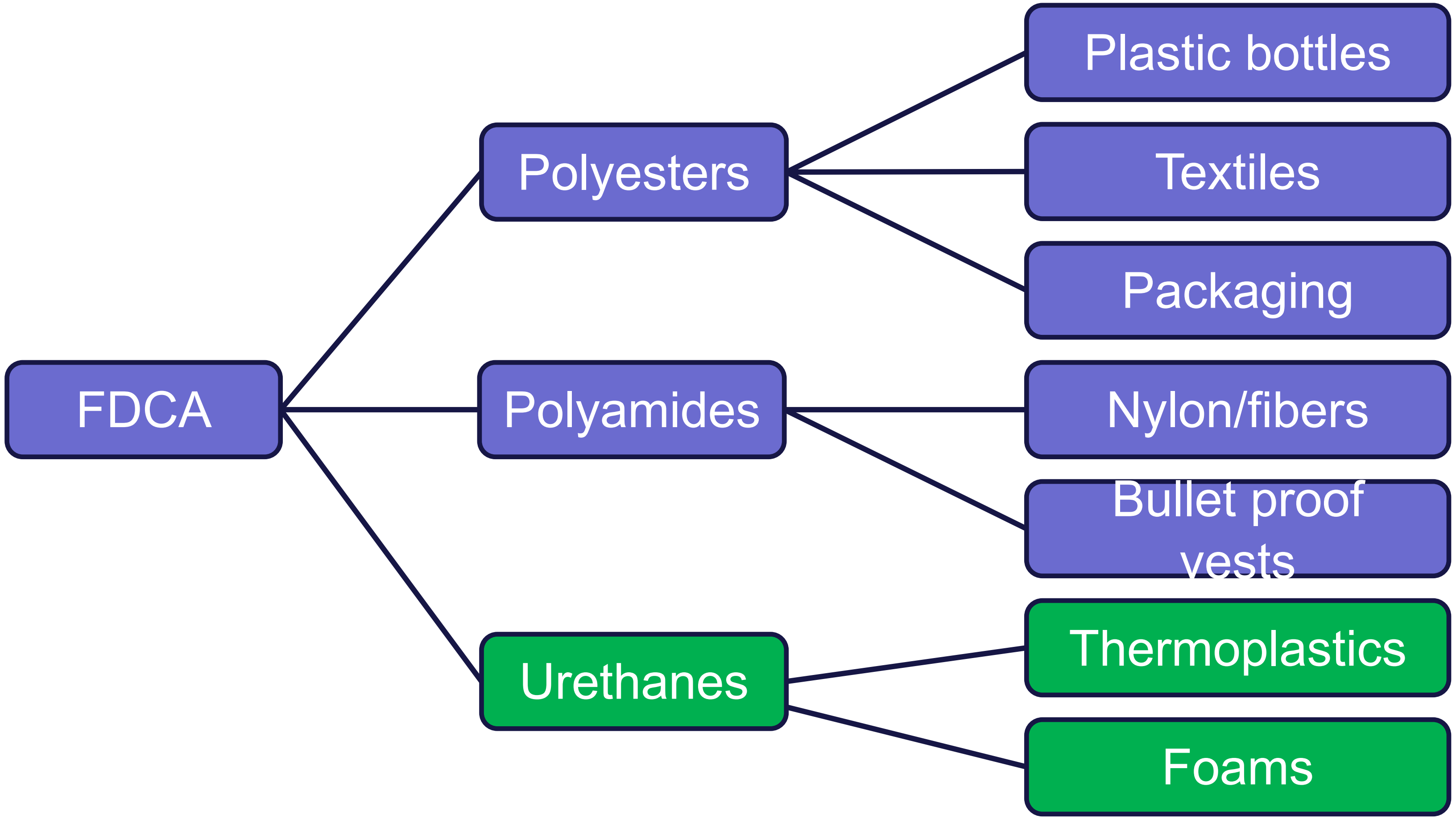
Chlamydomonas "402"
algae biomass



Pagán-Torres, Y. J.; et al. Production of 5-Hydroxymethylfurfural from Glucose Using a Combination of Lewis and Brønsted Acid Catalysts in Water in a Biphasic Reactor with an Alkylphenol Solvent. *ACS Catalysis* **2012**, *2* (6), 930–934.
Ramli, R. N.; et al. Extraction and Characterization of Starch from Microalgae and Comparison with Commercial Corn Starch. *IOP Conference Series: Materials Science and Engineering* **2020**, *716* (1), 012012.

2 – Progress and Outcomes

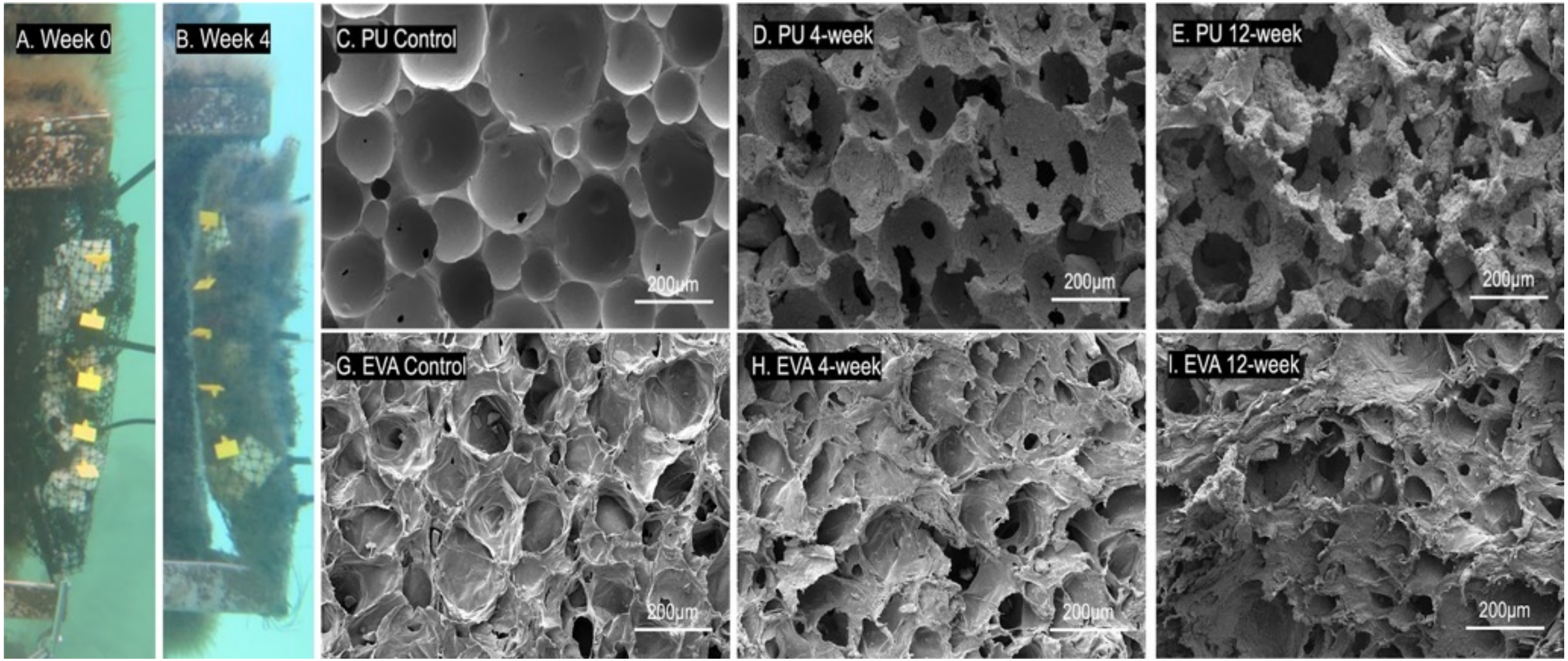
Potential uses for FDCA polymers



great_watson-int. FDCA & Next Generation Polyester. https://www.fcad.com/fdca-next-generation-polyester/ (accessed Mar 20, 2023).
Post, C.; Maniar, D.; Voet, V. S.; Folkersma, R.; Loos, K. Biobased 2,5-Bis(Hydroxymethyl)Furan as a Versatile Building Block for Sustainable Polymeric Materials. *ACS Omega* **2023**, *8* (10), 8991–9003.

2 – Progress and Outcomes

Next BP – Make PU products and test them for both physical metric and biodegradation



3 – Impact

- We have isolated, sequenced, characterized, and develop molecular and breeding tools for an extremophile green algae for use as bio-products production platform
- We have an extremophile cyanobacteria with some of the highest biomass productivity ever reported that we are also developing as a bio-products production platform
- ➢ We will make these strains and all of the developed tools available to the algae community without reservation
- To demonstrate the utility of these strains for commercial purposes, we are engineering these for the production of polyurethane precursors (PUPs), and using these PUPs to make biodegradable polyurethane products – *with commercial partners*

Summary

- World wide extremophile algae have been grown for thousands of years. - in very unsophisticated ponds - as supplemental food sources, and continue in that role today
- Spirulina, an extremophile cyanobacteria, is grown outdoors for months on end at reasonable costs, all over the world
- Breeding – the mating and selection of progeny - allowed agriculture to develop – and that is the main reason 8 billion people can live on this planet
- Strains and publications are great – Products change the world

➢ We have identified and are developing ***extremophile algae*** that can be genetically transformed AND breed and selected for improved traits

➢ We have made novel algae based polyurethane foam ***products*** using algae PUPs

➢ Biodegradable plastics may be our only hope to cure ocean plastic pollution

Quad Chart Overview

Timeline

- *Start of Project: 10/1/2021 (3/01/22)*
- *End of Project: 9/30/2024*

	FY22 Costed	Total Award
DOE Funding	*(3/01/2022 – 9/30/2022)* $475,476	*$3,200,000*
Project Cost Share *	$119,200	$800,000

TRL at Project Start: 1
TRL at Project End: 6

*Only fill out if applicable.

Project Goal

Combine genetic engineering, traditional breeding, high-throughput screening, chemical processing, and cultivation technologies that have been developed over the last 10 years at the California Center for Algae Biotechnology, to generate high quality biomass for the production of fuels and high value polyurethane (PU) co-products from commercial strains of algae and cyanobacteria.

End of Project Milestone

The primary goal of this project is to develop commercial algae strains and a suite of synthetic biology, breeding, and directed evolution tool, that can be used to enable these strains to produce significant amounts of high value PU precursors in a high salt and high pH raceway pond. Developing these strains and tools will demonstrate the utility of algae as a platform for the production of sustainable and recyclable polymers, as well as enable robust economic production of algae biofuels. We will demonstrate the success of this program by generating sufficient PU precursors at the 100s of grams to kilogram scales from these strains to generate a commercially relevant PU product that has > 50% algae-derived bio-content, while processing the remaining biomass into biofuels to enable a complete economic analysis of the production process.

Funding Mechanism

- DE-FOA-0002423 (2021) Topic 2a

Project Partners*

- Algenesis
- BASF

Additional Slides

Responses to Previous Reviewers' Comments

- During the initial verification, the project team should explicitly identify the baseline values, supporting data for these values, and strategies for achieving the FOA required metrics
- Areal Productivity
  - 20% improvement in baseline areal productivity under simulated conditions
  - 20% improvement in baseline areal productivity in outdoor environment
- Biomass Quality
  - Biomass quality translating to at least 85 gallons of gasoline equivalent per ton algae biomass for minimum target
  - Biomass quality translating to at least 85 gallons of gasoline equivalent per ton algae biomass for stretch target

Responses to Previous Reviewers' Comments

Cyanobacteria *Synechococcus sp* PCC 11901

- **Fastest growing** cyanobacteria published to date (doubling time ~2 hr under optimized conditions) can accumulate up to 33 gDCW/L; ~1.2 gDW/L/Day
- **Extremophile**: halotolerant (up to 10% NaCl; ~3x sea water), high temp tolerant (optimal = 38°C, tolerates up to 43°C), high light tolerant (optimal = 660 μE)
- **Naturally transformable;** Sequenced genome**;** demonstrated tools for accumulating up to 6mM free fatty acids in supernatants or cell extracts in 7 days

Chlamydomonas sp (402) was isolated from high pH algae trap located at UCSD Field Station

- **Fast growing** green algae with a baseline10 g/m2/day
- **Has mating type plus and minus and inducible mating**
- **Extremophile**: halotolerant (up to 1/2X sea water), high temp tolerant (up to 43°C), high light tolerant
- **Transformable; Sequenced genome;** demonstrated tools for accumulating of recombinant proteins

Publications, Patents, Presentations, Awards, and Commercialization

- None yet – many coming soon!