DOE Bioenergy Technologies Office (BETO)  
2021 Project Peer Review

Developing Advanced Genetic and Synthetic Biology Tools for Improved Algae Productivity

March 22, 2021  
Technology Area Session

Stephen Mayfield  
University of California, San Diego

This presentation does not contain any proprietary, confidential, or otherwise restricted information
Project Overview

• We will develop advanced genetic tools, high-throughput screening methods, and *breeding strategies* for industrial strains of green algae and cyanobacteria

• We will develop these tools by engineering recombinant proteins and polymer precursors as high value co-products in cyanobacteria and green algae, and then transfer these tools into new industrial strains

• We will grow industrially relevant strains under field conditions to demonstrate the utility of the developed tools – PEAK Challenge

• We will perform LCA and TEA of demonstrated products at green house scale

• Key Industrial Partners - Algenesis (polymers), Triton Health & Nutrition (proteins), Global Algae Innovations (GAI - engineered systems)
1 – Management

- **Stephen Mayfield – (PI) UCSD**
  - Develop Molecular Genetic, high throughput screening and breeding tools for Green Algae

- **Michael Burkart - UCSD**
  - Extraction and conversion of lipids to biofuels and polymer precursor co-products

- **Ryan Simkovsky - UCSD**
  - Develop Molecular Genetic tools and engineer protein and polymer precursor production in cyanobacteria

- **Frank Fields - UCSD**
  - Algae Biomass Production at Green House Scale – PEAK Challenge

- **Alissa Kendall - UCD**
  - Life Cycle Assessment and Techno Economic Modeling of biofuel and co-products

- **Peter Morgan - Algenesis**
  - Production of commercial polyurethane products using UCSD generate monomers

- **Dave Hazlebeck - GAI**
  - Integration of engineered production and harvesting system at pilot scale
2 – Approach

Strain Development Process

Synthetic Biology Paradigm

PEAK – Strain Development Process
2 – Approach
Production process and outcomes
3 – Impact

**Protein**

Triton – OPN – PEAK Challenge
Covid Spike Protein Antigen
Hochland SE – Milk Proteins

**Polyurethanes**

Algenesis – Polyols – Peak Challenge
Reef – flip flops
BASF – isocyanates
Pepsi – food packaging
Callaway – foam padding …
4 – Progress and Outcomes

Project Goals:
1) The primary goals of this project are to develop the advanced genetic tools, breeding, and high-throughput technologies that will advance the entire algae biotechnology field, and enable economic viability of algal biofuels ... through production of high value co-products.

2) Apply these tools to commercial production strains of algae and cyanobacteria

3) To advance the technology from their present baseline state (TRL3 - proof of concept), to demonstration at the pilot scale with our commercial partners (TRL6 - prototype demonstration in a relevant environment)
## 4 - Progress and Outcomes

<table>
<thead>
<tr>
<th>Objective</th>
<th>Description</th>
<th>Deliverable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Objective 1</td>
<td>Develop advanced genetic tools for improved nuclear transgene expression and advanced metabolic engineering to enable economic production of biofuels from algae.</td>
<td>A process for designing synthetic biology tools for industrial species of green algae. PEAK Process</td>
</tr>
<tr>
<td>Objective 2</td>
<td>Improve expression and secretion of recombinant proteins as co-products to improve the economics of biofuel production.</td>
<td>Validated sequences and vector design methods to increase recombinant protein expression from industrial strains.</td>
</tr>
<tr>
<td>Objective 3</td>
<td>Establish a rapid high throughput screening method for strains with improved protein production and secretion abilities.</td>
<td>Rigorous methods for rapidly isolating production strains from genetically diverse pools generated by mating or mutagenesis.</td>
</tr>
<tr>
<td>Objective 4</td>
<td>Develop genetic tools and genome editing strategies to enable metabolic engineering of commercially viable species of cyanobacteria.</td>
<td>A process for developing synthetic biology tools for novel cyanobacteria with industrial production potential that currently lack genetic tools</td>
</tr>
<tr>
<td>Objective 5</td>
<td>Develop a suite of highly controlled gene expression tools for metabolic engineering of cyanobacteria to produce diacids and diols as polyurethane precursors.</td>
<td>Methods for engineering high value co-products from cyanobacteria. Demonstrate by producing and extracting diacids and diols for polyurethane production</td>
</tr>
<tr>
<td>Objective 6</td>
<td>Develop improved targeted genome editing and transgene delivery methods for green algae to accelerate strain engineering.</td>
<td>Improved methods for delivering synthetic biology tools to green algae genomes.</td>
</tr>
<tr>
<td>Objective 7</td>
<td>Develop non transgenic improvement strategies using breeding, high throughput screening, and mutagenesis to develop new production strains.</td>
<td>A process for production strain improvement using tradition breeding strategy.</td>
</tr>
<tr>
<td>Objective 8</td>
<td>Develop genetic tools for commercial strains, based on learning from previous objectives, to demonstrate rapid adaptation of genetic tools to new strains. Covid-19 Spike protein</td>
<td>Generation of new genetic tools for increasing the economic viability of biofuel production for novel strains through generation of high value co products.</td>
</tr>
<tr>
<td>Objective 9</td>
<td>Grow unmodified and genetically modified strains under field conditions to determine impact of genetic modifications on biomass productivity and product yield.</td>
<td>Key data on biomass productivity and the viability of using co products to improve the economic viability of biofuel production.</td>
</tr>
<tr>
<td>Objective 10</td>
<td>Conduct a life cycle assessment and techno economic assessment (TEA) on GM and non-GM strain under outdoor cultivation conditions. Task 11</td>
<td>TEA/LCA and Socio-economic reports comparing unmodified and genetically modified strains under field conditions</td>
</tr>
</tbody>
</table>
4 - Progress and Outcomes
Can we combine synthetic biology, breeding, and in vitro evolution into a single process

A
Average fold change of soluble GFP relative to P1GFP

Synthetic Biology
Breeding
Mutagenesis

B
OPN final strain
(15x improvement from primary transformant)
4 - Progress and Outcomes

Can we translate these tools to develop new strain - rapidly

Binding to human ACE2 protein
## 4 - Progress and Outcomes

**New commercial candidate strain**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <strong>Within <em>Chlamydomonas</em> genus</strong></td>
<td></td>
</tr>
<tr>
<td>2. <strong>Capable of mating/breeding</strong></td>
<td>3. <strong>Transformable with Recombinant protein accumulation</strong></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Culture of sexually active gametes</td>
<td>B. Mating of gametes</td>
</tr>
<tr>
<td>C. Formation of a pellicle and harvesting of zygotes</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Expression of GFP</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>4. <strong>More productive than <em>C. reinhardtii</em> in alkaline ponds</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Cultured outdoors in both San Diego and Hawaii, ranging from 10-15 g/m²/d High Salt / pH 10.5</td>
<td></td>
</tr>
</tbody>
</table>
4 - Progress and Outcomes
Development of modular synthetic biology tools for commercial cyanobacteria

Rapidly-generated cloning and expression vectors for diverse cyanobacteria strains
- RBD
- sfGFP-tagged proteins
- Pimelic acid pathway
- Succinic acid pathway
- etc.

Natural NS expression, lower is better

>4x improvement over standard synthetic promoter (PconII)
4 - Progress and Outcomes
Developing high value polymer co-products

- Algae biomass
- Purification
- Free fatty acids
  - Omega-3 Fatty Acids (20:5)
- Isolate
  - C16-1
  - Cleavage with ozone
  - Polycondensation
- Polyester polyol
- Step polymerization with MDI

Flexible Polyurethane
4 - Progress and Outcomes

Renewable Isocyanates using Flow Chemistry

# 4 - Progress and Outcomes

## Bio-based Isocyanate Scope

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>![Chemical Structure]</td>
<td>![Chemical Structure]</td>
</tr>
<tr>
<td>![Chemical Structure]</td>
<td>![Chemical Structure]</td>
</tr>
<tr>
<td>![Chemical Structure]</td>
<td>![Chemical Structure]</td>
</tr>
<tr>
<td>![Chemical Structure]</td>
<td>![Chemical Structure]</td>
</tr>
<tr>
<td>![Chemical Structure]</td>
<td>![Chemical Structure]</td>
</tr>
<tr>
<td>![Chemical Structure]</td>
<td>![Chemical Structure]</td>
</tr>
</tbody>
</table>

- **Bio-sourced**
- **Carboxylic acids**
- **Bio-Isocyanates**
4 - Progress and Outcomes
Can we take this to a pilot commercial setting – PEAK challenge

Original wildtype:
- Nitrate –
- Salt tolerance –
- pH tolerance –
- OPN expression –
- Growth in ponds -

Improved wildtype:
- Nitrate +
- Salt tolerance +
- pH tolerance +
- OPN expression -
- Growth in ponds +

Improved recombinant:
- Nitrate +
- Salt tolerance +
- pH tolerance +
- OPN expression +
- Growth in ponds +

Cultivation in alkaline media in GAI open ponds
Harvest via GAI membrane filtration

Filtrate
Retentate
Summary

Our objectives were to develop a suite of synthetic biology, breeding, and HT screening tools for green algae and cyanobacteria, and to develop these as a “process” that could be transferred to any commercial strain – We have accomplished a significant portion of that goal.

We demonstrate this by:
1) Rapidly producing the SARS covid spike protein in algae
2) Obtaining high levels of protein expression in commercially relevant algae and cyanobacteria
3) Used our developed process to achieve sustained growth in open pond setting
4) Producing novel high value bio-based isocyanates!

With Algenesis we have turned the polyols into commercial products – Footbeds and Outsole of Flip Flops and Shoes, that will be commercially available in 2021.
Quad Chart Overview

### Timeline
- **Project start date**: 9/30/17
- **Project end date**: 6/30/21

### Project Goal
- The primary goals are to develop advanced genetic tools, breeding, and HT screening that will advance the algae industry, and enable economic viability of algal biofuels through production of high value co-products.

### End of Project Milestone
- Complete comparative environmental and economic impact of 1) A strain producing a high value protein product and 2) A strain producing a high value polymer product, under pilot scale production in outdoor ponds.

### Project Partners*
- Partner 1 UC Davis
- Partner 2 Global Algae Innovations

### Funding Mechanism
- **DE-FOA-0001628**
- Topic Area 1 – Strain Improvement

### FY20 Costed

<table>
<thead>
<tr>
<th>DOE Funding</th>
<th>Total Award</th>
</tr>
</thead>
<tbody>
<tr>
<td>(10/01/2019 – 9/30/2020) $1,001,336 (negotiated total federal share) $3,000,000</td>
<td></td>
</tr>
</tbody>
</table>

### Project Cost Share

<table>
<thead>
<tr>
<th>DOE Funding</th>
<th>Total Award</th>
</tr>
</thead>
<tbody>
<tr>
<td>$65,040</td>
<td>$422,257</td>
</tr>
</tbody>
</table>
Additional Slides
Responses to Previous Reviewers’ Comments

Comments: No in depth discussion of the FOA technical target to achieve biomass productivities exceeding 18g/m-2/day or 80 GGE per ton biomass potential. The proposed development of co-product (secreted proteins) and specialty branched chain wax esters may facilitate achieving these targets and a final TEA and LCA analysis following biomass composition evaluation is proposed. However, an upfront assessment of the feasibility of hitting these key targets, through this workplan, would strengthen the technical merit of the proposal.

Response: The proposed plan was to build the synthetic biology, breeding, and HT screening tools, first in lab strains, and then transfer those tools to potential commercial strains in this project. The biomass productivity of the commercial strains was the true baseline to address.

- We have now started that process and have good baseline biomass data for both a green algae and cyanobacteria commercial strain.
- We have deployed several of our developed tools in these commercial strains, and will analyze the entire system during the PEAK challenge.
Publications, Patents, Presentations, Awards, and Commercialization

- **Engineering Polyketide Synthase Machinery in Cyanobacteria**
  Patent Application Number: 62643370
Publications, Patents, Presentations, Awards, and Commercialization


• Algae Biomass Summit 2020 Presentations
  – Anthony Berndt, “Production of a Recombinant Proteins in Different Subcellular Compartments in Green Algae can Alter Post-translational Modifications and Hence Protein Biological Activity” September 30, 2020.