NREL
NATIONAL RENEWABLE ENERGY LABORATORY

DOE Bioenergy Technologies Office (BETO) 2019 Project Peer Review

1.3.1.301 Cyanobacteria Photosynthetic Energy Platform

Advanced Algal Systems

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Jianping Yu

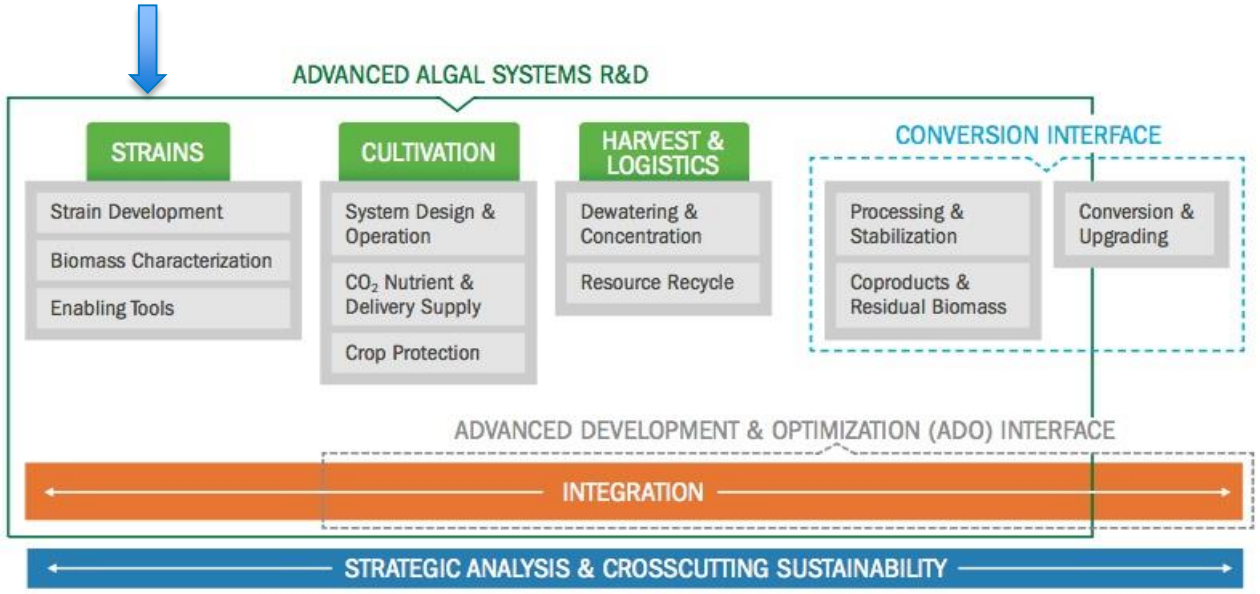
National Renewable Energy Laboratory

Goal Statement

Goals: Develop cyanobacteria genetic tools to improve photosynthetic efficiency through carbon pathway engineering to improve biomass productivity, leading ultimately to lower cost for fuels and chemicals

Outcome: Unique strategies impacting cyanobacteria photosynthetic processes that can be extended to other species, including eukaryotic species

Relevance: Developing tools in model organisms allow for rapid hypothesis testing and then provide options to application to production cyanobacteria and eukaryotic species



Quad Chart Overview

Timeline

- Start: FY2019
- Merit review cycle: FY2019-2021
- 10% complete of review cycle

	Total Costs Pre FY17**	FY 17 Costs	FY 18 Costs	Total Planned Funding (FY 19-Project End Date)
DOE Funded				1200k

Barriers addressed

Aft-C. Biomass genetics and development.

Aft-A. Biomass availability and cost.

We develop genetic and biochemical tools to increase photosynthetic productivity.

Objective

Develop cyanobacteria genetic tools to improve photosynthetic efficiency through carbon pathway engineering to improve biomass productivity, leading ultimately to lower cost for fuels and chemicals

End of Project Goal

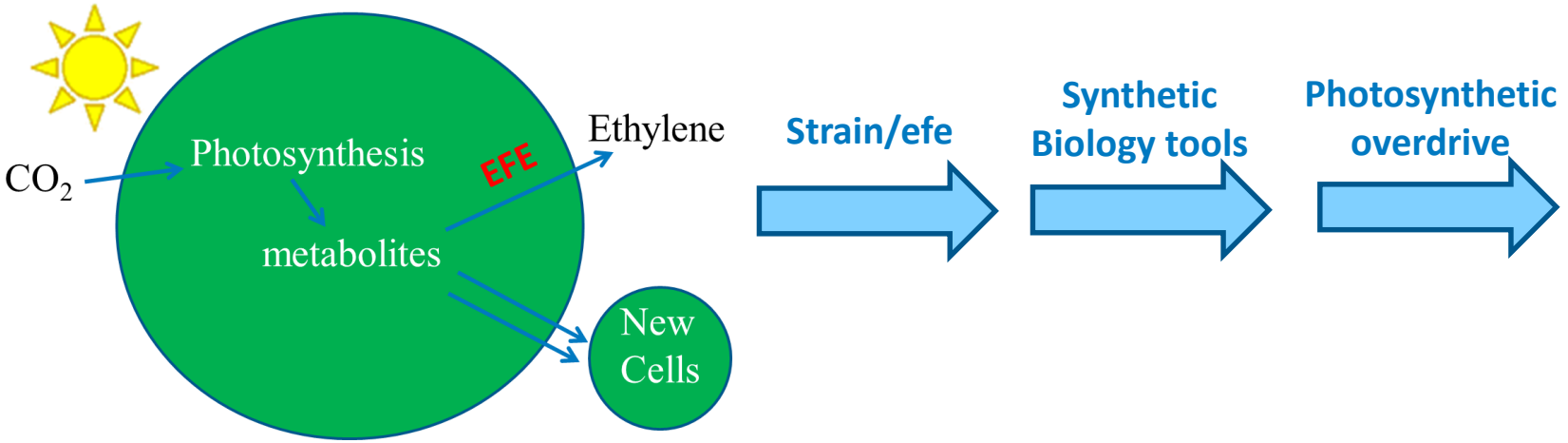
Improved biomass productivity in cyanobacteria by 25%. Translates to $2/GGE reduction in MFSP in 2018 SOT model. Translate lessons to other algae.

Previous project provided foundation for the current project:

- Title: Producing Transportation Fuels via Photosynthetically Derived Ethylene.
- FY10-FY18, total cost 2423K.
- Project developed ethylene producing cyanobacteria, genetic and photosynthetic toolbox, and the concept of photosynthetic overdrive.

Photosynthetic Overdrive
Ethylene production leveraged to increase photosynthesis flux

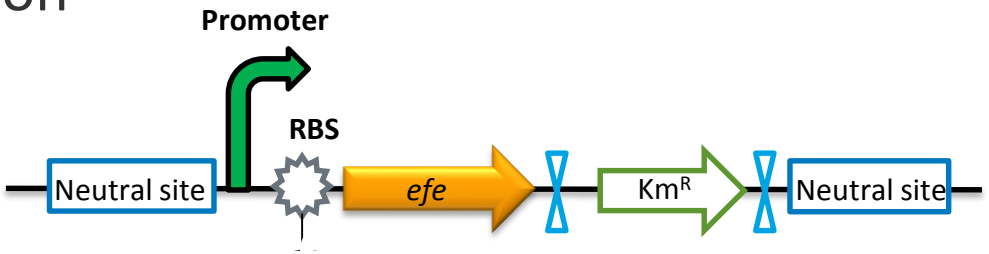
1. Improving strain stability. Accomplished by redesigning ethylene forming enzyme (*efe*) gene sequence and using a more robust strain.
2. Improving EFE expression. Accomplished by developing synthetic biology tools such as promoters and ribosome binding sites. EFE protein reached 12% of soluble protein, with 20% fixed carbons go to ethylene. Improved ethylene productivity by 1000X and showed increased photosynthetic flux.
3. Improving substrate supply. This is the current bottleneck, requires more knowledge on photosynthesis and carbon metabolism, the focus of this work.



Project overview: Tools and capability developed in *Synechocystis* 6803 will be used for this project

Genetic manipulation toolbox:

- high-efficiency transformation
- promoters
- ribosome binding sites
- up- or down-expression
- gene deletion
- in-house mutant library with 200+ strains affecting various metabolic processes



Photosynthetic phenotyping toolbox:

- PSI photobioreactors and inline gas analysis
- ATP/ADP levels
- carbon concentrating and carbon fixation parameters
- multi-omics analysis including flux analysis using isotope

Approach - Management

Manipulate energy management and source-sink relationship to improve biomass productivity. Tools and lessons will be transferred to other BETO algae projects.

Team members, weekly updates, monthly meetings, quarterly milestones

Bo Wang – Photosynthesis and genetic engineering.

Damien Douchi – Carbon uptake and fixation.

Wei Xiong – Metabolic flux analysis.

Industry collaborations

Celanese, Nova, SABIC, Nzyme2HC: chemical production.

Pond Technologies: photobioreactor system for biomass and chemical production.

Compact Membrane System: bio-ethylene harvesting.

Academic Collaborators

TAMU and Miami University Ohio: proteomics.

Oklahoma St Univ., CEA (France), and Uppsala Univ (Sweden): photosynthesis.

Nottingham University (UK): bio-ethylene production

National lab collaboration

LANL: Transcriptomics data collection.

Technical Approach: Challenges and Critical Success Factors

Challenges

- Photosynthetic productivity and energy conversion processes in algae systems are not fully understood.
- Transcriptional regulation of photosynthetic machinery and metabolic control by energy balancing system has not been engineered towards dramatic improvements in algae.

Critical success factors

- Identify and manipulate regulatory factors to increase biomass productivity.
- Apply the tools and knowledge to other algae.

Approach - Technical

Genetic manipulation of carbon sink

Expand sink via ethylene production

Block sink in glycogen synthesis

I. Photosynthetic overdrive

- The initial observation
- The theory
- The proteins
- The switch
- Engineering strategy

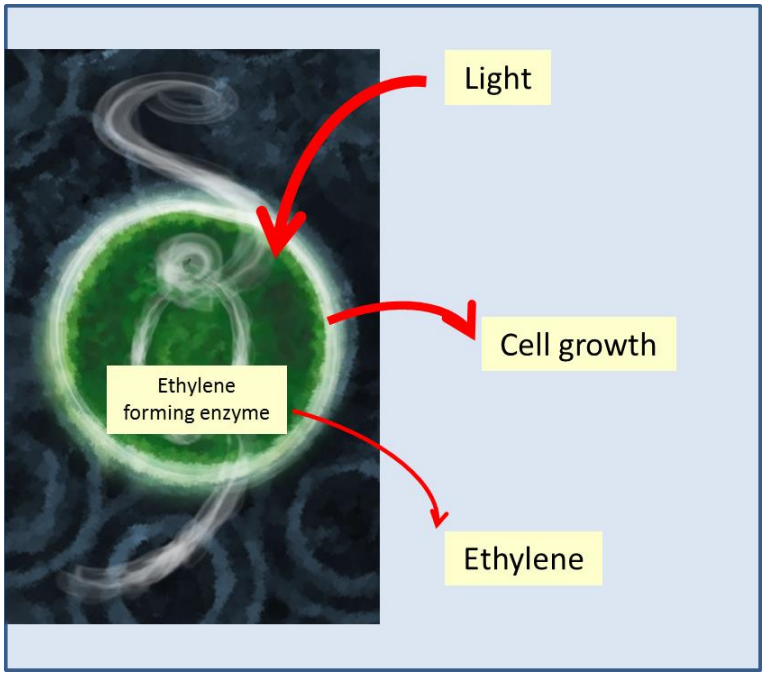
II. Energy balancing

- The initial observation
- The proteins
- The switch
- The theory
- Engineering strategy

Tool and strategies to improve algal biomass productivity

Technical Accomplishments and Progress

Previous Research: Ethylene production stimulates photosynthetic productivity



- 1000X higher ethylene productivity improvement
- R&D 100 Award, Editors Choice Award, 2015
- 20% fixed carbons go to ethylene
- Synthetic biology and photosynthesis tools that help other researchers

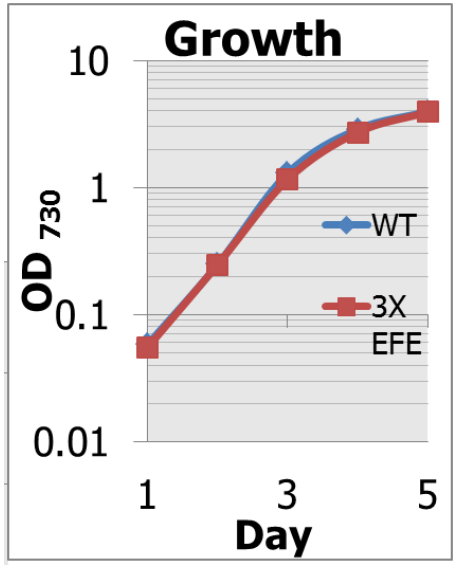
- Ethylene production stimulates photosynthesis. We are taking lessons from the stimulation to increasing biomass productivity

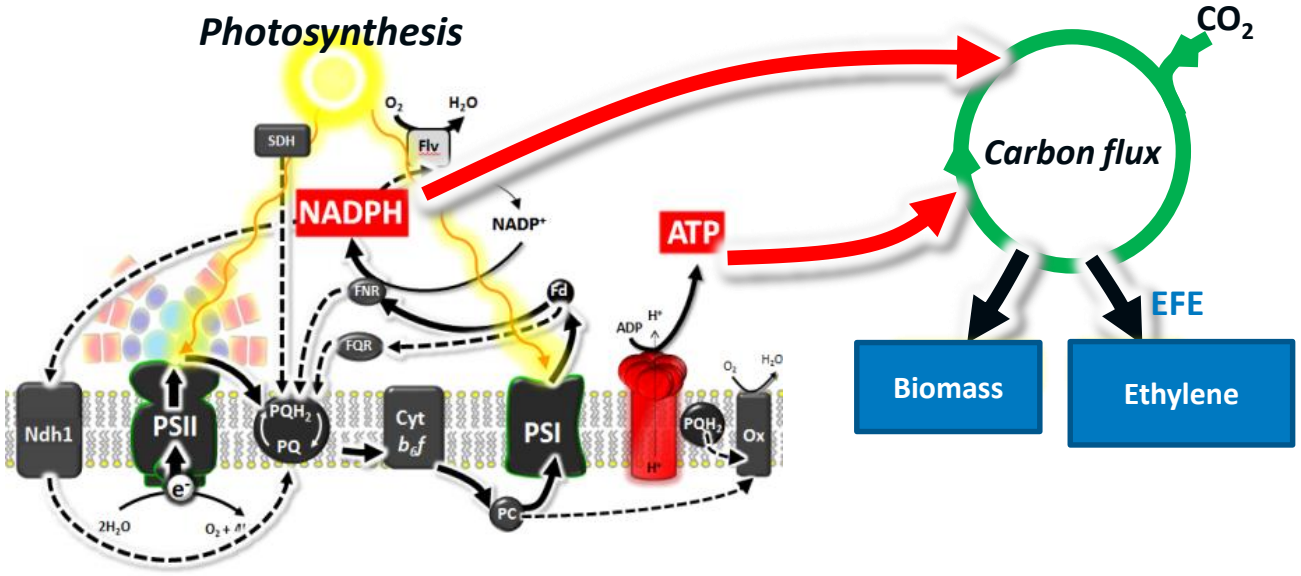
- Ungerer *et al* 2012 *Energy & Environmental Science*
- Xiong *et al* 2015 *Nature Plants*
- Holland *et al* 2016 *Algal Research*
- Wang *et al* 2017 *ACS Synthetic Biology*

I. Photosynthetic Overdrive
Ethylene production stimulates photosynthesis

Production of ethylene expands carbon/energy sink, causing higher rate of photosynthesis (i.e. photosynthetic overdrive). The underlying molecular mechanism can be exploited to increase biomass productivity.

- Growth was not affected while cells produce ethylene; light energy was used to fix more CO_2, support growth plus ethylene production.



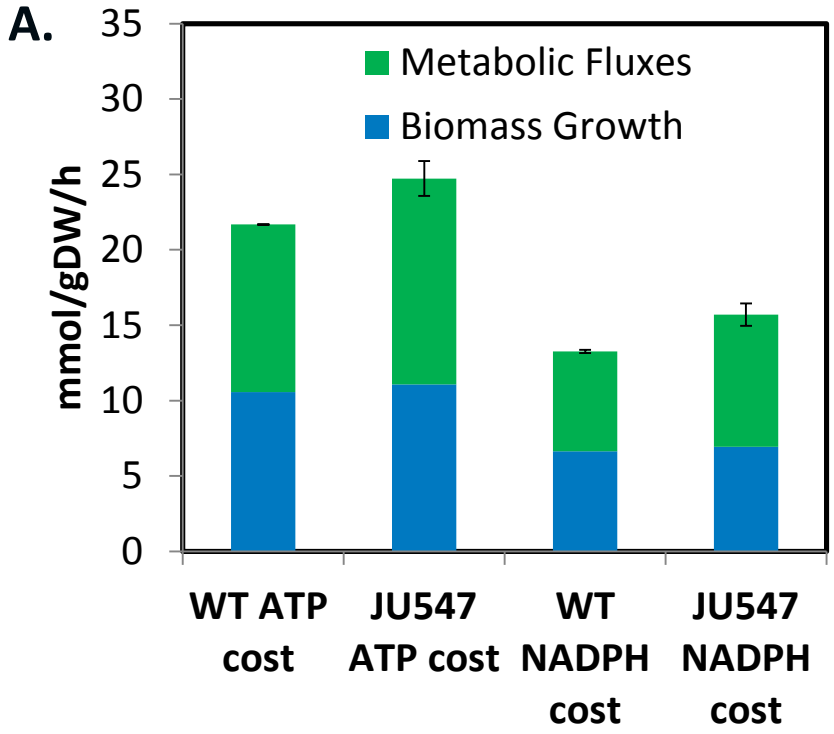


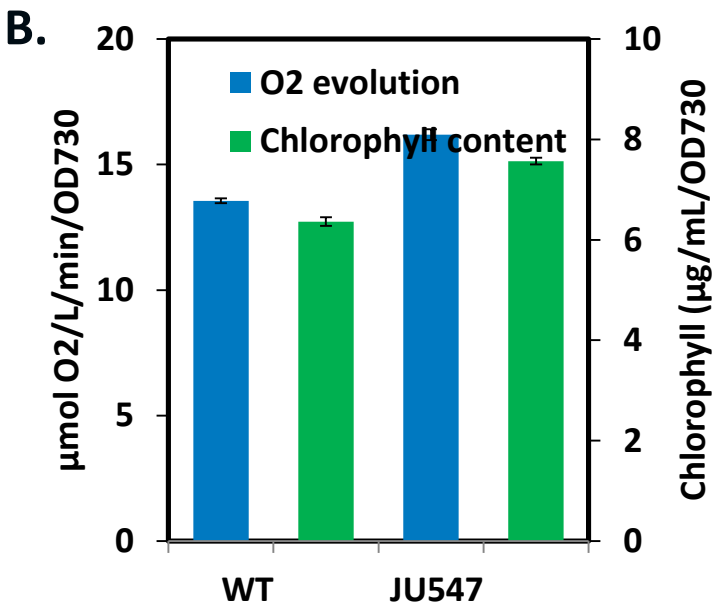
- Xiong *et al* 2015 Nature Plants
- Xiong *et al* 2017 Current Opinion in Chemical Biology

I. Photosynthetic Overdrive
Photosynthetic overdrive parameters

Multiple parameters increase by 10-20% in the ethylene strain JU547 over WT:

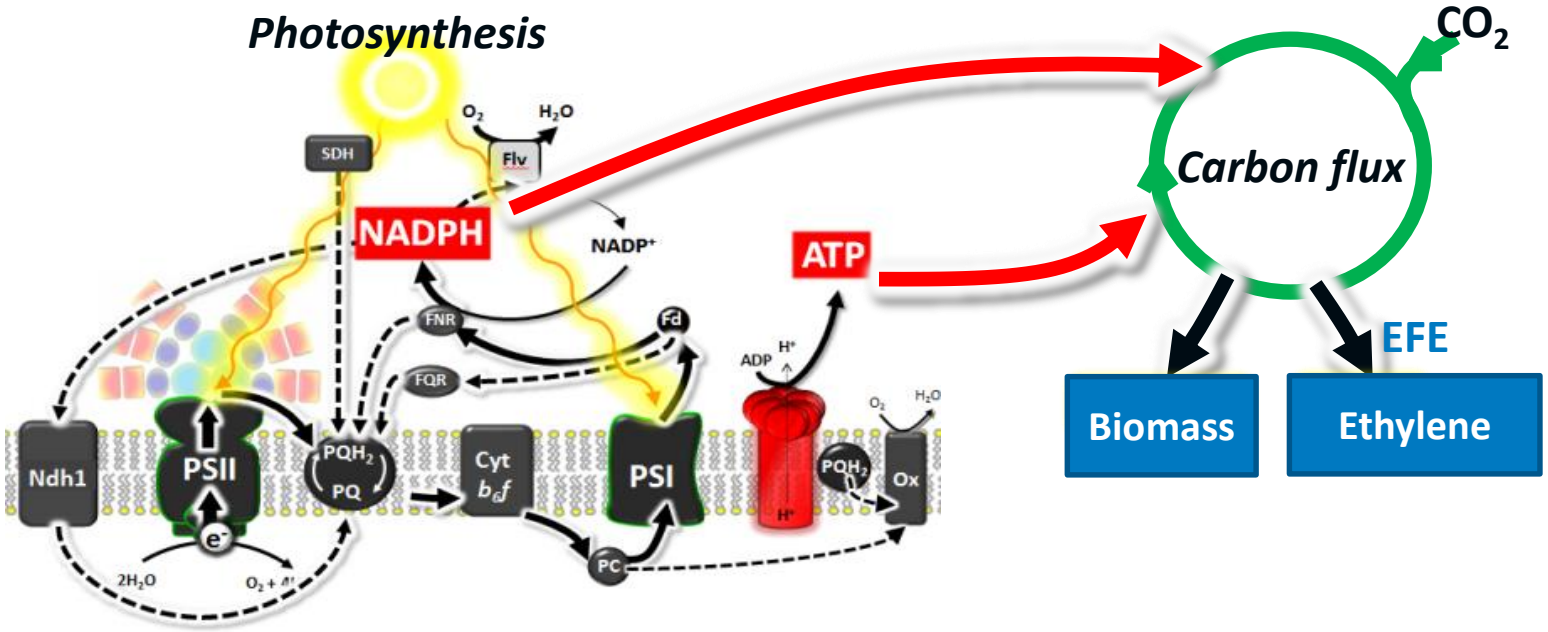
- A. Energy demand for ATP and NADPH.
- B. Chlorophyll content; O_2 evolution rate.
- PSII efficiency; CO_2 fixation rate; Electron transfer capacity (P700 reduction kinetics)





- Xiong *et al* 2015 Nature Plants.
- Holland *et al* 2016 Algal Research

I. Photosynthetic Overdrive
Proteome changes identify molecular machinery



Increased proteome allocation in photosynthesis in ethylene strain versus WT:

Chlorophyll synthesis

Cytb6f complex **ATPase** **Ci transporter**

Carboxysome

I. Photosynthetic Overdrive
NtcA/PII may be the switch to photosynthesis overdrive

Hypothesis: Transcriptional regulation of photosynthesis genes responds to carbon sink expansion to increase photosynthesis proteome allocation and energy conversion.

- Protein level of the transcriptional regulator PII increased in ethylene strain versus WT, while the level of another transcriptional regulator NtcA decreased.

Protein abundance in the ethylene strain vs WT (100 %)

	12h in culture	60h in culture
PII	119%	148%
NtcA	60%	No difference

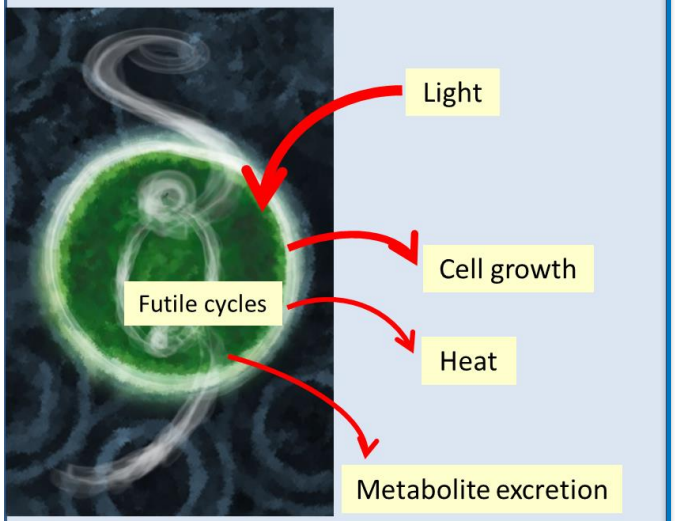
- These two transcriptional regulators are conserved in cyanobacteria; they regulate expression of many photosynthesis genes in response to metabolic changes including ATP and AKG. NtcA is a repressor for some photosynthesis genes. Lower NtcA abundance thus could unleash expression of photosynthesis genes.

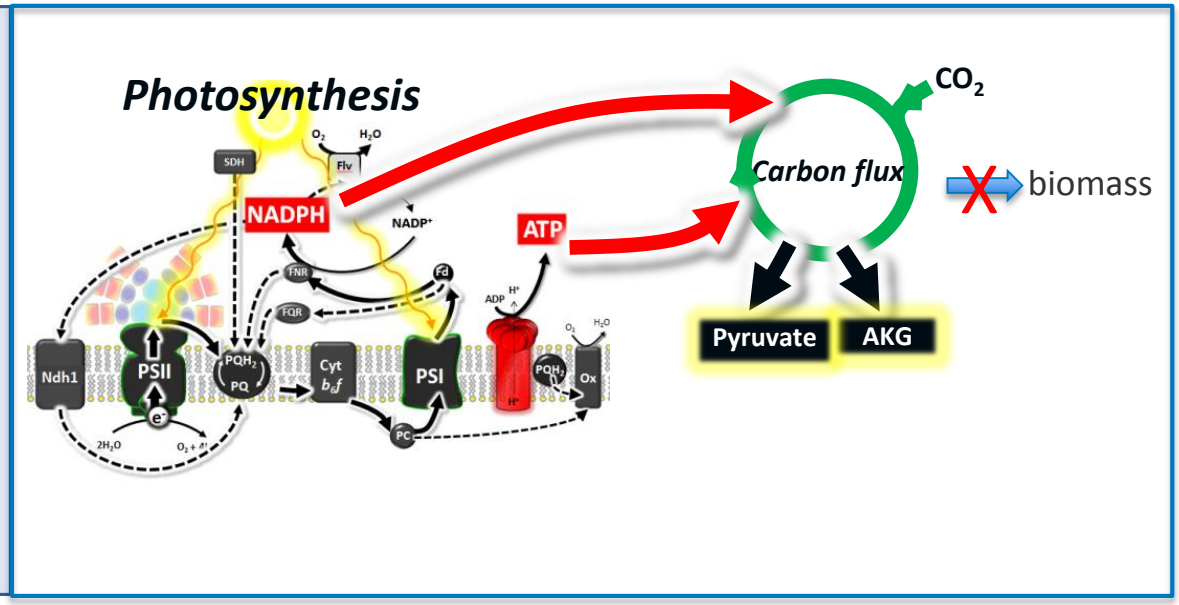
- Future work: Recreate photosynthesis overdrive in WT background by manipulating the putative switch PII/NtcA.

II. Energy balancing
Regulation of growth and carbon flux is energy driven

Futile cycles, and associated energy loss, are involved in energy balancing

- A glycogen synthesis mutant was generated to direct more carbon flux to ethylene but redirected carbon from biomass to organic acids instead
- This provides an experimental system to understand the regulation of carbon partitioning
- **The carbon partitioning is primarily regulated by cellular energy**
- **High cellular energy levels leads to faster growth.**

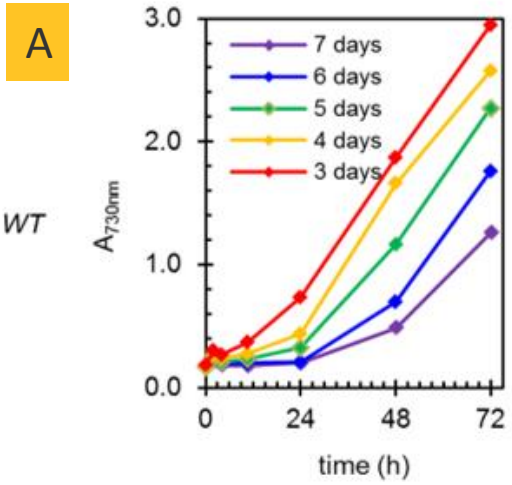


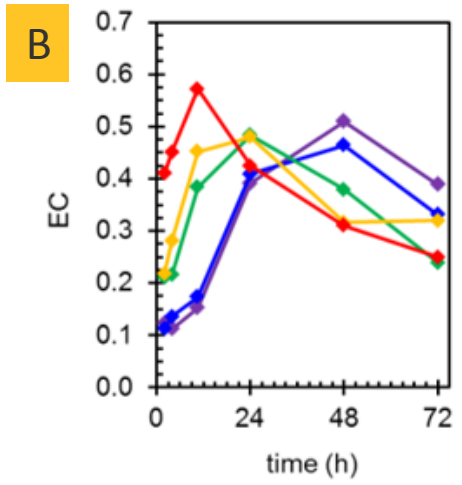


Carrieri *et al* 2012; 2015; 2017; Cano *et al* 2018

II. Energy Balancing
An energy charge threshold triggers rapid cell growth

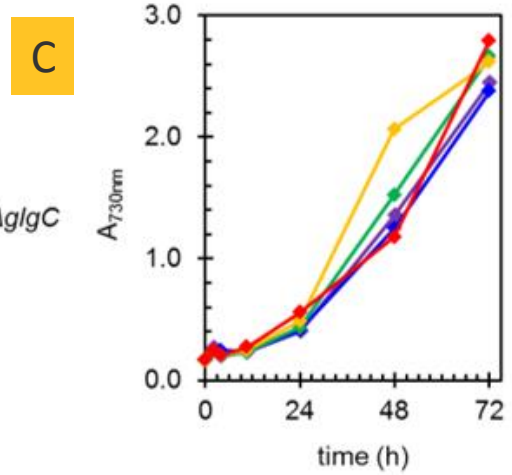
WT shows lag phase according to inoculant age

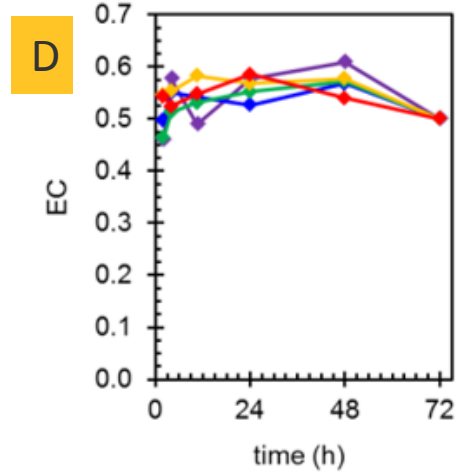




WT shows variable Energy Charge (EC):
ATP / ATP+ADP

Glycogen mutant shows rapid growth irrespective of inoculant age

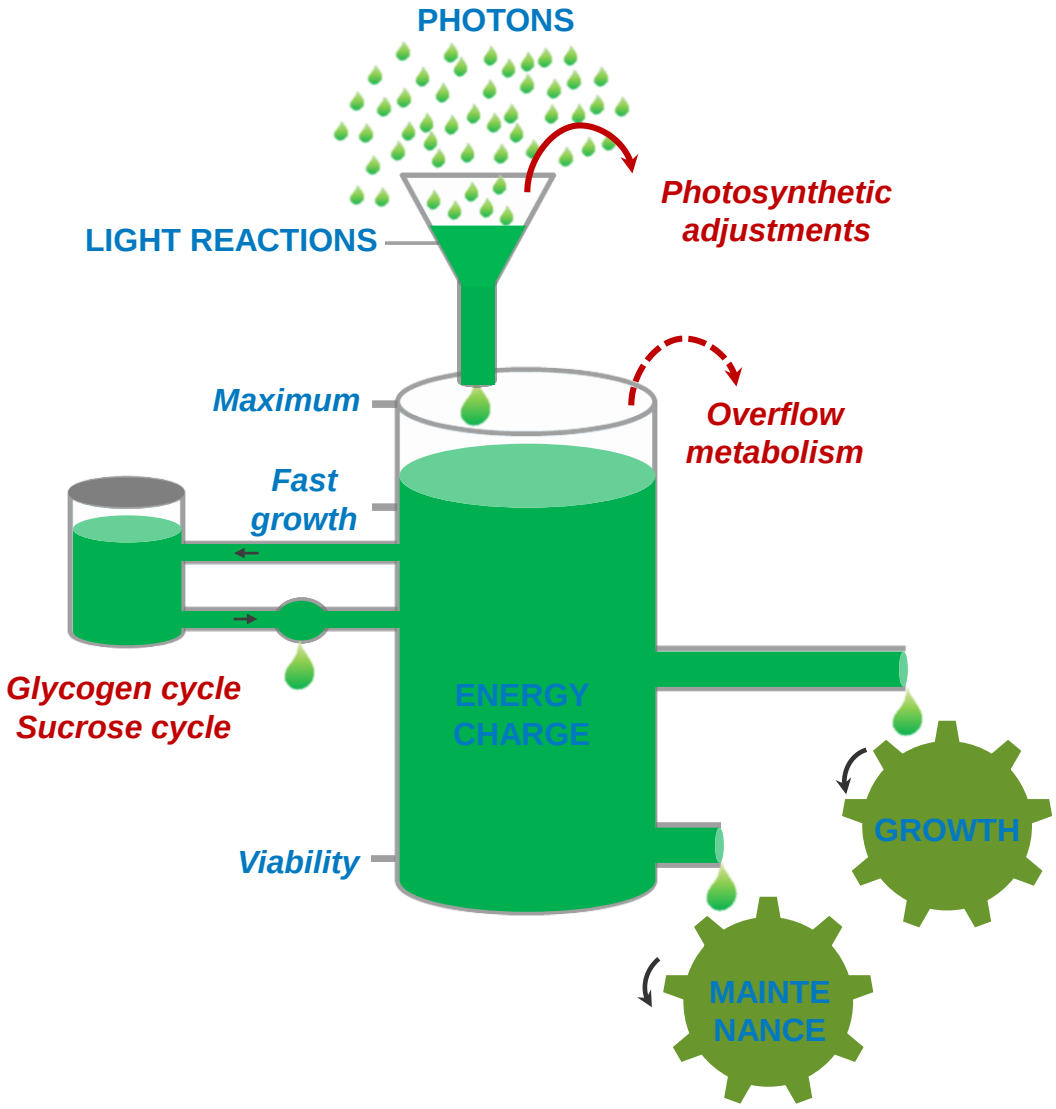




Glycogen mutant shows high Energy Charge

- Cano *et al* 2018 Cell Reports
- Future work: Manipulate energy balancing mechanisms, glycogen cycle and sucrose cycle, to increase biomass growth.

II. Energy Balancing:
How cells lose energy in photosynthesis



1. Glycogen cycle and sucrose cycle are ATP-consuming, energy balancing mechanisms, and have impact on biomass growth.

2. Knocking-down *glgC* expression will decrease the level of the committed enzyme AGPase, restrict glycogen cycle and its energy loss, and enhance biomass growth.

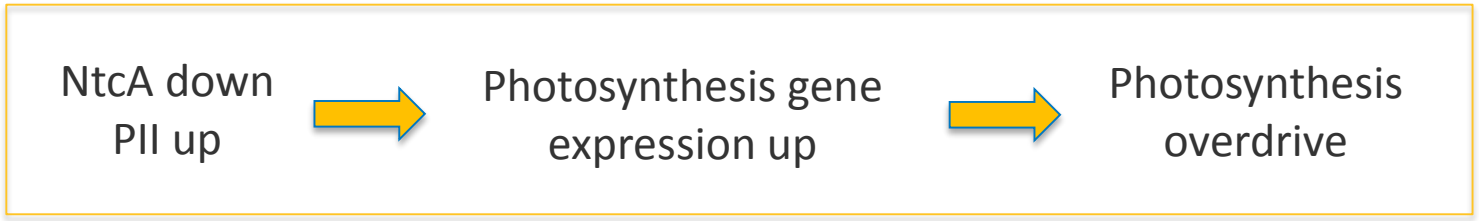
3. Similarly, knocking-down or knocking out *sps* gene encoding sucrose phosphate synthase, the committed step for the sucrose cycle, may reduce energy loss and enhance biomass growth.

Relevance

- Address 2019 MYP. 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.
- The objective of the work is to develop a cyanobacteria platform with the goals of exploiting photosynthetic energy conversion and carbon utilization to ultimately be in a position to use the fundamental biochemical targets to improve biomass productivity
- This project leverages years of fundamental research and an excellent publication track record at NREL in photosynthesis, carbon and energy metabolism in the model strain Synechocystis and will translate and project this knowledge base to cyanobacteria of industrial interest such as species from the Synechococcus and Arthrospira genera.
- The end of the project will be a successful platform for rapid testing of photosynthesis and biomass improvement hypotheses in cyanobacteria, including strains of industrial relevance, as well as develop a route to implement these strategies to selected eukaryotic systems.
- The project targets a 25% increase in biomass productivity, which translates to $2/GGE reduction in MFSP in 2018 SOT model

Future Work:
I. Photosynthetic Overdrive

- Complete integrated analysis of proteomics and transcriptome data to evaluate the roles of NtcA and PII in regulating photosynthetic output.
- Test the effects of PII overexpression and NtcA knockdown on growth and photosynthesis in WT background.
- Test additional hypotheses derived from omics analysis.
- Stack beneficial mutations to further increase biomass productivity.



Future Work:
II. Energy Balancing

- Down-regulate glycogen or sucrose flux using weaker/inducible promoter to drive *glgC* and *sps* expression.
- Test growth, energy charge, glycogen content, carbon overflow, high light, darkness, simulated natural light to identify beneficial mutations.
- Stack beneficial mutations to further increase biomass productivity.
- Transfer successful strategies to other algae and cyanobacteria.

Low glycogen/sucrose → high energy charge → fast growth

Go/No-go Decision

3/31/2020: Demonstrate successful 20% increase in biomass productivity under outdoor conditions.

Summary

Objective
Manipulate transcriptional regulators NtcA and PII, and energy balancing mechanisms glycogen cycle and sucrose cycle, to increase biomass productivity in cyanobacteria.

Approaches

1. Identify and manipulate putative metabolic switches underpinning photosynthetic overdrive, based on studies in ethylene producing cyanobacterium.
2. Manipulate cellular energy balancing system which controls growth and carbon partition, based on studies in glycogen and sucrose synthesis mutants.

Outcome: Tools and strategies that improve algal biomass productivity.

End of Project Goal
Improved biomass productivity in cyanobacteria by 25%. Translates to $2/GGE reduction in MFSP. Translate lessons to other algae.

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Jianping.Yu@nrel.gov

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Response to Reviewers' Comments 2017

Comments

The production of ethylene does not appear to directly advance the goals of the MYPP in terms of biofuel or biomass production (although ethylene can be converted to liquid fuels that is not the highest value product). However, if this technology can be advanced to the commercial stage the benefits will be meaningful in terms of reducing petroleum usage for the production of products and providing an economically sustainable avenue for the development of methods and facilities for cultivating and processing cyanobacteria.

Response

The current project takes the tools and knowledge developed in the ethylene research towards increasing biomass productivity. The new focus aligns well with BETO algae MYPP, and will use cyanobacterial genetics to develop strategies towards higher biomass productivity and lower MFSP.

Publications 2016-2019

- **Damien Douchi, Feiyan Liang, Melissa Cano, Wei Xiong, Bo Wang, Pin-Ching Maness, Peter Lindblad, Jianping Yu (2019) Membrane-Inlet Mass Spectrometry enables a quantitative understanding of inorganic carbon uptake flux and carbon concentrating mechanisms in metabolically engineered cyanobacteria. In revision at *Frontiers in Microbiology*.**
- **Melissa Cano, Steven Holland, Juliana Artier, Rob Burnap, Maria Ghirardi, John A. Morgan, and Jianping Yu (2018) Glycogen synthesis and metabolite overflow contribute to energy balancing in cyanobacteria. *Cell Reports,* DOI 10.1016/j.celrep.2018.03.083.**
- **Jianping Yu (2018) Nitrogen goes around. *Nature Chemical Biology* (News & Views), DOI 10.1038/s41589-018-0058-8.**
- **Bo Wang, Wei Xiong, Jianping Yu, Pin-Ching Maness, Deirdre R. Meldrum (2018) Unlocking the photobiological conversion of CO2 to (R)-3-hydroxybutyrate in cyanobacteria. *Green Chemistry*, DOI: 10.1039/c8gc01208c.**
- **Bo Wang, Carrie Eckert, Pin-Ching Maness, Jianping Yu (2017) A genetic toolbox for modulating the expression of heterologous genes in the cyanobacterium *Synechocystis* sp. PCC 6803. *ACS Synthetic Biology*, DOI: 10.1021/acssynbio.7b00297.**
- **Wei Xiong, Melissa Cano, Bo Wang, Damien Douchi, and Jianping Yu (2017) The plasticity of cyanobacterial carbon metabolism. *Current Opinion In Chemical Biology*, 41: 12-19. DOI: 10.1016/j.cbpa.2017.09.004**
- **Steven C. Holland, Juliana Artier, Neil Miller, Melissa Cano, Jianping Yu, Maria L. Ghirardi, Robert L. Burnap (2016) Impacts of genetically engineered alterations in carbon sink pathways on photosynthetic performance. *Algal Research* 20: 87–99. DOI: 10.1016/j.algal.2016.09.021.**
- **Jennifer N. Markham, Ling Tao, Ryan Davis, Nina Voulis, Largus T. Angenent, Justin Ungerer and Jianping Yu (2016) Techno-Economic Analysis of a Conceptual Biofuel Production Process from Bioethylene Produced by Photosynthetic Recombinant Cyanobacteria. *Green Chemistry* DOI: 10.1039/C6GC01083K.**

Presentations 2017-2019

1. **Algal Biomass Summit, speaker**
2. **American Chemical Society Annual meeting, poster**
3. **Western Photosynthesis Conference, speaker**
4. **South Dakota State University, seminar speaker**
5. **University of Nebraska, seminar speaker**
6. **Danforth Plant Science Center, invited speaker**
7. **Colorado School of Mines, seminar speaker**
8. **Nova Chemicals, webinar.**

In addition, bio-ethylene production is taught at North Carolina State University, College of Textiles.

Energy regulates growth and carbon flux

1. Prior work found that a glycogen synthesis mutant strain can behave as catalyst, converting CO_2 to organic acids, alpha ketoglutarate and pyruvate, without cell growth – *Photocatalytic conversion* (Carrieri, EES 2012)
2. Photo-damaged catalyst can be self-repaired, potentially supporting catalysis-repair cycle (Carrieri, Microb Biotech 2015)
3. The catalyst functions in mixotrophic conversion of sugars, glucose and xylose, to organic acids with superior carbon efficiency over heterotrophic systems (Lee, Met Eng 2015)
4. Acetate is produced as well, via phosphoketolase pathway which conveys high carbon efficiency but low energy yield (Xiong, Nature Plants 2015)
5. Photosynthetic adjustments help adapt to the loss of glycogen synthesis (Holland, Algal Research 2016)
6. The dramatic transition of carbon flux from building cell biomass to photo-catalysis is not accompanied by increases in pathway enzymes (Carrieri, Algal Research 2017)
7. It is regulated by energy charge; so is growth transition from lag to log phase. Glycogen synthesis, overflow metabolism, and photosynthetic adjustments are part of energy regulation system- Energome (Cano, Cell Reports 2018)

Academic collaborations

TAMU: NREL gene expression toolbox enabled 100X limonene productivity improvements (Wang *et al* 2017 PNAS). In return, TAMU helped us with proteomics work leading to the identification of a novel guanidine degradation enzyme (Wang et al manuscript in preparation).

Oklahoma St Univ: photosynthesis measurements (Holland et al 2016; Cano et al 2018).

Uppsala Univ (Sweden): carbon fixation improvements (Douchi et al 2019).

SDSU: Guanidine production from nitrogen-fixing cyanobacteria (Wang et al manuscript submitted).

Purdue Univ: Metabolomics and fluxomics analysis (Xiong et al 2015; Cano et al 2018).

ASU: High efficiency transformation (Wang et al 2015); 3HB production for biodegradable plastics (Wang et al 2018).

Vanderbilt Univ: Biological clock controls ethylene production.

LANL: Transcriptomics data collection.

Nottingham Univ: Bioethylene production using NREL gene expression toolbox.

Project overview: Novel approaches to increase biomass productivity in cyanobacteria

Objective
Manipulate transcriptional regulators (NtcA and PII), and energy balancing mechanisms (glycogen cycle and sucrose cycle), to increase biomass productivity in cyanobacteria.

Approaches

1. Identify and manipulate putative metabolic switches underpinning photosynthetic overdrive, based on studies in ethylene producing cyanobacterium.
2. Manipulate cellular energy balancing system which controls growth and carbon partition, based on studies in glycogen and sucrose synthesis mutants.

Outcome: Tools and strategies that improve algal biomass productivity.

End of Project Goal
Improved biomass productivity in cyanobacteria by 25%. Translates to $2 reduction in MFSP.

Approach - Technical

Novel strategies are being developed to improve algal biomass productivity through optimizing photosynthetic energy pathways

1. Identify and manipulate putative metabolic switches underpinning photosynthetic overdrive, based on studies in ethylene producing *Synechocystis*
2. Manipulate cellular energy balancing system which controls growth and carbon partition, based on studies in glycogen and sucrose synthesis mutants

- *Target transcriptional regulators NtcA and PII, and energy balancing mechanisms glycogen cycle and sucrose cycle, to increase biomass productivity in cyanobacteria.* ***Building on a wealth of existing data and strains will increase our chance of success.***