DOE Bioenergy Technologies Office (BETO)  
2019 Project Peer Review

Developing Advanced Genetic and Synthetic Biology Tools for Improved Algae Productivity

March 7, 2019
PEAK -

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This presentation does not contain any proprietary, confidential, or otherwise restricted information
Goal Statement

• The **goal** of this project is to develop a process for making advanced genetic tools using: genomics, synthetic biology, high-throughput screening methods, and breeding technologies.

• The **outcome** of this project will be a process in which a genome sequence from any algal strain is the input and a designed set of genetic tools and high throughput screening technologies are the output.

• **Relevance to bioenergy industry**
  – Drop-in hydrocarbon fuels can be produced directly from algae; however, it is generally acknowledged that wild type algal strains do not have the productivity, robust growth characteristics, and *value added co-products* that will allow algal biofuels to compete economically with fossil fuels.
  – Improved molecular and synthetic biology tools coupled with classic domestication processes - could enable biofuels from algae to be cost competitive with fossil fuel prices of today in as little as five to ten years.
Quad Chart Overview

Timeline
- Project start date 09/30/2017
- Project end date 12/31/2020
- Percent complete 20%

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<th>FY 18 Costs</th>
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Barriers addressed
- Aft-C. Biomass Genetics and Development
- Ct-K. Developing Methods for Bioproduct Production

Objective
Developing advanced genetic tools and HT screening protocols for commercial algae production strains

End of Project Goals
- A suite of advanced genetic tools, high-throughput screening, and breeding protocols for green algae and cyanobacteria.
- Commercially relevant strains producing a high value protein co-product for TAI, and improved lipid accumulation for GAI.
- A process for developing genetic control systems that can be applied to any species of algae as they are identified by academic or commercial enterprises.

*Partners:
- Global Algae Innovations, 5%
- Triton Algae Innovations, Cost share
- Algenesis Materials, Cost share

*Only fill out if applicable. If there are multiple cost-share partners, separate rows should be used.
**Only fill out if applicable.
1 - Project Overview

- This project is a continuation of 10 years of DOE funded programs to develop algae as an economic platform for biofuel production

- Genetic modification of production strains will be required for improving yields and productivity, as well as allowing for the production of high-value co-products, that will enable economic biofuel production from algae

- This project will develop advanced genetic engineering tools and methods for use in commercial production strains, both algae and cyanobacteria

- The outcome of the project will be a process in which a genome sequence from any algal strain is the input and a designed set of genetic tools and high throughput screening technologies are the output
Project Overview – Objectives

- **Objective 1** - Develop advanced genetic tools for improved nuclear transgene expression and advanced metabolic engineering to enable economic production of biofuels from algae.
- **Objective 2** - Improve expression and secretion of recombinant proteins as co-products to improve the economics of biofuel production.
- **Objective 3** - Establish a rapid high throughput screening method for strains with improved protein production and secretion abilities.
- **Objective 4** - Develop genetic tools and genome editing strategies to enable metabolic engineering of commercially viable species of cyanobacteria.
- **Objective 5** - Develop a suite of highly controlled gene expression tools for metabolic engineering of cyanobacteria to produce methyl-branched fatty acids and wax esters.
- **Objective 6** - Developed improved targeted genome editing and transgene delivery methods for green algae to accelerate strain engineering.
- **Objective 7** - Develop *non-transgenic* improvement strategies using breeding, high throughput screening, and mutagenesis to develop new production strains.
- **Objective 8** - Develop genetic tools for commercial strains, based on learning from previous objectives, to demonstrate rapid adaptation of genetic tools to new strains.
- **Objective 9** - Grow unmodified and genetically modified strains under field conditions to determine impact of genetic modifications on biomass productivity and product yield.
- **Objective 10** - Conduct a life cycle assessment and techno-economic assessment (TEA) on GM and non-GM strain under outdoor cultivation conditions.
2 – Project Approach (Management)

Milestones and Deliverables – Key Personnel

**Year 1:** Validate baseline genetic tool set and develop approaches for advanced genetic tool construction for algae (Mayfield) and cyanobacteria (Golden)

**Year 2:** Create synthetic gene control system for recombinant protein expression (Mayfield/TritonHN) and metabolic engineering of polyurethane precursors (Burkart, Golden/Algenesis). Adapt tools for commercial strain engineering

**Year 3:** Outdoor field trials of improved strains – GAI and Mayfield lab TEA and LCA of GM strains to demonstrate economic and environmental improvements (UCSD & Kendal UC Davis)
2 – Project Approach (Technical)

**Approach**

Characterized algal strain THN-101 → Synthetic biology tool building process → New genetic tools for commercial strain development

**Challenge**

Domestication of corn took 6,000 years

Target High Value Products

Algae are the most diverse organisms on the planet
3 - Technical Progress – Genetic Tools for Microalgae

- Improved recombinant protein expression in *Chlamydomonas*
  - Developed new vector that achieves higher expression of recombinant proteins than previous expression vectors – and much better control over gene expression

![Diagram of Promoters, 5' UTR, 3' UTR, and Codon optimized coding region]

- Poor recombinant protein expression has been a problem
- Need many transformation events to get good expression
- Lack the viral promoter elements that other systems have
- Can target proteins to subcellular location including export
Sequences preceding highly expressed genes from *C. reinhardtii* genome

25 unique synthetic promoters were generated
Synthetic promoters show increased transcript abundance over best endogenous promoter.
Sap11 only required 150 nt contained six elements – only 2 essential
New synthetic promoter library of 2 X 1,000 members

Oligo pools from TWIST

Add constant regions from sap11

Variable region
160bp each

1000 Unique sequences per pool

Full promoter region
340bp

Reduced to 300 nt and 5 elements per promoter
New vector results in higher expression of GFP reporter protein

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New vector results in higher expression of GFP reporter protein

- Double antibiotic selection of Hygromycin + Zeocin identifies transformants with up to 3X higher GFP expression than using Hygromycin alone
Identifying conditional promoter elements

Library conditions

Minimal media
Expression of mCherry driven by synthetic promoters under light and dark growth

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3- Technical Progress - reporter gene expression in commercial cyanobacteria

J. Golden

- Cyanobacteria GAI-220 containing a broad host-range plasmid with a spectinomycin selectable marker and expressing a *yfp* reporter gene driven by a synthetic constitutive promoter
Assembly strategy for the modular construction of vector systems

1. Selection donors vectors
2. Restriction digest
3. Column purification
4. Assembly reaction: Gibson or Seamless (Life Tech)

Constitutive Promoter Library

Mutagenesis of PconII promoter

- 300 promoters first tested in *S. elongatus* PCC7942
- Cloned into RSF1010 plasmid for testing in multiple cyanobacterial strains
- Sorted into groups by strength
- Currently testing in GAI-220 for reporter gene expression
Synthetic theophylline-responsive riboswitches

- Engineered 5' UTR of mRNA transcript
- Theophylline binding -> riboswitch conformation change allows translation
- Panel of 6 theophylline riboswitch variants (Topp et al., AEM 2010)

(Lynch et al., Chem Biol 2007)

(Ma et al., 2014. Appl. Envir. Microbio.)
3 – Technical Progress - Genome Recombination Mating – Cell Fusion

1. Done through sexual recombination or protoplast fusion to introduce genomic variation and create unique progeny

2. Shuffles multiple genes (or variants) in the genome and combines multiple traits into a single line
Parent GFP mt+ strain (A) crossed with diverse set wt C. reinhardtii isolates
Mutagenesis to introduce genetic diversity
Resulting in increased GFP expression
We can go from a wild type strain to an optimized strain in 17 weeks
3 – Technical Progress - Algae oil extraction and conversion methods – M. Burkart

- *Isolation and conversion of lipids from algae and cyanobacteria will prove critical to development of products for real-world applications*
- *Initial focus has been placed on polyols for flexible polyurethanes*
Polyurethane Chemistry

\[
\text{polyol} \quad \xrightarrow{\text{diisocyanate}} \quad \text{polyurethane}
\]
TAGs from Autotrophic Algae Cultivation

Nannochloropsis salina

Qualitas Inc.
Columbus, NM
GCMS of FAMEs

Abundance

16:0
16:1
18:1
18:3

Time ->
Diacid and Diol Preparation

\[
\begin{align*}
\text{Ozonolysis:} & \quad \text{H}_2\text{O}_2 \\
\text{Ozonolysis:} & \quad \text{NaBH}_4
\end{align*}
\]
Qualitas waste oil

1. saponification
2. acidification

Fatty acid 90%

Urea complexation

Monounsaturated fatty acid
Yield: 85% Purity 85%

Ozonolysis with O3, Work up with H2O2

Azelaic acid

Mixture of products

C9 - Diacid
C7 - monoacid

Distillation

Extraction

Crystallization
Polyurethanes produced with algae oil diols and Methylene diphenyl diisocyanate

\[ \text{1, 9 nonanediol} + \text{MDI} \]
4 – Relevance

Developing advanced genetic tools, high-throughput screening methods, and breeding technologies for microalgae

• This project will enable algae biofuels to be more cost competitive with fossil fuels by improving yields and developing high value co-products
• Directly supports BETO’s mission “to enable the cost-effective and sustainable production of drop-in biofuels”

Relevance to Bioenergy Industry

• Metabolic engineering and co-products will both be required to enable economic fuel production from algae
• We are developing a process that can be applied to any algae species, especially those that are commercially relevant
• We are working directly with cost share partners to develop algae based co-products including recombinant proteins and renewable polymers
5 – Future Work

• **Identify improved synthetic promoters for recombinant protein expression**
  – Screening of promoter library
  – Condition-specific inducible promoters
  – Develop site-specific integrase tools

• **Strain improvement and commercial production**
  – Mutagenesis and breeding for strain improvement
  – Production of commercial strains at Cal-CAB field station

• **Develop genetic tools for commercial strains**
  – Transcriptomics under different growth conditions
  – Promoter library and regulated expression systems

• **Optimization of metabolite production**

• **100% algae polyurethane**
Accumulation of OPN in chloroplast or secreted by nuclear expression and protein targeting
Overlay Synthetic Biology and classical strain improvement for commercial production strains

Strain improvement process developed for Task 5.

GM and non-GM strains will be grown in greenhouse using paddle wheel ponds allowing characterization of transgenic strains without any EPA permitting.
Summary

1. Overview – We are developing advanced genetic tools and high throughput screening processes that will enable increased productivity and value added co-products for commercially relevant strains

2. Approach – We are using a design – build – test – learn approach to develop tools that will enable the algae industry, not just a few select labs or companies

3. Technical Accomplishments – We have demonstrated the utility of the approach to increase recombinant protein production up to 15 fold for one reporter proteins, and have begun development of cyanobacteria vectors for commercial strains. We have also been able to advance a key product (renewable polyurethanes) that are already on their way to becoming a commercial product

4. Relevance – The bioeconomy is rapidly approaching and these technologies will allow algae to play a significant role in this new era

5. Future work – The potential for algae as a bioproducts platform is clear, we need to develop the enabling tools that will allow this potential to be realized
Additional Slides
Responses to Previous Reviewers’ Comments

• This is a new project that was not previously reviewed
• Also provide highlights from any Go/No-Go Reviews
Publications, Patents, Presentations, Awards, and Commercialization

Publications

Presentations

Patents