NREL
NATIONAL RENEWABLE ENERGY LABORATORY

Producing Transportation Fuels via Photosynthetically Derived Ethylene

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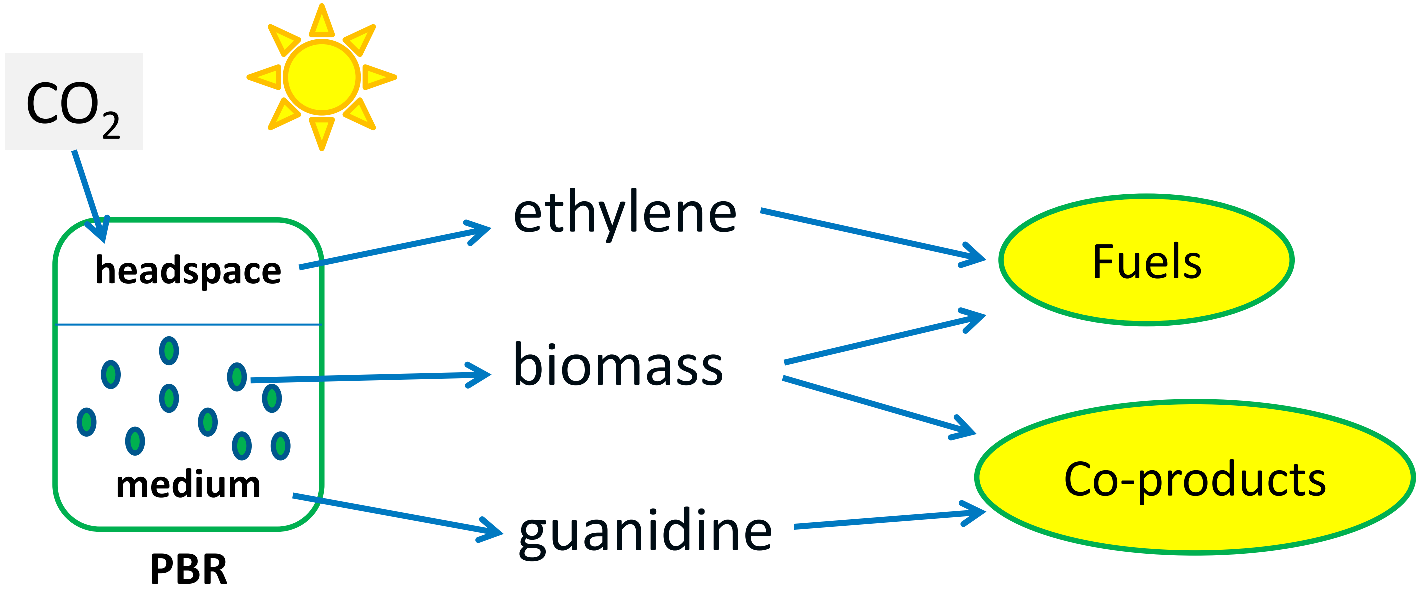
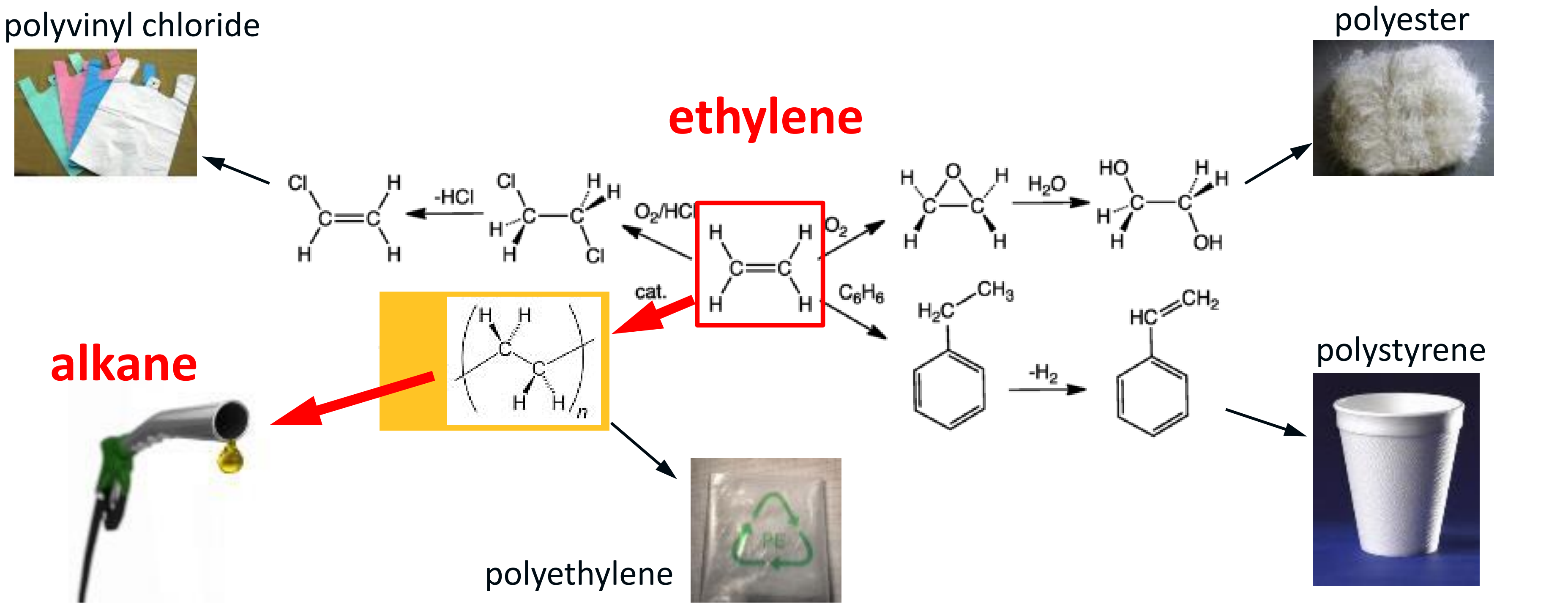
1. Overview: Goal Statement

Develop cyanobacterial biofuel technology via the production of ethylene, biomass and co-products



Overview: Ethylene

- Ethylene is the most produced petrochemical (>150 million tons per year).
- It is a versatile feedstock for conversion to both chemicals and fuels.
- The current method of producing ethylene, steam cracking of petroleum, is the largest CO_2 emitting process in chemical industry.

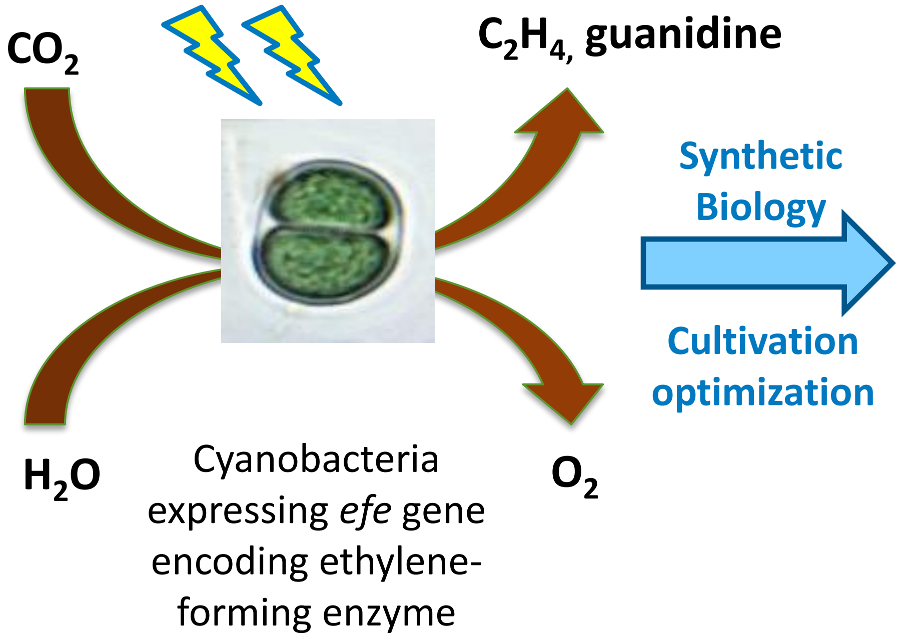


Overview: Cyanobacteria

- **Powerful genetic tools**, some developed in this project, allow us to redirect carbon flux in cyanobacteria by design, toward production of specific fuel intermediates and chemicals.
- Cyanobacteria have demonstrated the **highest photosynthetic conversion efficiency** of any microalgae.
- **Cyanobacterial ethylene: let sunlight do more work—biomass plus two products**
    - Produces ethylene, which self separates from the culture, and is the only organic compound in headspace.
    - Produces guanidine, a platform chemical and potential nitrogen fertilizer, as co-product from ethylene-forming reaction.
    - Protein-rich (half of CDW) biomass has potential as animal feed or feedstock for HTL.
    - High value co-products may include pigments such as phycocyanin.
    - The best part: stimulates photosynthesis.

Overview: Project History

- Project started in FY10.
- Rapid productivity improvement enabled by synthetic biology tools.
- Demonstrated ethylene production with seawater.



- 1,000X productivity improvement
- Ungerer *et al.*, *Energy & Environmental Science* 2012
- R&D 100 Award, Editor's Choice Award 2015
- NREL FY16 Outstanding Achievement Award
- US patent awarded in 2016


2015
R&D 100
EDITORS CHOICE
Mechanical/Materials
Cyanobacterial bioethylene
NREL
Presented to
National Renewable Energy Laboratory
for the development of
Cyanobacterial bioethylene
Selected as
One of the 100 Most Technologically Significant
New Products of the Year in Mechanical/Materials
R&D 100 AWARDS Winner
2015
Bea Riemschneider
R&D 100 Awards Editorial Director

Quad Chart Overview

Timeline

- Start date: 10/1/2015
- End date: 9/30/2018
- 50% completion
- Ongoing

Budget

	Total Costs FY 12 –FY 14	FY 15 Costs	FY 16 Costs	Total Planned Funding (FY 17-Project End Date)
DOE Funded	602K	321K	400K	800K

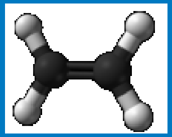
Barriers

- **Aft-C. Biomass Genetics and Development.** We develop genetic and biochemical tools to increase photosynthetic productivity.
- **Aft-D. Sustainable Harvesting.** Ethylene self-separates from the culture; guanidine is excreted into medium.

Partners

- Academic and industry collaborations

2 – Approach (Management)



Single lab project with two tasks:

1. Strain development for ethylene-based chemicals
2. Process development and techno-economic analysis

Team members, monthly meetings

Bo Wang, Lauren Magnusson, Wei Xiong – molecular biology, engineering;

Ling Tao, Jennifer Markham – process development, TEA

Damien Douchi – Swiss National Science Foundation Postdoc Fellow; molecular biology.

Academic collaborations

Sharing of strains and DNA

Joint publications

Grant applications

Industry interactions

Webinars and onsite visits

Grant applications

Tech licensing

2 – Approach (Technical)

Potential challenges

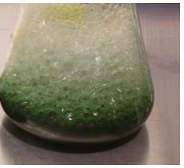
- Low photosynthetic productivity.
- High cost of PBR cultivation system and high energy consumption.
- Ethylene, guanidine and biomass harvesting.

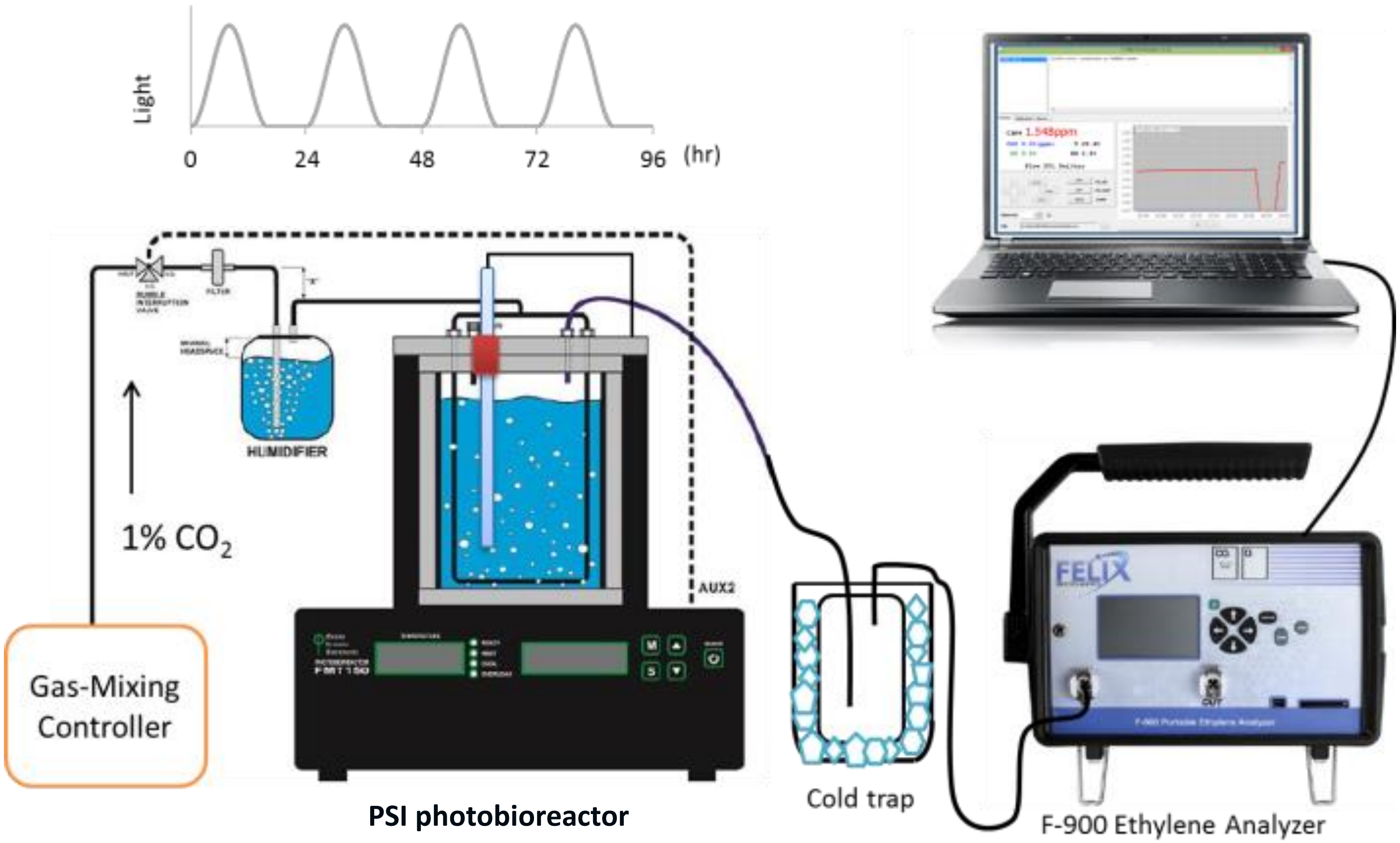
Critical success factors

- Demonstrate cost effective production of fuel and chemicals based on ethylene, biomass, and co-products.
- Develop low cost photobioreactor development suitable for biomass growth and ethylene/guanidine harvesting.

Approach (Technical), continued

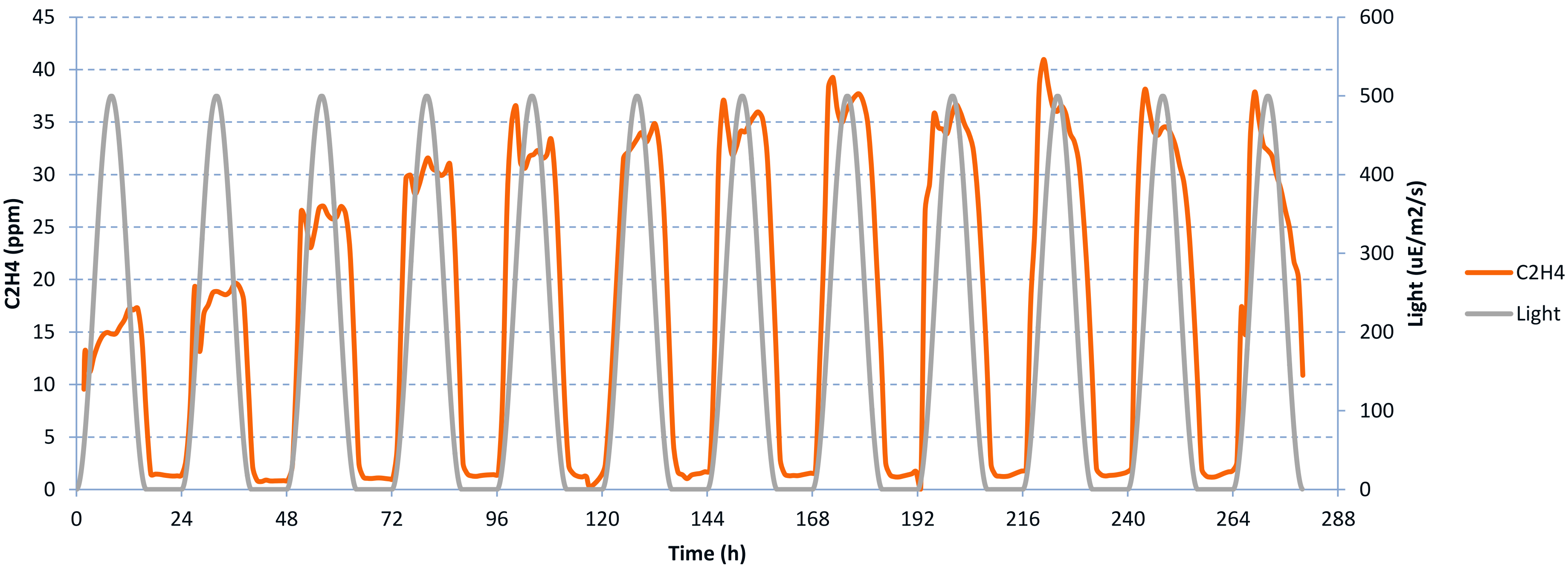
- Establish continuous ethylene production process in photobioreactor, capable of operating under simulated diurnal conditions. Obtain a baseline productivity and improve from there. This addresses prior reviewers' comments.
- Increase productivity by identifying and overcoming limiting factors in ethylene production
- Identify co-products including biomass valorization to help with economics
- Develop bioethylene process and cost model to drive research.





Continuous ethylene production and online monitoring system, enables analysis under simulated outdoor conditions

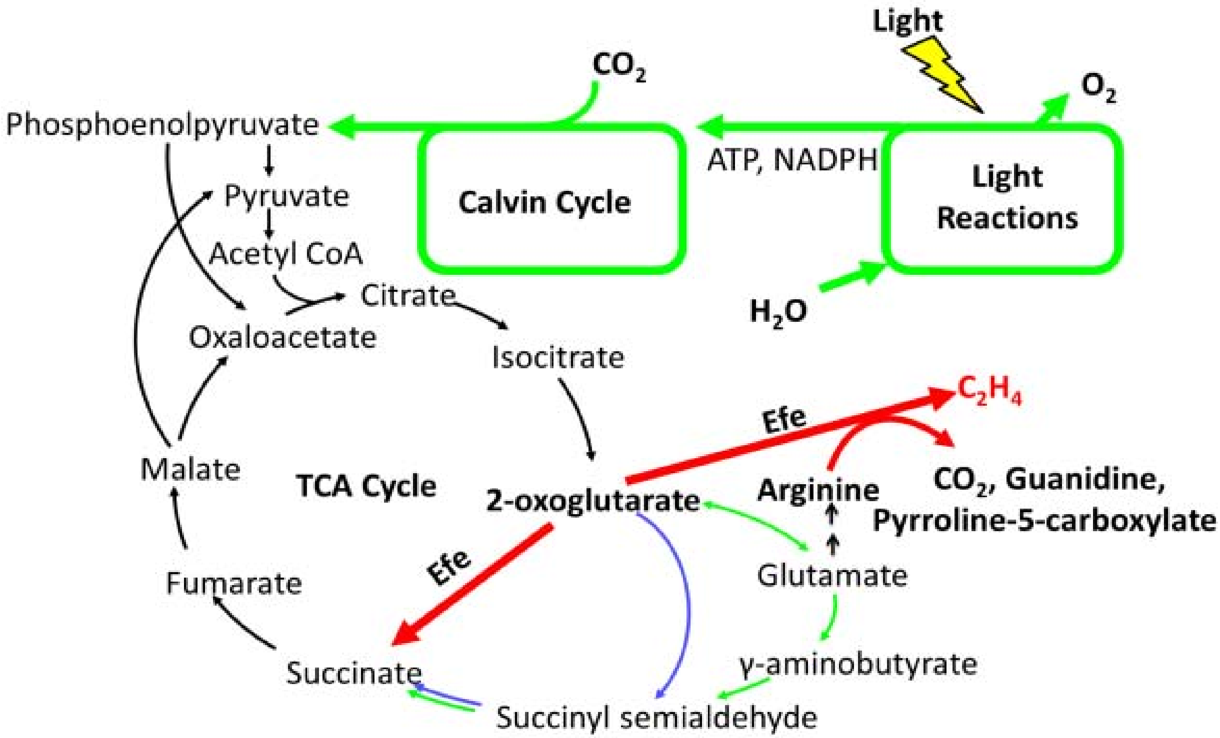
Real Time Monitoring of Ethylene Production Under Simulated Outdoor Conditions



New process relevant reactor configuration provides key insights for further process improvements

- Baseline ethylene productivity from PB646 strain is 15 mg/l/day.
- Carbon partition ratio (ethylene vs fixed carbon) is about 20%.
- Light energy is underutilized in the middle of the day. There is low level of ethylene production at night at the expense of biomass.
- How can these be improved? – Smaller antenna? Manipulate glycogen degradation rate in the dark? Identify limiting factors in current strain.

Ethylene production sheds light on photosynthesis



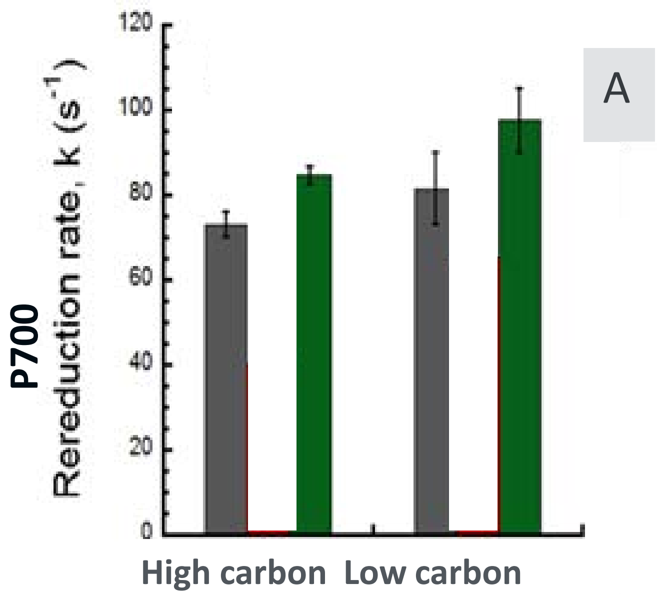
- Introduction of a novel pathway could cause metabolic imbalance or generate toxic intermediate, thus slowing down photosynthesis and carbon flux.
- Yet the ethylene strains grow as fast as WT in high carbon conditions despite losing carbons via EFE reaction.
- Using metabolic flux analysis, we showed that ethylene production rewires central carbon metabolism, with cyclic TCA operation and 3X flux into TCA cycle, and stimulates photosynthesis by up to 19% in strain JU547.

Xiong *et al*., Nature Plants, 2015

Both Light Reactions and Carbon Uptake Are Stimulated

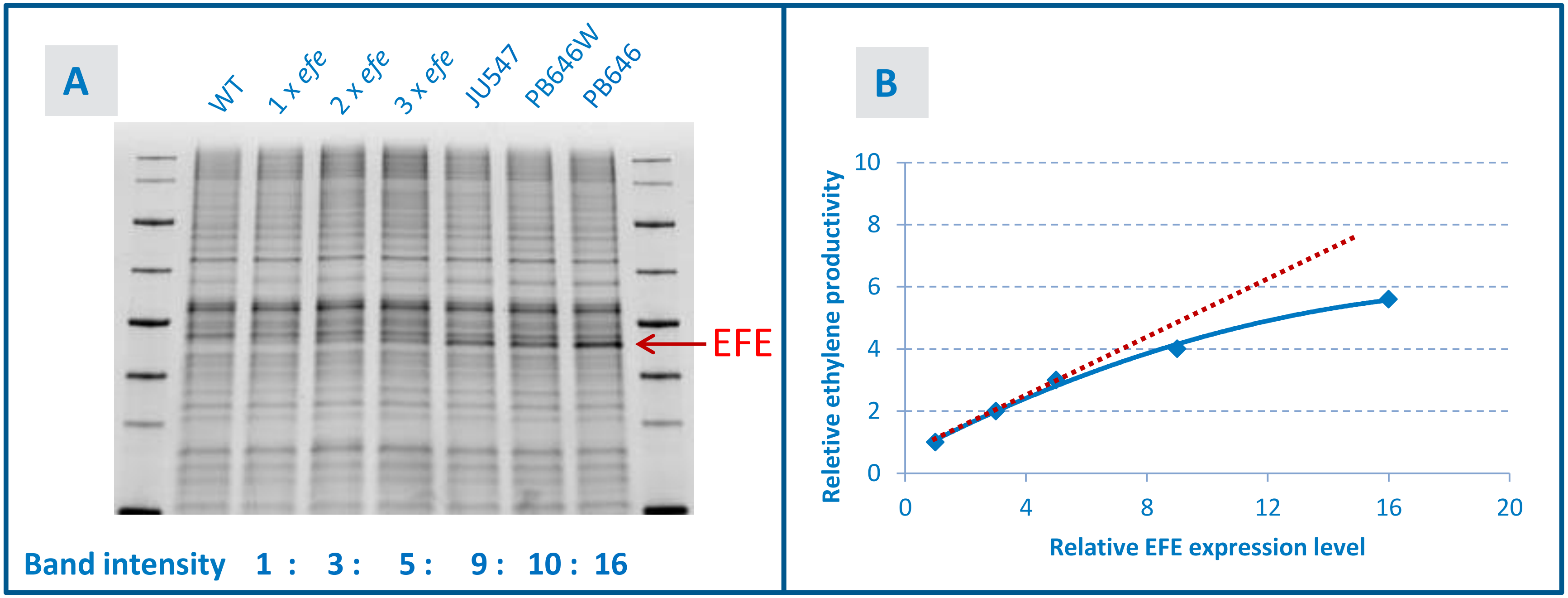
A. Increase in electron transfer capacity in EFE strain (JU547, green columns) versus WT (gray columns)

B. Increase in a bicarbonate transporter SbtA under low carbon conditions.



- We are getting more out of photosynthesis. How much further can we go?
- Can we apply the lessons to eukaryotic algae and plants?
- Holland *et al*., Algal Research, 2016.

Rate Limiting Step in Ethylene Production



- Enzyme or substrate?
- Initially EFE level was limiting, and was linear to ethylene productivity.
- That has changed as we steadily increased EFE levels (A, B).
- AKG did not appear to be limiting.

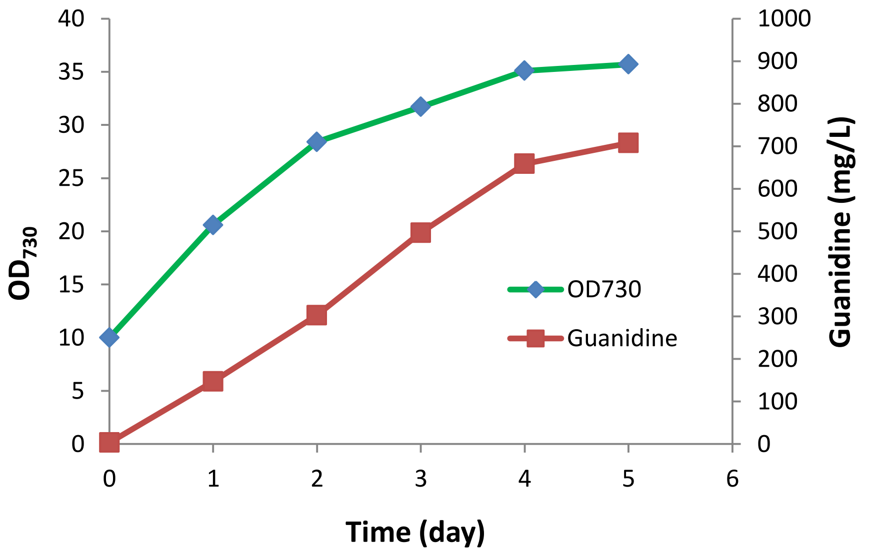
EFE

$3\ AKG + 3\ O_2 + Arginine \rightarrow 2\ Ethylene + Succinate + P5C + Guanidine + 7\ CO_2 + 3\ H_2O$

Arginine Supply May Limit Ethylene Productivity

- We are looking into arginine synthesis and degradation pathways
- Is guanidine recycled back to arginine? Appears not.
- We identified a putative agmatinase that could recycle guanidine to arginine in cyanobacteria. Working on its expression.

Meanwhile, significant amount of guanidine is found in the medium! Intracellular concentration is 100 X higher. This could be a co-product that help with economics and to accelerate commercialization.



Guanidine Is a Platform Chemical

Guanidine

Pharmaceuticals

Sulfaguanidine
Sulfamethazine
Pyrimethamine
Trimethoprim
Sulfadiazine
Folic acid
Triamterene
Methotrexate

Pesticides

Imidacloprid
Thiamethoxam
Sulfonylurea herbicide
Bensulfuron-methyl
Clothianidine
Halosulfuron-methyl

Propellants/ Explosives

Guanidine picrate
Guanidine nitrate
Guanidine perchlorate
Nitroguanidine

Denaturants

Guanidine hydrochloride
Guanidine thiocyanate

Flame Retardants

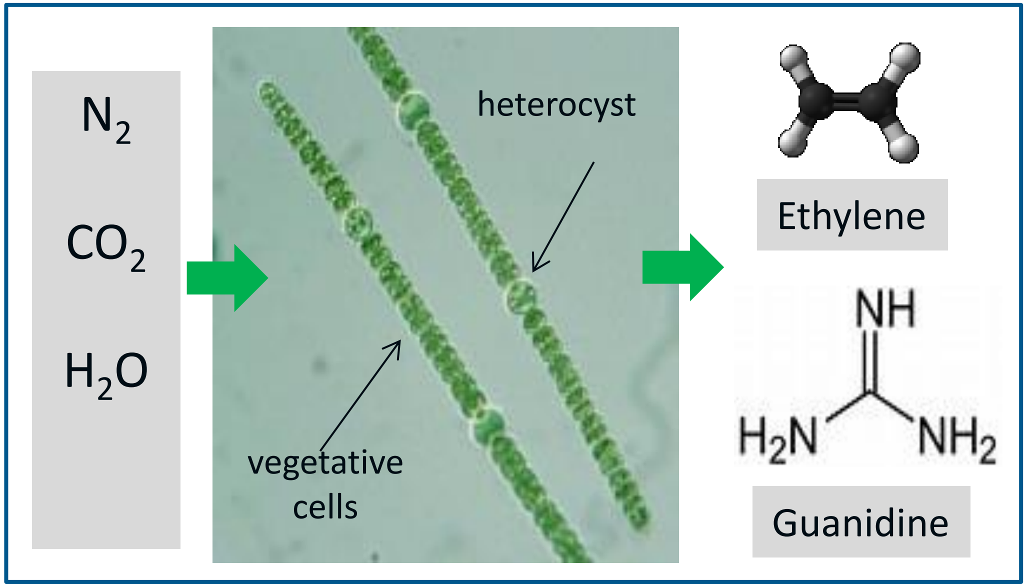
Guanidine stearate
Guanidine sulfamate
Guanidine hydrochloride
Guanidine Phosphates

Other Derivatives

Alkylguanidines
Aminoguanidine
Pyrazoles
Biguanide
1,2,4-triazines
Organoguanidines
Arylguanidines
1,2,4-triazoles
thiadiazoles
Robenidine
nitroguanidine
pyrimidines
tetrazoles
Triaminoguanidine

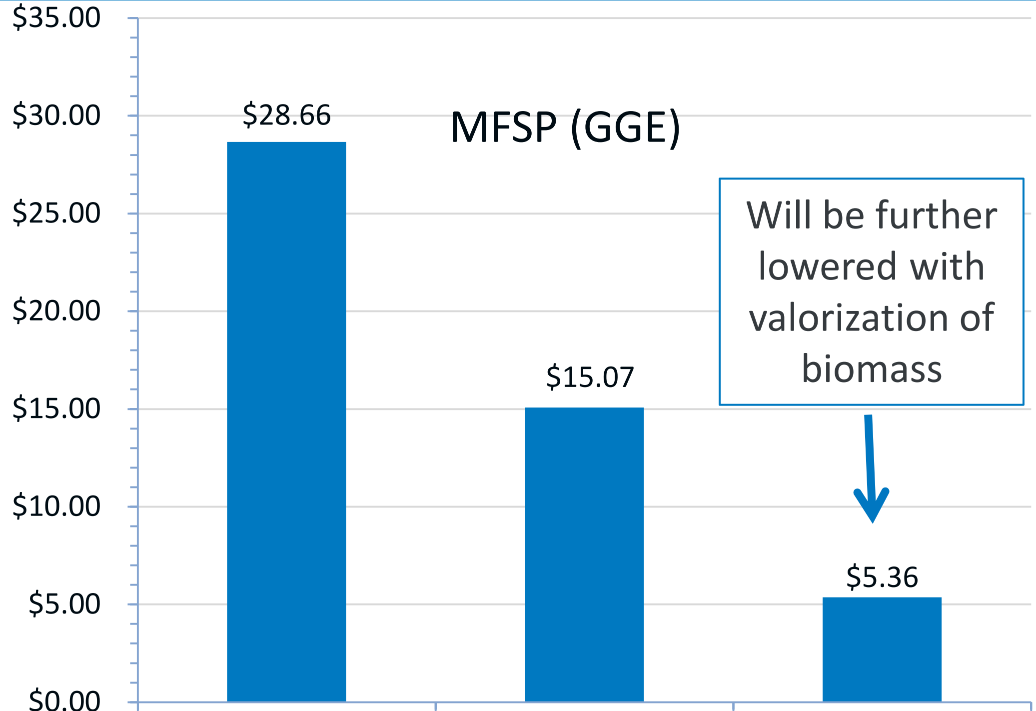
Producing Guanidine by Nitrogen Fixation

- If we are to produce a nitrogen-rich platform chemical or fertilizer from cyanobacteria, why not take advantage of their capability of N_2 fixation?
- In collaboration with South Dakota State University, we generated *Anabaena* strains expressing EFE. Under N_2-fixing condition, ethylene and guanidine production was observed.
- This could be developed into an alternative to the energy-intensive Haber-Bosch process, which is responsible for 3% of carbon emissions worldwide.



Preliminary Bioethylene Process Design and Cost Model

- First TEA on photosynthetic ethylene process. Focused on ethylene alone.
- Photosynthetic productivity has the largest impact.
- Path to low cost biofuels should include guanidine as well as valorization of biomass such as pigments.



	Near-Term Target	Mid-Term Target	Long-Term Target
Ethylene Productivity [g/m²/day]	3.15	7.19	18.78
% of Total Irradiance to Biomass & C_2H_4	11%	11%	13%
Energy Partitioning to Ethylene	75%	78%	90%
Photon Transmission Efficiency	86%	86%	95%
Biomass & C_2H_4 Accumulation Efficiency	50%	55%	60%
Quantum Requirement	48	24	16
Cost of CO_2 ($/metric ton)	$40	$40	$0
Turnover Time	60 days	60 days	330 days
Separation Method	CD	CD	ABS
Type of Cultivation System	PBR	PBR	Covered Pond-No Liners

4 – Relevance

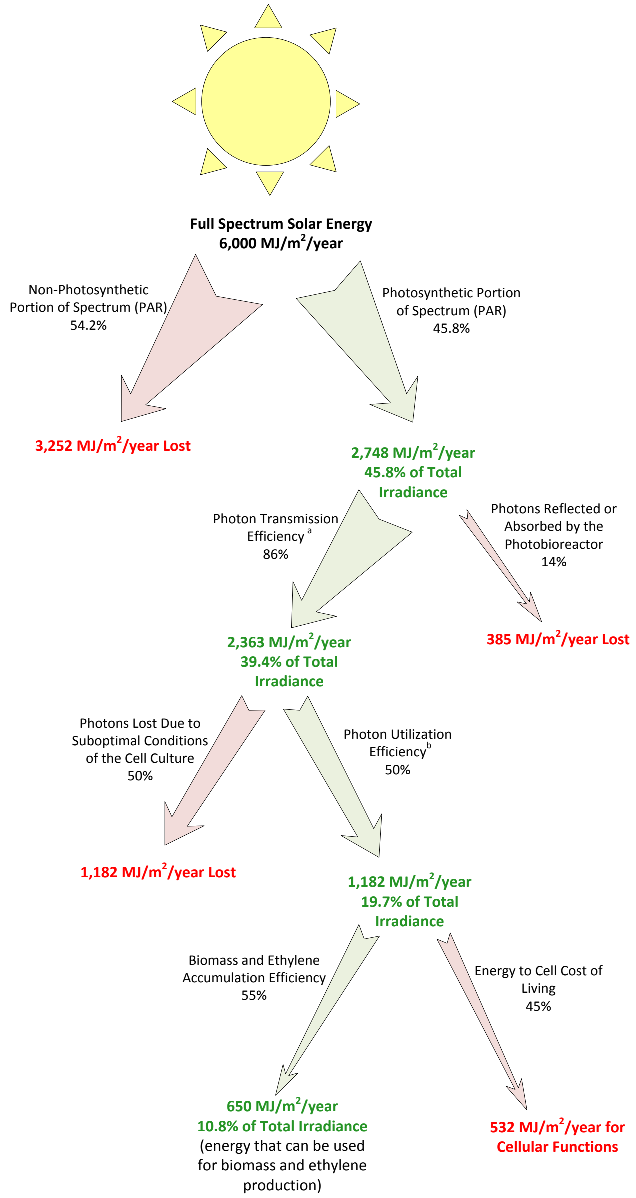
- This is a different paradigm that reduces risk in algal biofuels development.
- Ethylene production is getting more out of photosynthesis; lessons may be transferred to other algae and plants.
- The findings attracted many researchers who can help us develop strategies to enhance productivity.
- We develop advanced genetic and biochemical tools such as promoters and fluxomics that are used by others.
- Membrane separation system for bioethylene is being developed by industry.
- We continue to have dialogue with industry on technology licensing.

5 – Future Work

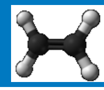
- Increase ethylene productivity by directing more carbon (than 20%) away from biomass growth and toward ethylene synthesis.
  - Overcome substrate supply limitation by enhancing arginine synthesis and recycling pathways, and by clock manipulation.
  - Overexpress the putative agmatinase in *efe* strains to improve arginine supply (FY 17 Q2 Go/NoGo milestone)
  - Achieve 50% increase in ethylene productivity over baseline (FY17 year-end milestone), and further increase in out years.
- Use PBR system to identify new targets for productivity improvements.
- Update TEA model to include guanidine and biomass valorization.
- Collaborate with industry on ethylene/guanidine harvesting using membranes.
- **Apply insights on photosynthesis improvement to this and other systems**

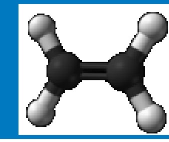
Understanding Energy Flow in Photosynthetic Productivity

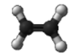
- Revisit theoretical maximum photosynthetic energy conversion efficiency by looking at energy conservation vs loss (diagram on right).
- Measure and optimize efficiency in ethylene-producing cyanobacteria. In WT, the amount is up to 12.5% (absorbed light) in literature.
- High efficiency leads to low cost.
- Markham *et al*, *Green Chemistry*, 2016.



Summary







- We have demonstrated continuous photosynthetic CO_2 to ethylene production and online monitoring system.
- Current best strain converts about 20% of fixed carbon to ethylene in simulated diurnal cycles. Still a large room for improvement.
- Enhanced EFE expression to the point that something else in metabolism (likely arginine supply) is becoming rate limiting.
- Ethylene production stimulates photosynthesis at both light reactions and carbon metabolism.
- Identified guanidine as a co-product, and produced by N_2 fixation.
- Developing bioethylene process design and cost analysis to include ethylene, guanidine, and biomass valorization.
- Working with industry on ethylene/guanidine harvesting.
- Working with academia on regulation of photosynthesis.

Acknowledgements

Team members

Collaborators (listed in Additional Slides)

BETO funding

Thank you for attention!

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Additional Slides

Responses to Previous Reviewers' Comments

- Comment: More work needs to be done to characterize what this system may look like at scale, and research efforts should be tailored to best promote the establishment of a viable system.

- Response: We have set up continuous ethylene production and monitoring system, operating under simulated diurnal light conditions. Results from this system are guiding further studies.

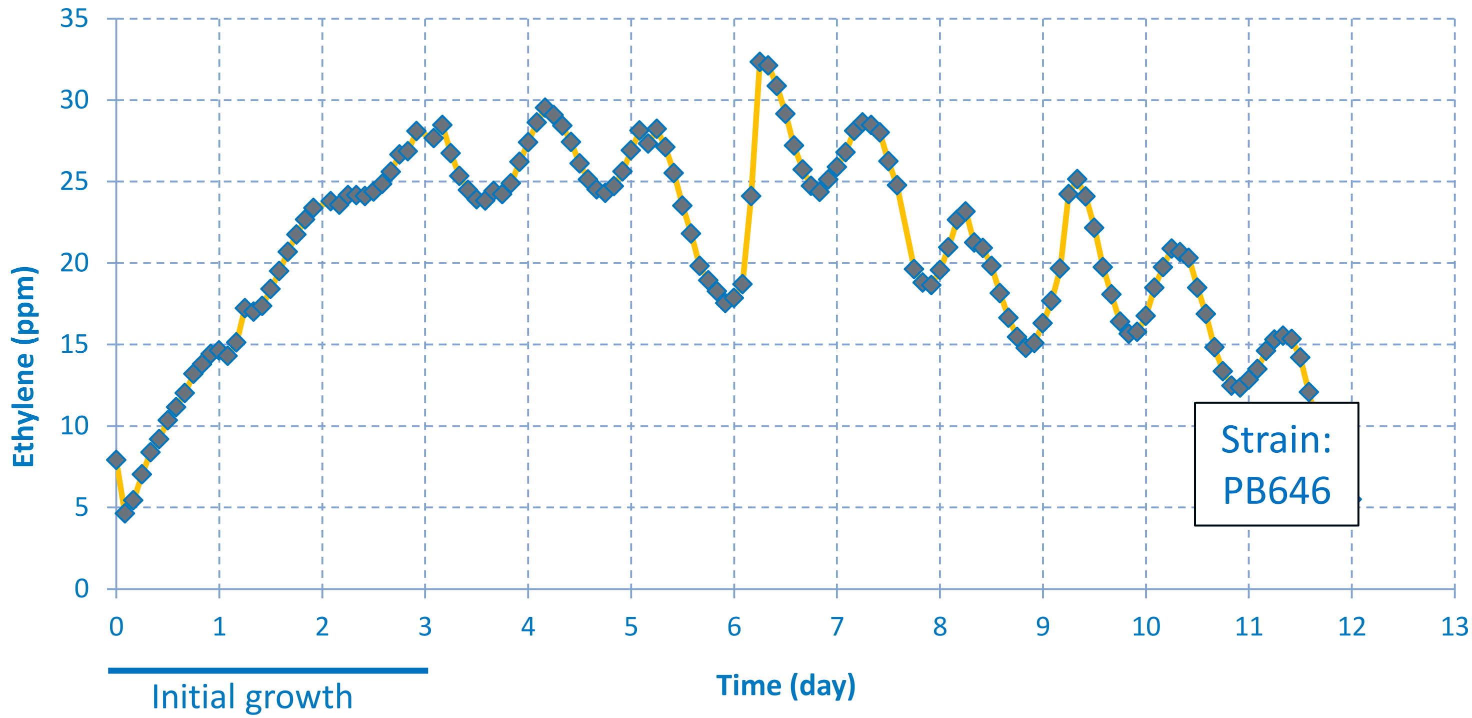
Publications, Patents, Presentations, Awards

- **R&D 100 Award and Editor's Choice Award in the Mechanical/Materials Category, 2015.**
- **FY16 Outstanding Achievement Award, NREL**
- **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.**
- **Bo Wang, Jianping Yu, Weiwen Zhang, Deirdre R. Meldrum. Pre-methylation of Foreign DNA Improves Integrative Transformation Efficiency in *Synechocystis* sp. PCC 6803.*Applied and Environmental Microbiology* 2015. doi:10.1128/AEM.02575-15**
- **Tai-Chi Lee, Wei Xiong, Troy Paddock, Damian Carrieri, Ing-Feng Chang, Hui-Fen Chiu, Maria Ghirardi, Justin Ungerer, Suh-Hang Hank Juo, Pin-Ching Maness, Jianping Yu. Engineered xylose utilization enhances bio-products productivity in the cyanobacterium *Synechocystis* PCC 6803. *Metabolic Engineering*. 2015. DOI 10.1016/j.ymben.2015.06.002.**
- **Wei Xiong, John A. Morgan, Justin Ungerer, Bo Wang, Pin-Ching Maness, and Jianping Yu (2015) The plasticity of cyanobacterial metabolism supports direct CO_2 conversion to ethylene. *Nature Plants*, DOI 10.1038/nplants.2015.53**
- **US patent. Biological Production of Organic Compounds. 2013-0203136.**
- **Presentations at Algal Biomass Summit, American Chemical Society, Gordon Conference, Michigan State University, South Dakota State University, Workshop On Cyanobacteria, International Conference on CO_2 Utilization, Colorado School of Mines.**
- **Major chemical companies and a membrane separation company reviewed NREL TEA draft and provided inputs on ethylene harvesting technology. A PBR company studied continuous ethylene production. There is ongoing discussion with investors on technology licensing.**

Academic collaborations

- Lars Angenent, Cornell University. PBR development; 1 joint TEA paper, 4 other papers using our ethylene-producing strain.
- Ryan Gill, University of Colorado. *E. coli* model for ethylene production and ethylene-forming enzyme mutagenesis. 2 joint papers.
- John Morgan, Purdue University. Metabolic flux analysis. 1 joint paper on the analysis of our ethylene-producing strain.
- PSI, PBR development (Czech). 1 paper using our ethylene-producing strain.
- Rob Burnap, Oklahoma State University. 1 joint paper on the stimulation of photosynthesis by ethylene production.
- Diana Kirilovsky, CEA (France). Photosynthetic energy dissipation in ethylene-producing strains. Joint proposal successful.
- Joshua Yuan, TAMU. 1 paper (PNAS) using our promoter. Proteomics analysis that helped identify a putative agmatinase involved in arginine metabolism.
- Ruanbao Zhou, South Dakota State University. Generated *efe* strains of the nitrogen-fixing cyanobacterium *Anabaena* 7120, with potential in converting N_2 to guanidine.
- Robert Hausinger, Michigan State University. Ethylene-forming enzyme reaction mechanism. 1 paper using our *efe* gene.
- Ken Reardon, Colorado State University. Thin film photobioreactors.

Ethylene Productivity Is Regulated by Biological Clock



- We may learn about limiting factors by operating PBRs under simpler conditions.
- We saw cycling of ethylene productivity in continuous light.
- Which component(s) of ethylene production is regulated by the clock?
- Can we increase ethylene productivity by engineering the clock?
- We are collaborating with Vanderbilt University on this topic.