DOE Bioenergy Technologies Office (BETO) 2019 Project Peer Review

1.3.5.270 Rewiring Algal Carbon Energetics for Renewables (RACER)

Advanced Algal Systems

March 6, 2019

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National Renewable Energy Laboratory

Goal Statement

RACER

Goals: Improve the overall carbon-to-fuel intermediate productivity for a biorefinery using *D. armatus* as a production species to reach at least 3,900 gal $acre^{-1}$ yr^{-1}

- Improvements in photosynthetic carbon conversion efficiency through random mutagenesis and targeted engineering
- Cultivation management advances through implementation of informed permutations of operations and nutrient management
- Tailoring and optimizing conversion processes to extractable lipids, carbohydrate-derived fuel intermediates, and HTL biocrude from protein residue

Relevance: Carbon conversion efficiency improvements by coordinating photosynthesis and carbon sink engineering is the basis of biomass accumulation and biofuels production, core to BETO's AAS program

Outcome: This project will yield a high-impact holistic process integration approach to demonstrate that improvements in strain engineering, cultivation operations and conversion engineering, can yield productivity improvements and cost reductions

Quad Chart Overview

Timeline

- Start: FY2018
- 3-year competitive award
- 40% complete

	FY 17 Costs	FY 18 Costs	Total Planned Funding (FY 19-Project End Date)
DOE Funded	$23K	$1.2M	$2.3M
Project Cost Share*	$1.1M	$157K	$315K

Partners: CSU (26%), CSM (5%), ASU (16%), POS Biosciences (2%)

Barriers addressed:

Aft-A. Biomass Availability and Cost: need for data on potential price, location, seasonality, environmental sustainability, quality, and quantity of available algal biomass feedstock creates uncertainty

Aft-C. Biomass Genetics and Development: improvement of productivity and robustness of algae strains against such factors as temperature, seasonality, predation, and competition

Aft-E. Algal Biomass Characterization, Quality, and Monitoring: composition of fundamental components (lipids, carbohydrates, and proteins) of algal biomass need to be reported and controlled

Aft-H. Integration: Integration of co-located inoculation, cultivation, primary harvest, concentration, and preprocessing systems

Aft-I. Algal Feedstock On-Farm Preprocessing: processing or fractionation into lipids, bio-oils, carbohydrates, and/or proteins before these individual components can be converted into the desired fuel and/or products.

Aft-J. Resource Recapture and Recycle: recycling valuable nitrogen, phosphorus, carbon, or micronutrients, replacing fresh fertilizer inputs

Objective

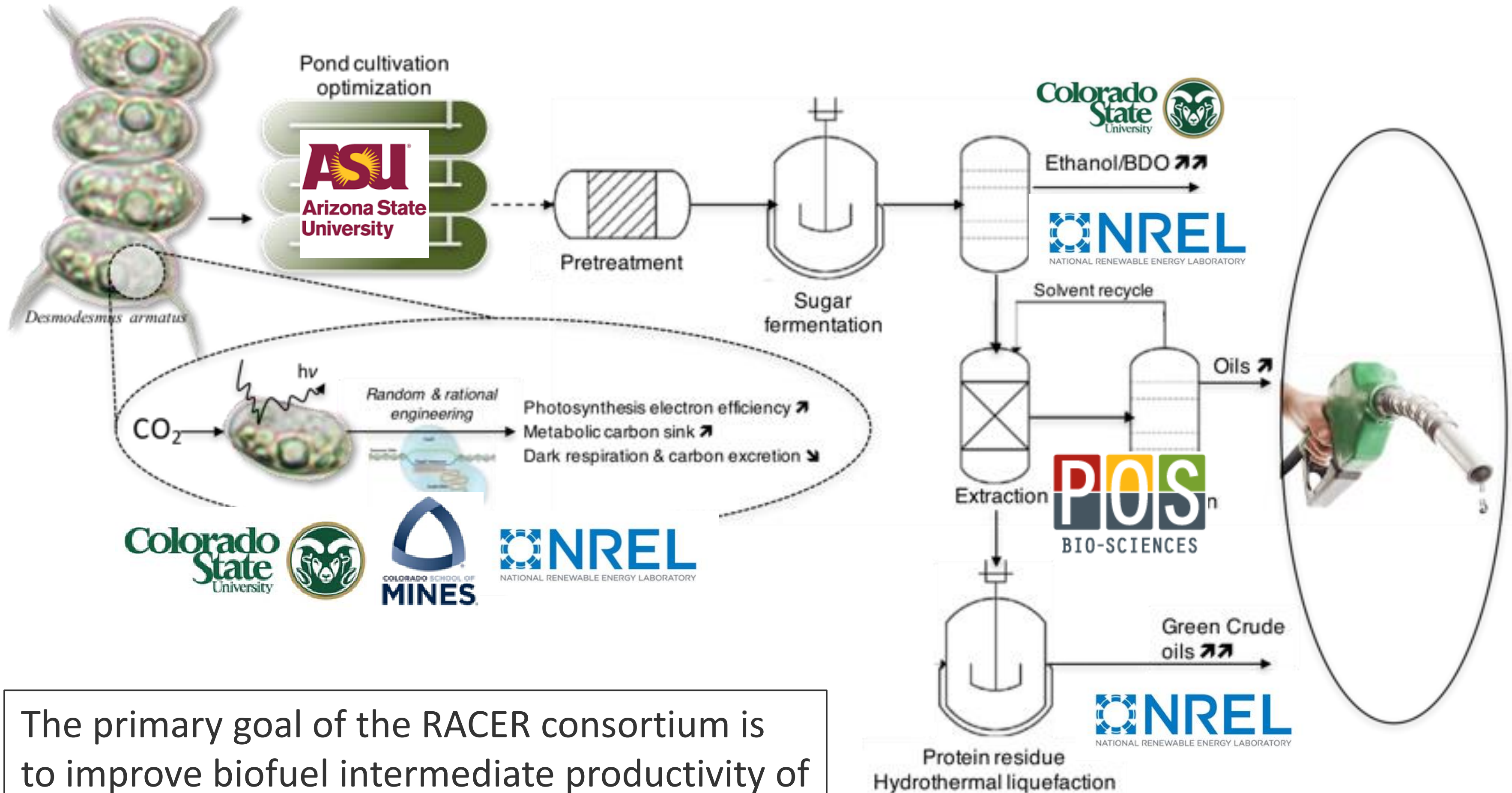
Improve biofuel intermediate productivity of the commercially-relevant ***Desmodesmus armatus*** by addressing critical carbon conversion efficiencies towards a trifecta of fuel intermediates; ethanol or butane diol, lipids, HTL green crude oils

End of Project Goal

Achieve overall biofuel intermediate yield of over 3900 gal acre^{-1} yr^{-1} based on both carbon assimilation and conversion improvements (FY20 MYP goal)

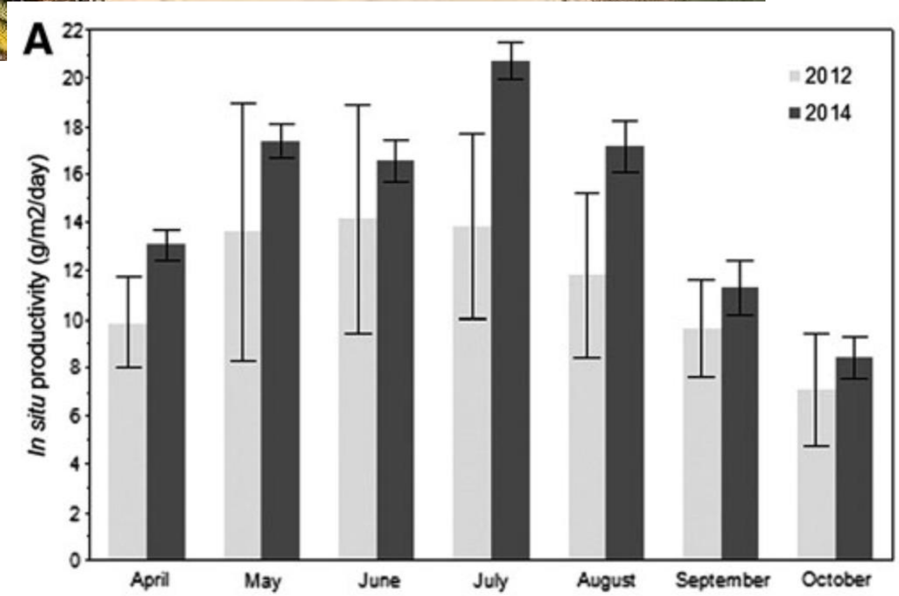
Overview

RACER



The primary goal of the RACER consortium is to improve biofuel intermediate productivity of ***Desmodesmus armatus*** by addressing critical carbon conversion efficiencies towards a trifecta of fuel intermediates, *ethanol or BDO, lipids and green crude oils*

Baseline Productivity – Las Cruces, NM



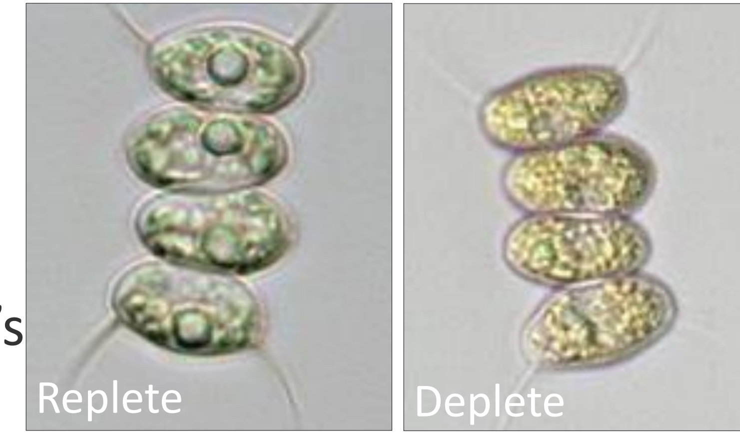
White and Ryan, 2015, Ind. Biotech. 11(4):213

Volume 30,000L outdoor ponds, measured AFDW (g/L) by volume of pond	2014 SE00107 Average of Validated In Situ Productivity (g/m2/day)	2015 SE00107 Average of Validated In Situ Productivity (g/m2/day)
January		3
February		8
March		11
April	13	
May	17	
June	17	
July	21	
August	17	
September	11	
October	9	
November	5	
December	5	
Overall Average:	**11.4**	

Sapphire Energy

Desmodesmus armatus SE000107

- Robust growth, year over year demonstrated productivity at Sapphire's test site (achieving >11 g/m2/day)
- **Available draft genome** (76% annotated) – informed selection of target genes (>9892 genes)
- Transformation and mutagenesis protocols made available
- **Attractive biomass composition for conversion***:



	Desmo SE000107 (Sapphire)	Chlamy v5.5
Contig number	1413	1495
***N50* Contig length**	233.5kb	218.4kb
Genome size	132.5Mb	111.1Mb

	Days of Growth	Biomass (g/L)	Lipid (%DW)	Carbs (%DW)	Protein (%DW)	Ash (%DW)
D. armatus - early	4	0.7	**7.2**	**36.0**	28.0	5.1
D. armatus - mid	9	3.2	**10.8**	**52.3**	13.6	2.5
D. armatus - late	22	6.6	**19.4**	**60.6**	7.7	2.1

*NREL indoor photobioreactor data (simulated Arizona conditions)

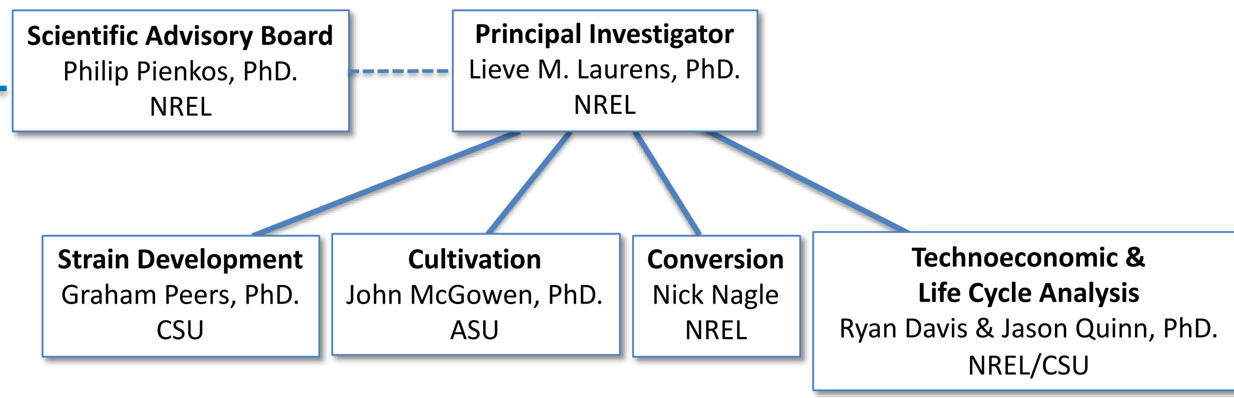
Approach - Management

- Project split into 4 subtasks aligning with project objectives, with biweekly subtask meetings and quarterly all-hands meeting
- Progress tracked through quarterly milestones and internal project deliverables
- **Scientific Advisory Board**: Support for direction and major changes in scope, feedback on approach and commercial relevance
- **Commercialization strategy**: Team has Intellectual Property plan in place and will work with partners to identify and develop opportunities
- **Deliverable**: Successful completion of tasks and milestones will contribute to FY18, FY19 and FY20 State of Technology Assessment

Advisory board:

Lou Brown (SGI)
Shaun Bailey (SGI)
Rebecca White (Qualitas)
Colin Beal (TEA consultant)
Nancy Dowe (NREL Fermentation)
Olaf Kruse (Bielefeld)
Thomas Sharkey (Michigan State)

**Advisory board meeting conducted in December 2018*



Approach - Technical

Objective 1: Strain Development

1.1. Metabolic Engineering Tool Development
1.2 Photosynthesis Engineering for Reduced Alternate Electron Transport
1.3 Metabolic Rearrangement for Increasing Carbon Storage Sinks
1.4 Engineering for Reduced Dark Respiration and Excreted Carbon Losses

Objective 2: Pond Operational Management

2.1 Increased productivity through nutrient and pond operational management
2.2 Submission of TERA Protocols

Objective 3: Conversion to Fuel Intermediates

3.1 Pretreatment for Increased Feedstock Recovery
3.2 Improved fermentation carbon conversion efficiency
3.3 Optimize lipid extraction from fermentation stillage
3.4 Optimization of HTL conversion of protein-rich residue

Objective 4: Techno-economic and Sustainability Analysis

Technical Approach

Demonstrate improvements in overall productivity and fuel yields for *D. armatus*

Critical Success Factors	Technical Challenges	Strategy
Establish metabolic engineering tools	Transient unstable expression of transgenes	Build robust strain development toolbox
Demonstrate physiological phenotype improvement	Variable gene expression may not lead to measurable improvement in cell carbon physiology	Engineering multiple targets using range of promotors and gene-expression elements and developing strong screening tools for mutagenesis approach as alternative for identifying improved strains
Pretreatment of biomass to supply conversion process with high yielding fractions	Biomass component interactions during acid pretreatment, biomass compositional variability	Optimization of small-to-large scale translation of pretreatment with different biomass material
Demonstrate carbon conversion improvements to biofuel streams	Liquors generated vary based on conversion conditions thus yielding different compositional fractions	Use optimized conversion or pretreatment approach at sufficiently large scale from one biomass harvest to supply consistent material to downstream conversion

Project Schedule

March 2019

Targets:

- At least 10% improved biomass productivity for each of strain improvement categories (PBR) and 15% increase in carbohydrate content
- Demonstrate outdoor productivity improvement of 18% to >13 g/m2/day over at least 2 seasons
- At least 10% improvement in CCE for fermentation and 20% increase in carbon yield in fuel intermediates (including 95% extraction efficiency of lipids)

TEA/LCA of intermediate achievements (SOT)

TEA/LCA of final achievements (SOT)

	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9	Q10	Q11	Q12	Q13
Validation Phase	x												
Phase I: Strain and Process Engineering							G/NG						
Phase II: Strain and Process Targeted Optimization													G/NG

TERA Application submitted

TEA/LCA of baseline assumptions

September 2020

Targets:

- At least 50% improvement relative to parent strain in outdoor ponds (1000L) for at least 2 stacked trait strains with increase of 25% carbohydrates over at least 2 seasons
- At least 15% additional increase in CCE above interim baseline combining process improments with improved strain

Accomplishments

Strain Development and Deployment 'Funnel'

RACER

NREL NATIONAL RENEWABLE ENERGY LABORATORY

COLORADO SCHOOL OF MINES

Colorado State University

Vector design and transformation optimization

Oct 2017

9 overexpression targets
3 knock-out targets (RNAi + CRISPR)

Outdoor baseline WT cultivation

Summer 2018 AzCATI Arizona Center for Algae Technology and Innovation

Genotyping and stability testing

Oct 2018

Confirm transformation and genotype – **select 4**

Controlled cultivation mimicked conditions

Feb 2019

Demonstrate indoor phenotype – **select 2**

Outdoor improved strains cultivation

Summer 2019 AzCATI Arizona Center for Algae Technology and Innovation

Outdoor deployment

Combining best traits selected from performance testing

Jan 2020

Trait Stacking – **select 1**

Final demonstration

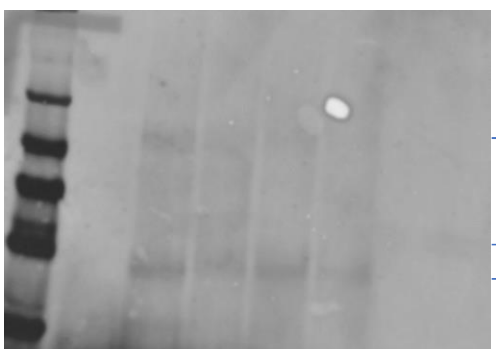
Summer 2020 AzCATI Arizona Center for Algae Technology and Innovation

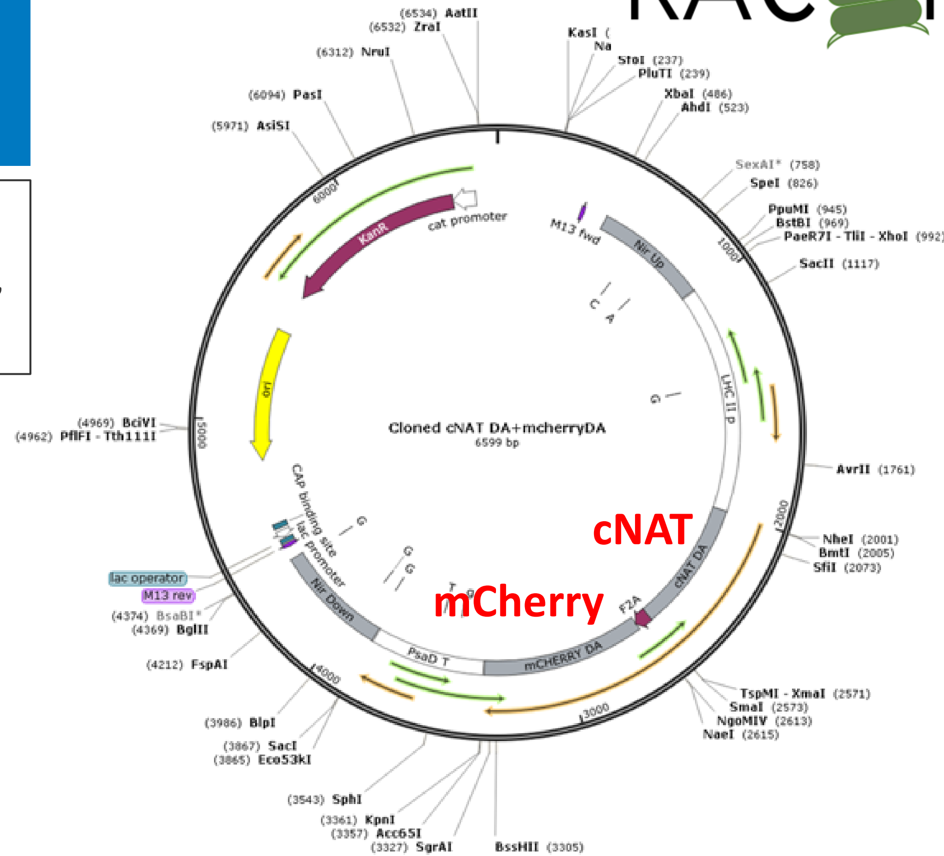
Outdoor

Molecular Biology Tool Development

Over 136 different conditions tested, biolistics, electroporation, Agrobacterium, across 3 different laboratories

First demonstration of genomic transformation of *D. armatus* – electroporation, cNAT resistance and mCherry reporter by Western blot

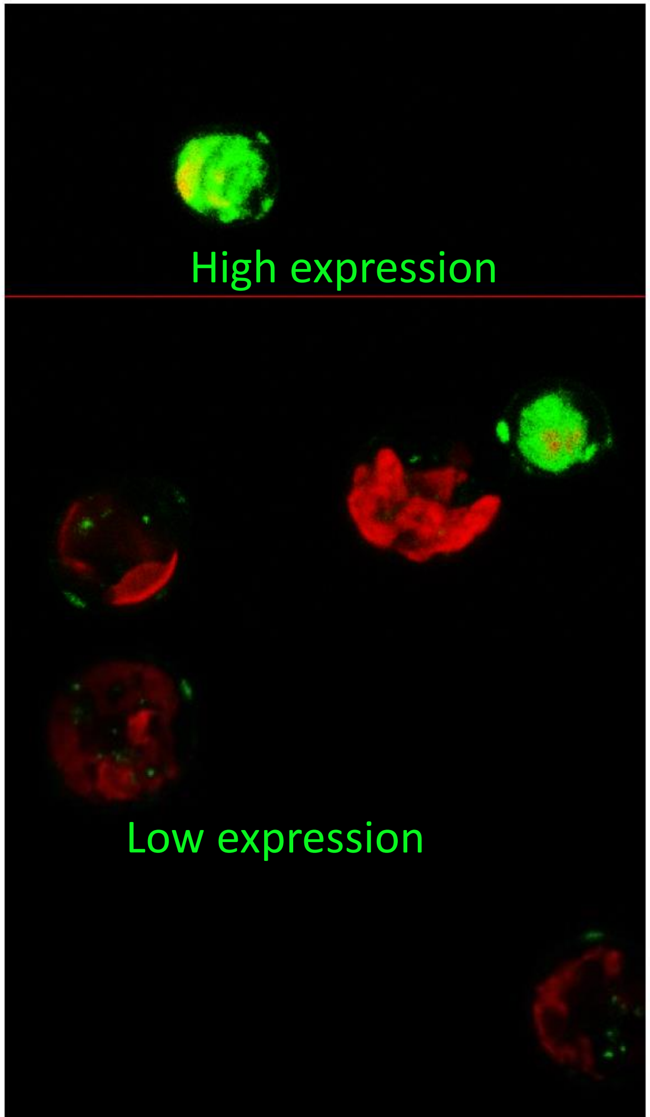




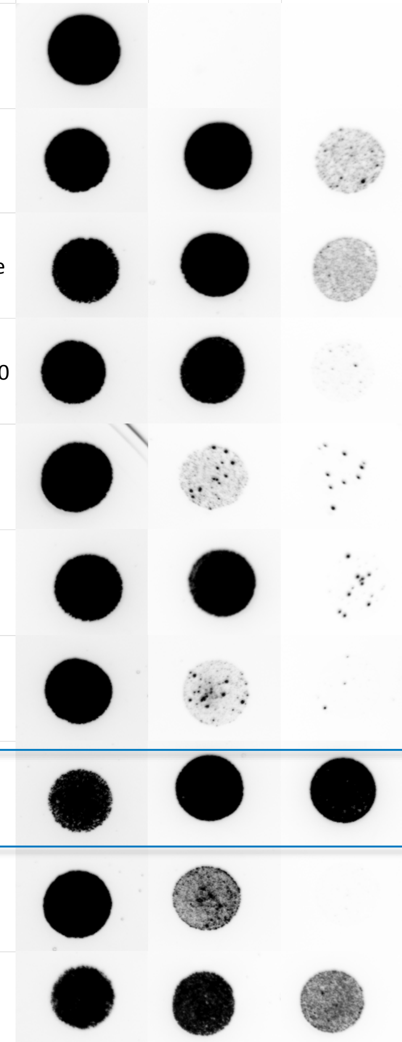
D. armatus genome sequence assembly - improvement

Assembly Type	Contigs	Nucleotides	N50
Illumina	1,413	132 Mb	233 Kb
PacBio	906	116 Mb	342 Kb
hybrid	528	115 Mb	670 Kb

Expression Optimization



Promotor screening and selection based on CNAT antibiotic resistance indicates at least 3 strong promotors – forming the basis of strain engineering efforts:



	control	cNAT 200	cNAT 400	prom
wt				
c6				LHCII g5945
45ae				LHCII g5945
72-10				LHCII g5945
73a				CaMV
74a				Rbs40
75b				RbcS
76c				El Fact
77a				LHCII g137
77b				LHCII g137

Strongest promotor – Elongation Factor

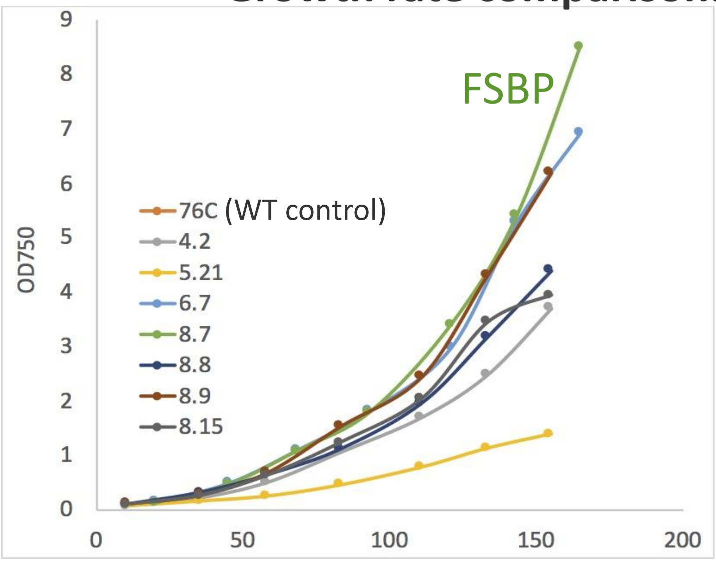
Overexpression targets

#1 = Levansucrase in the vacuole – no transformation yet
#2 = Cellulose synthase – no transformation yet
#3 = Fbp-Sbp (D-fructose 1,6-bisphosphatase class 2/sedoheptulose 1,7-bisphosphatase)
#4 = Levansucrase in the cytoplasm – 2 transformant lines
#5 = PGM (Phosphoglucomutase) – 2 transformant lines
#6 = g7207 - 7 transformant lines
#7 = fbaA (Fructose-bisphosphate aldolase class 2)
#8 = FSBP (Fructose-1,6-/sedoheptulose-1,7-bisphosphatase) – 15 transformant lines
#9 = ADP-glucose pyrophosphorylase – no transformation yet
#10 = Isoamylase – no transformation yet

**Calvin cycle carbon assimilation*
**Storage carbon sink*

cNAT (nourseothricin acetyl transferase) resistance marker confirmed insertion:

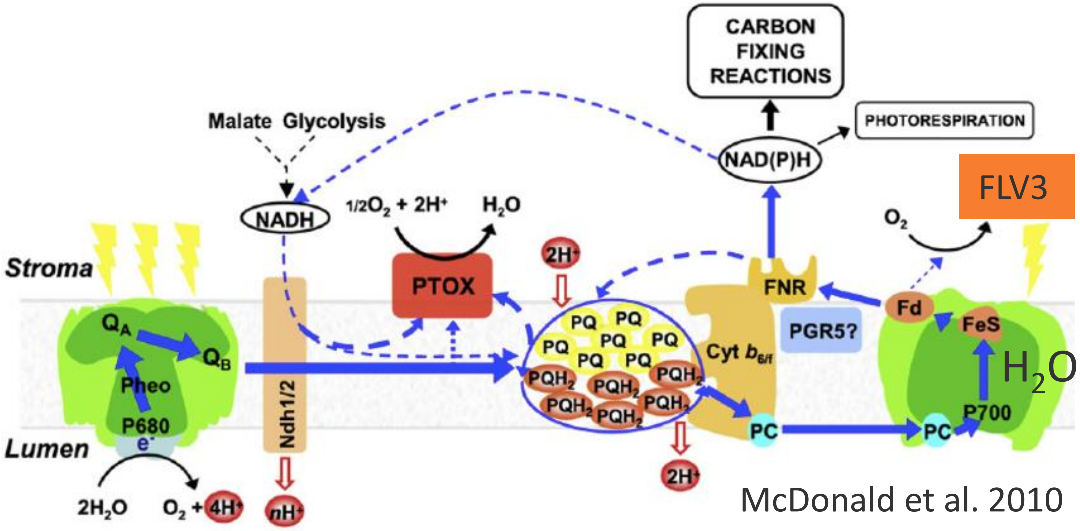
Growth rate comparison:



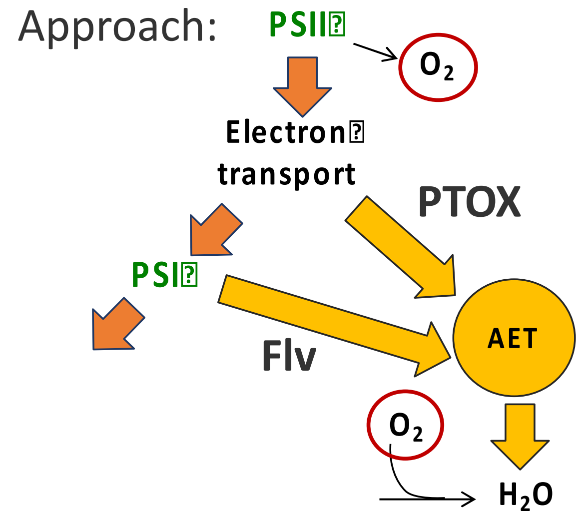
Photosynthesis Engineering

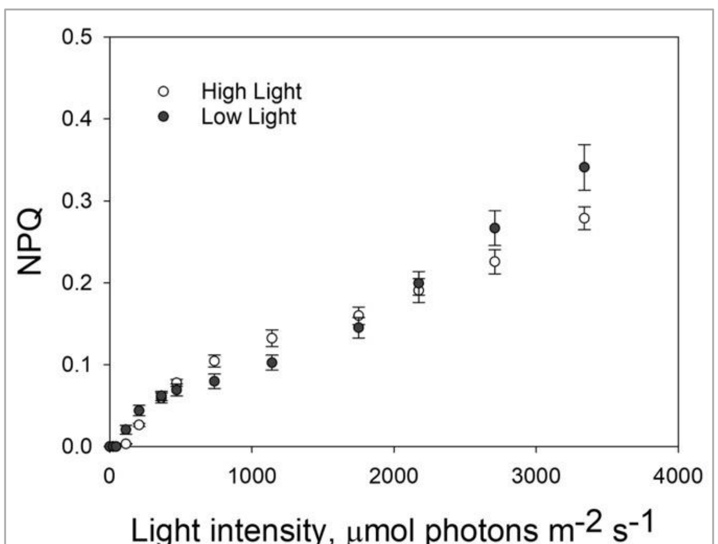
Recapturing energy losses associated with photoprotection:

- Non-photochemical Quenching (NPQ)
- Alternate Electron transport (AET)



McDonald et al. 2010



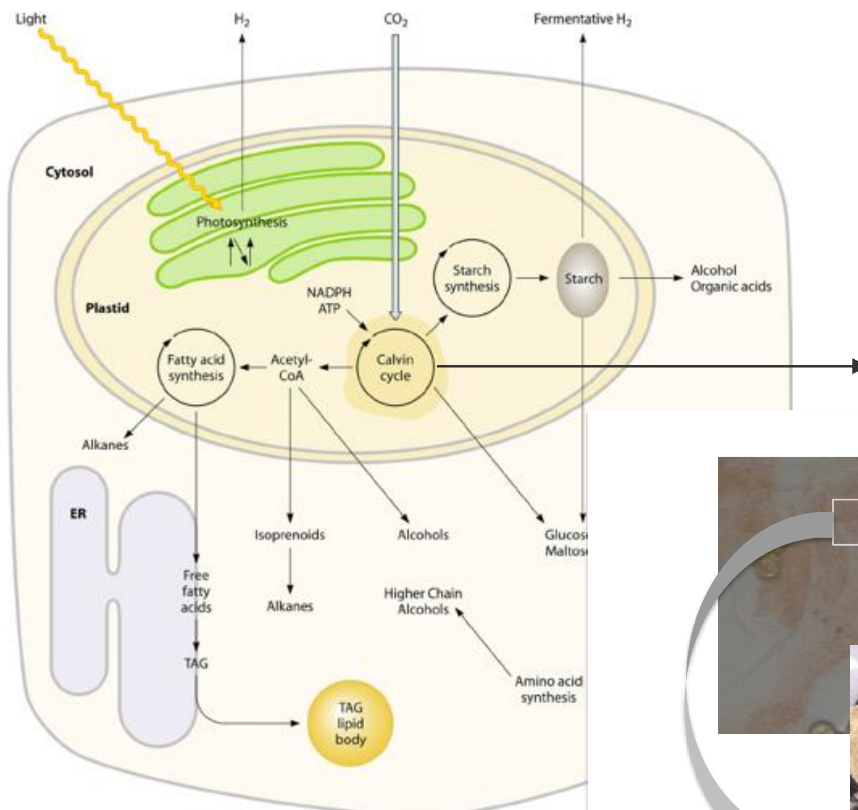


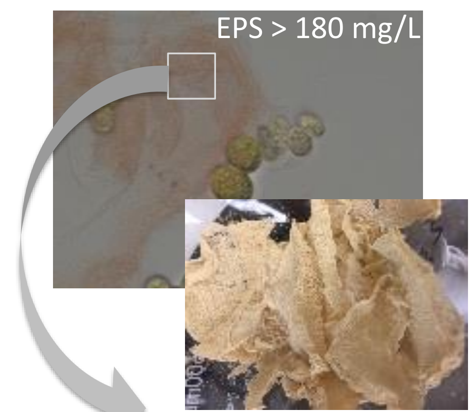
Majority of energy losses during photosynthesis happen through Alternate Electron Transport (AET) Eliminating PTOX and FLV genes is likely to increase biomass productivity

Dynamic Cell Carbon Metabolism Engineering

Can storage carbon metabolism be exploited to drive up carbon efficiency, photosynthesis and ultimately productivity?

How does the alga distribute carbon between different sinks in (and out) of the cell in response to nutrient limitation?



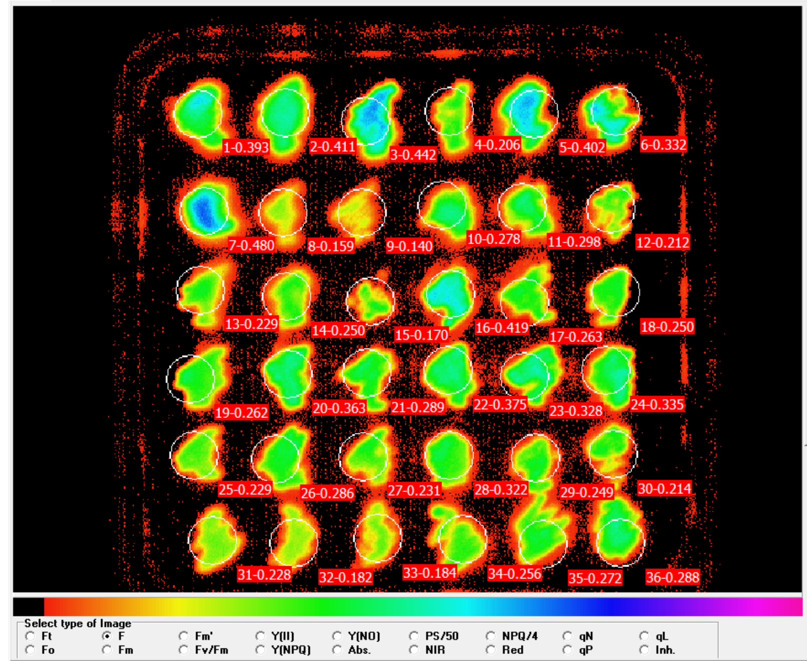


EPS Composition:

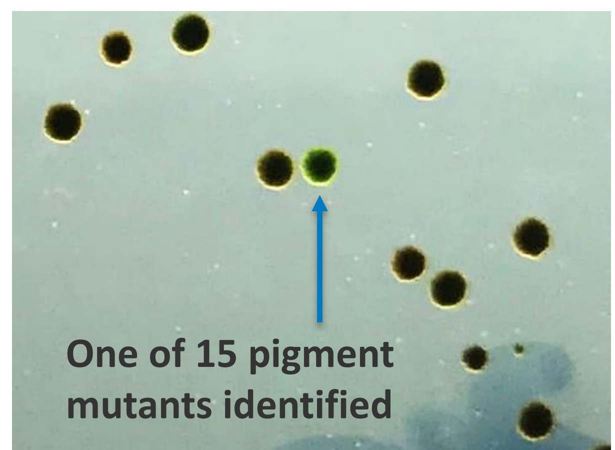
	Fuc	Gal	GlucN	Gluc	Man
early	17.2	6.1	12.0	16.9	42.0
mid	28.0	7.4	13.8	2.6	39.7
late	30.8	2.6	12.0	13.2	34.1

Mutagenesis

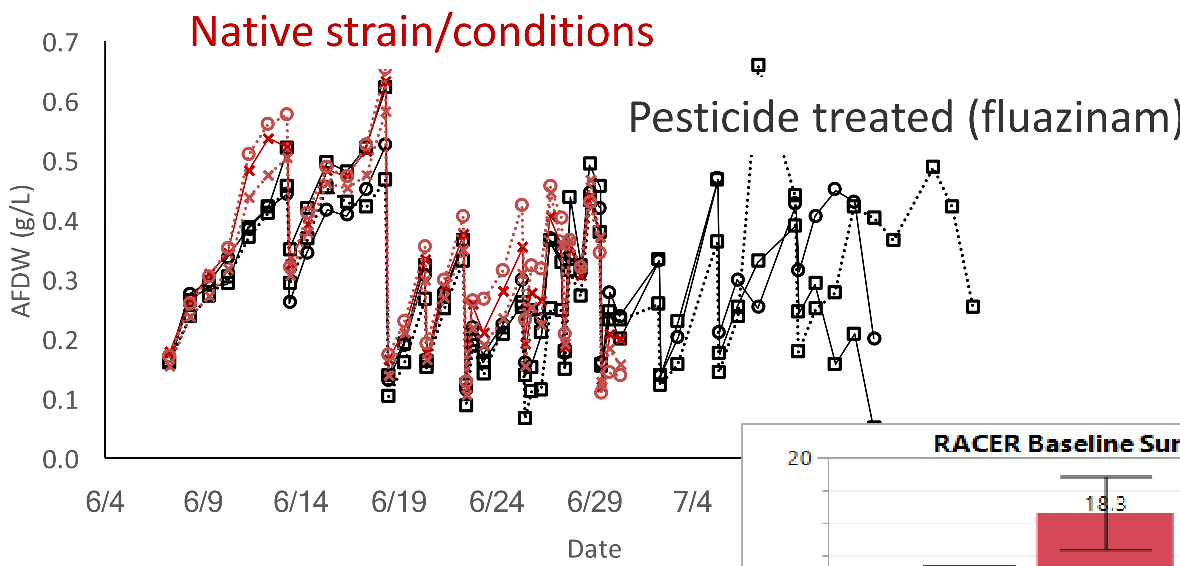
- Ethyl methanesulfonate (EMS) and UV mutagenesis treatment of *D. armatus* yielded > 7000 mutants
- Over Screening for:
  - ➢ mutants with suppressed AET, impaired NPQ (VAZ cycle de-epoxidation/ epoxidation cycle) and PTOX function
  - ➢ Growth rate and carbon metabolism
  - ➢ Reduced dark respiration
- One UV mutant identified with 50% increase in lipid content with no reduction in growth rate until OD > 2

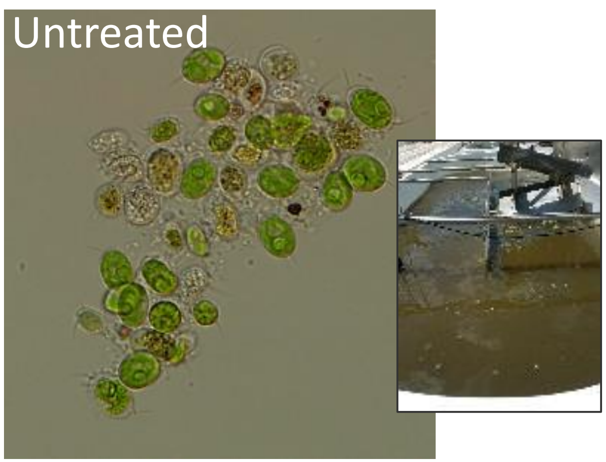


Traces of chlorophyll fluorescence have identified 147 candidates for photosynthetic phenotyping with 21 confirmed changes in photoprotection phenotypes

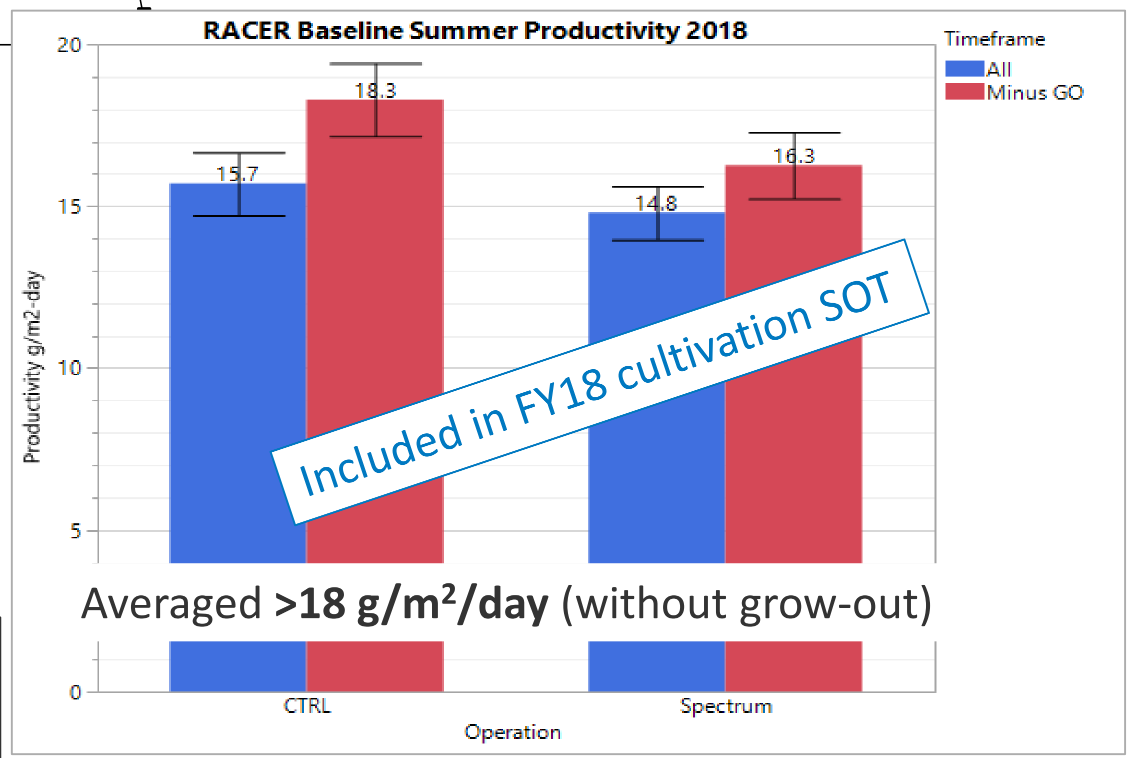


Outdoor Summer Productivity





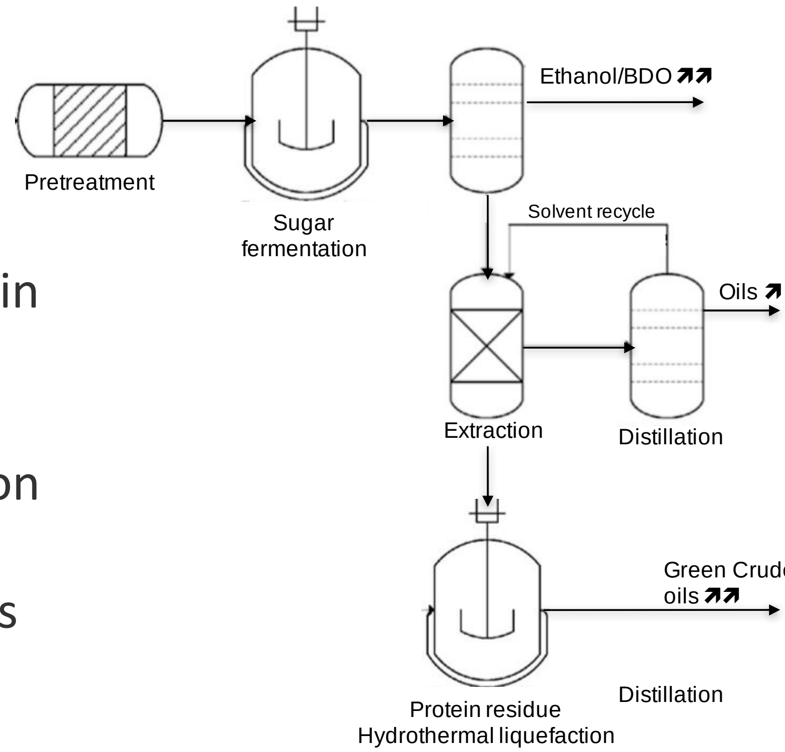
Completed EPA prenotice meeting in October 2018 prior to TERA preparation and submission

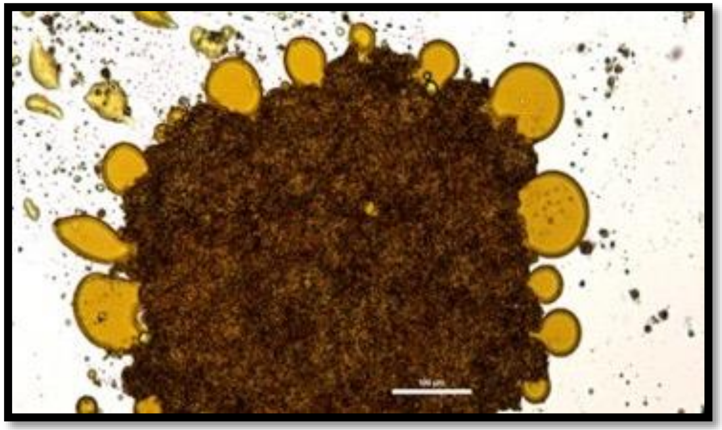


Conversion Engineering

RACER

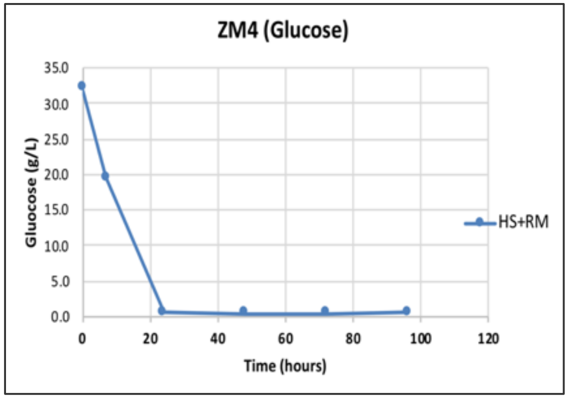
- Optimized small-scale pretreatment for *D. armatus* strain to achieve over 90% yield on fermentable carbohydrates – challenges in scaling pretreatment up beyond 75% yields in ZipperClave (~0.5L)
- Engineered *Zymomonas mobilis* to include mannose as a carbon source for fermentation to 2,3 BDO
- Demonstrated ethanol fermentation kinetics and CCE to demonstrate 80 wt% yield on solubilized carbohydrates, with the goal of demonstrating improvements on CCE by immobilized ethanol fermentation
- Demonstrated >95 wt% extractable oil yield with sequential extraction with co-solvent in process-relevant pathway at small scale
- Demonstrated > 30 wt% yield on HTL of protein-rich stillage

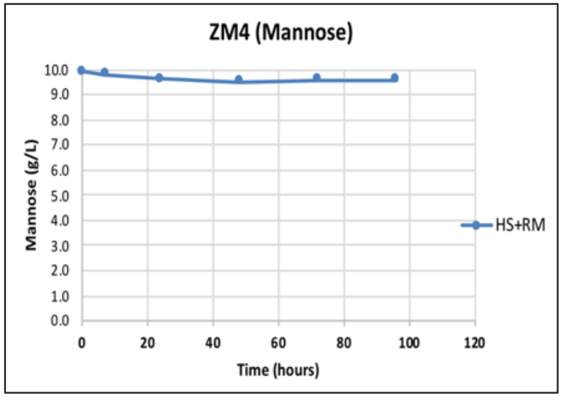


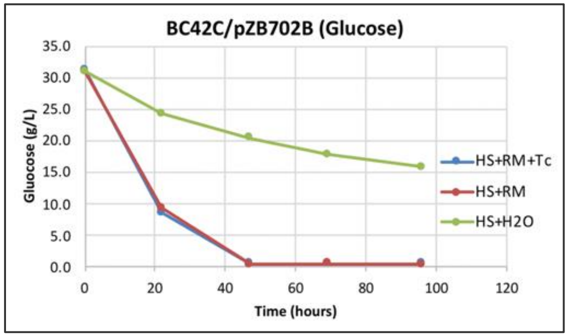


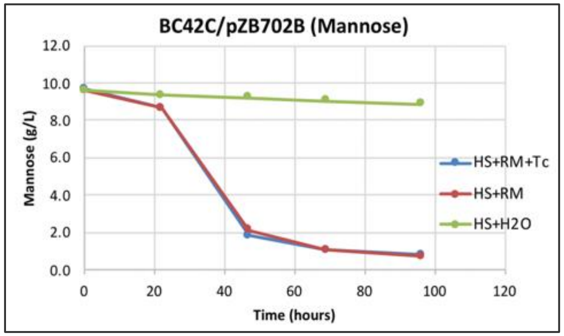
BDO fermentation on pretreatment liquor

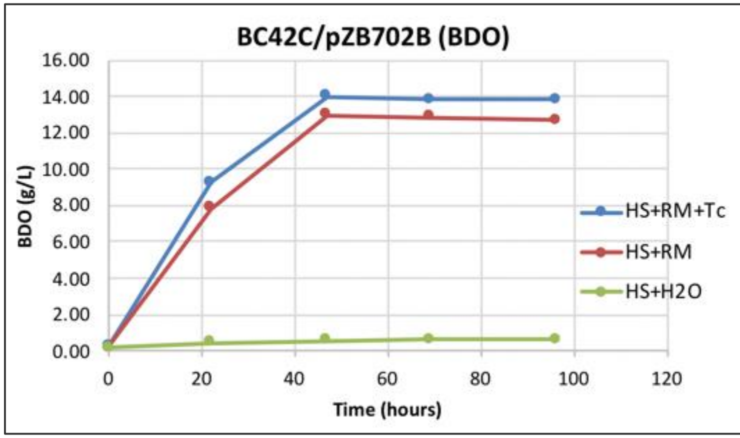
%DW		% profile				
Lipid	**Carbs**	Gal	Gluc	Man	Xyl	Rib
32.8	**37.4**	3.5	69.8	22.6	1.3	1.7
13.3	**43.2**	3.5	71.2	22.5	0.0	1.9











*BDO production demonstrated with novel mannose utilizing *Zymomonas* strain on hydrolysate liquor from closely related *Desmodesmus* sp. To date, this same performance has not been achieved with *D. armatus* liquors, suggesting interfering components

Zymomonas strain engineered for mannose utilization and BDO production – first demonstration of mannose utilization from algae pretreatment liquors in BDO strain, leveraging BETO Biochem platform

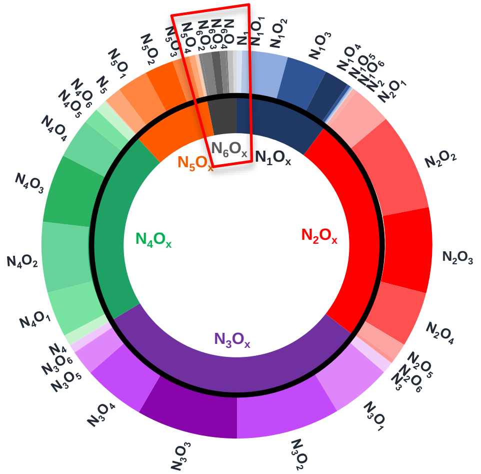
HTL Conversion of Protein Residue and Nutrient Recycling

	Bio-oil yield (%)*	Aqueous phase yield (%)	Char yield (%)
260C	23.8 ± 1.1	32.5 ± 3.9	9.2 ± 3.2
340C	30.4 ± 0.2	18.0 ± 2.1	14.8 ± 1.1

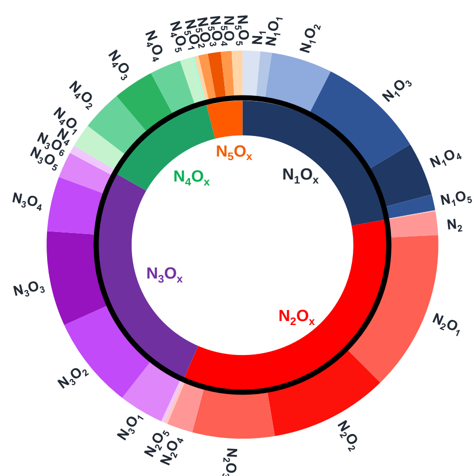
Biocrude oil Composition:

Temperature	Nitrogen %	Carbon %	Hydrogen %
260°C	6.38	75.65	9.20
340°C	6.17	76.18	9.10

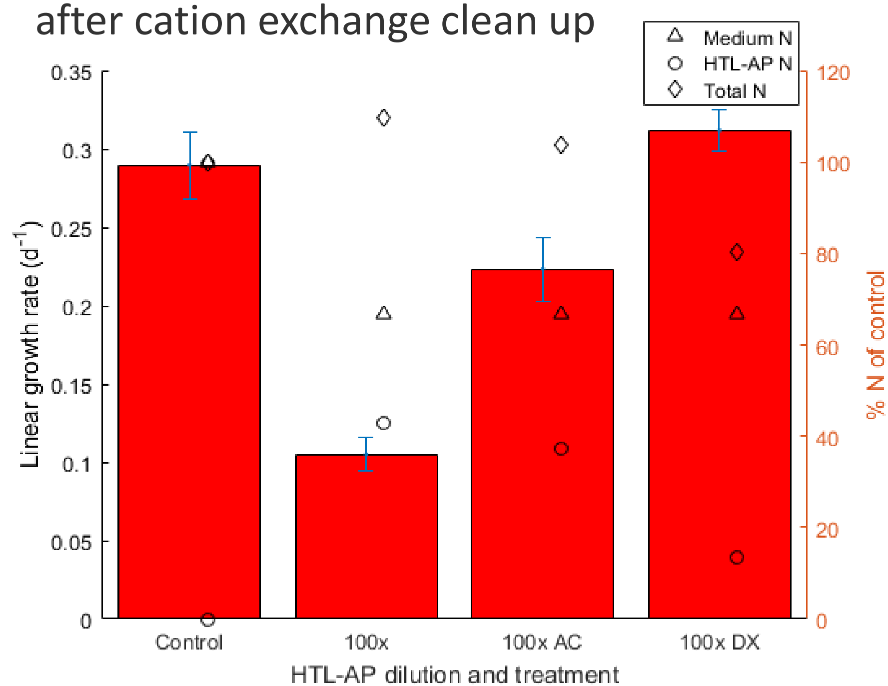
Untreated HTL Aqueous Phase Extract



Dowex Treated HTL Aqueous Phase Extract



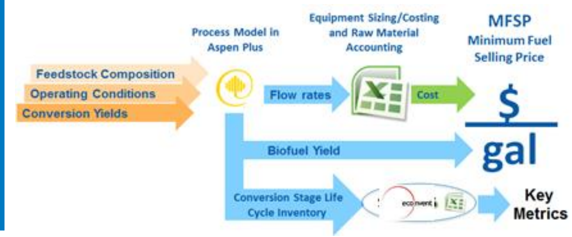
Nutrient Recycling from aqueous phase (AP)* improved to allow full N replacement after cation exchange clean up

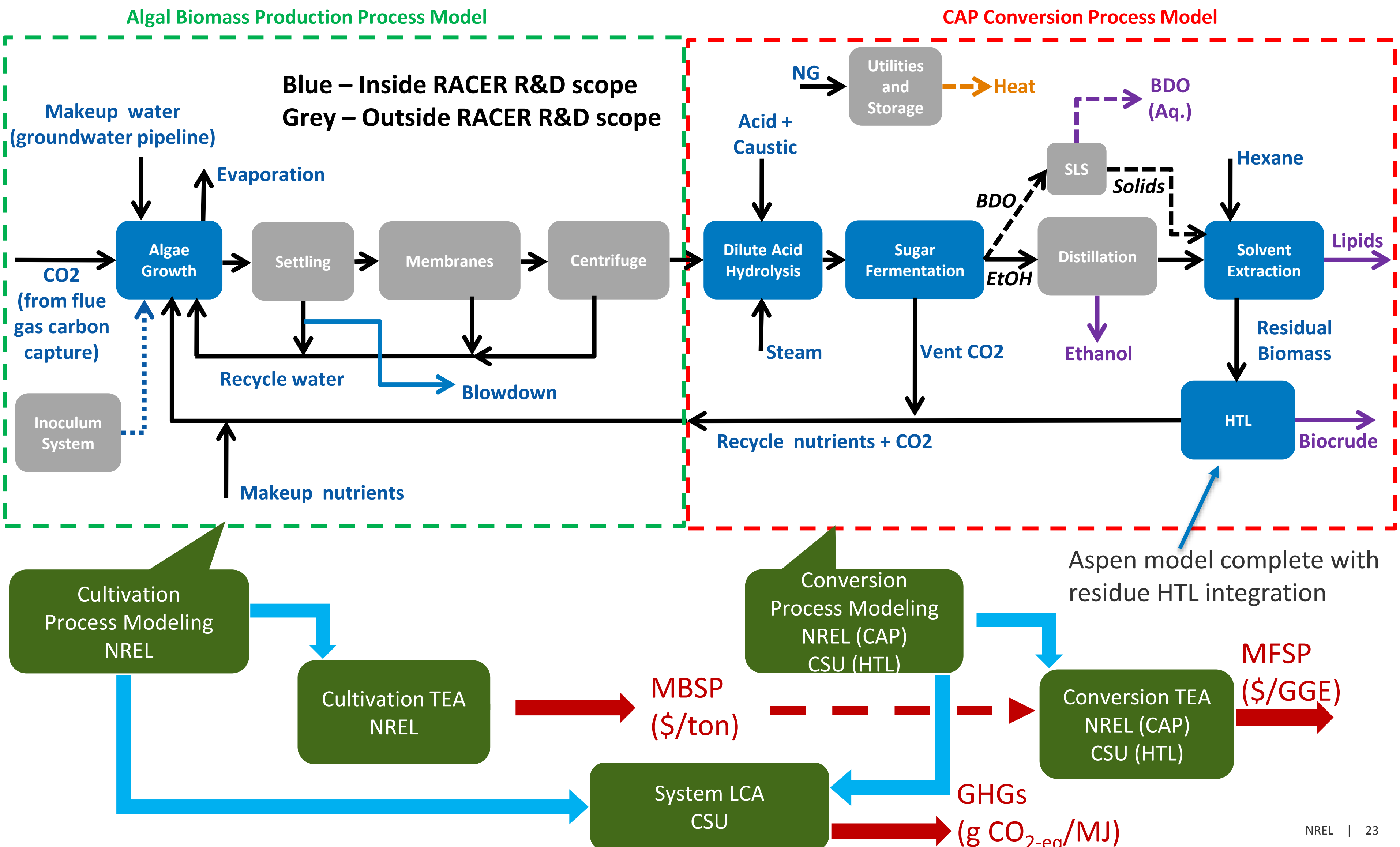


*Aqueous phase from Chlorella sp. direct HTL, provided by D. Anderson, PNNL

Molecular and elemental elucidation of composition of AP* by high resolution MS (FT-ICR MS) shows reduction in polyaromatic heterocyclic components (e.g. N_6O_x)

Process TEA/LCA: Integration & Data Flow

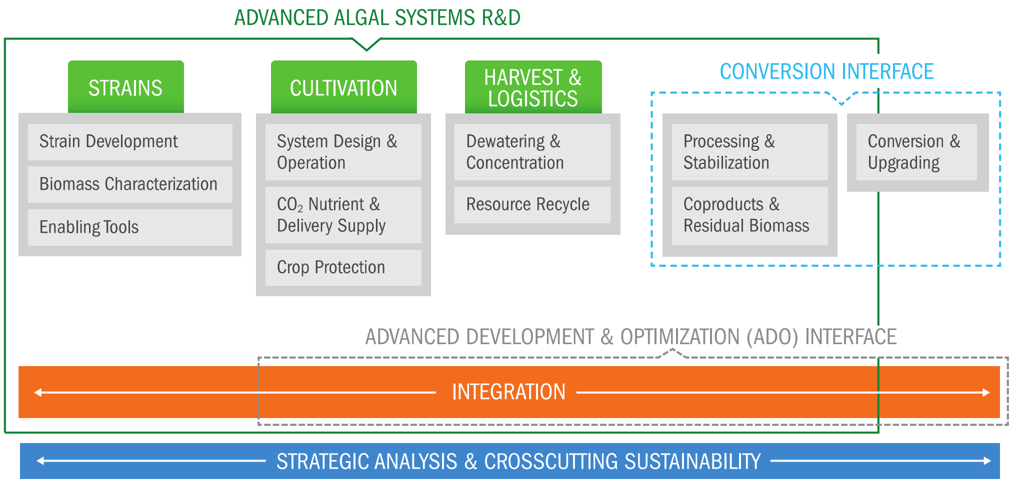




Relevance

Relevance

- Directly contributes to the Advanced Algal Systems directive of increasing biofuel-intermediate yields by removing key barriers that currently limit overall process carbon efficiency and biomass productivity
- Project approach covering strain development, cultivation and conversion focused on increasing biofuel intermediate yields provides unique position



Technical accomplishments feed directly to AAS annual targets:

- Cultivation SOT: summer productivity of *D. armatus* exceeded that of comparative species, summer productivity was **included in the FY18** productivity SOT assessment
- Conversion SOT: included the conversion of sugars, including mannose, to BDO fermentation as a novel tool and was **included in the FY18 SOT**

Future Work

March 2019 **Go/No-Go Decision**:

Demonstrate improved biomass productivity (measured as g/L/day) of at least 10% for at least two of the three strain development subtask approaches (starch metabolism manipulation, photosynthesis engineering, and dark respiration and excreted carbon loss mitigation) based on demonstrated laboratory-scale phenotype of engineered strains relative to the WT *D. armatus* baseline
Demonstrate an at least 10% improvement in CCE (sugar to fuel fermentation product yields) comparing ethanol and 2,3 BDO on the basis of fermentation kinetics of optimum immobilized fermentation strain on WT *D. armatus* pretreated slurry, combined with an at least at least 20% increase in carbon yield in fuel intermediate green crude oils after HTL of fermentation stillage

FY20 – 21 **Focus on outdoor deployment, demonstration of integration of improvements**:

Demonstrate combined increase in biomass productivity (g/m2/day) of at least 50% relative to parent strain in optimized outdoor cultivation protocol at 1000L scale for at least two (2) of the available improved (at least two stacked traits) strains of D. armatus, demonstrating an increase of at least 25% in carbohydrate content
Demonstrate increased CCE of fuel fermentation, lipid extraction and green crude conversion of protein residue by at least an additional 15% relative to the interim baseline using optimized lab-scale integration of immobilized cell fermentation, continuous counter-current lipid extraction and thermochemical conversion of fermentation residue

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March 2019

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- Demonstrate outdoor productivity improvement of 18% to >13 g/m2/day over at least 2 seasons
- At least 10% improvement in CCE for fermentation and 20% increase in carbon yield in fuel intermediates (including 95% extraction efficiency of lipids)

TEA/LCA of intermediate achievements (SOT)

TEA/LCA of final achievements (SOT)

	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9	Q10	Q11	Q12	Q13
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- At least 15% additional increase in CCE above interim baseline combining process improments with improved strain

Summary

- **Overview**: Demonstrate an integrated approach to develop a carbon-to-fuel pathway based on one, highly productive, commercially-relevant algae species
- **Approach**: *D. armatus* is being used as outdoor-relevant strain and chassis for rapid and iterative metabolic engineering and outdoor deployment supporting the concept of building strain improvement platform on outdoor production species
- **Technical Accomplishments**: Rapid development of successful and highly efficient strain development tools, targets of carbon assimilation pathways engineered; Outdoor cultivation exceeding SOT productivities; Conversion research targeted improvements in pretreatment to soluble sugars and fermentation to 2,3 BDO and ethanol as platform chemical; HTL of process residue further improving biofuel intermediate yields and ultimately cost reductions
- **Relevance**: Focusing on carbon conversion efficiency, by coordinating photosynthesis engineering with carbohydrate sink manipulation driving the basis of biomass accumulation and maximizing atmospheric carbon assimilation to biofuels, one of the major focus areas of BETO AAS
- **Future work**: Demonstrating improvements of modular process steps; strain phenotype improvements, cultivation and conversion; with the aim of a fully integrated demonstration run in FY20-21 from improved strain outdoor deployment to carbon conversion to fuels and nutrient recycling

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Thank You

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Publications / Presentations

- Chen, P., Venegas-Jimenez, J. L., Rowland, S.M., Quinn, J.C., Laurens, L. ML., 2019, Algae nutrient recycle from hydrothermal liquefaction aqueous phase through novel selective remediation approach, Submitted
- Knoshaug, E.P., Nag, A., Laurens, L. ML., 2019, Draft genome and chloroplast sequences of the biofuel-relevant microalga *Desmodesmus armatus*, Submitted

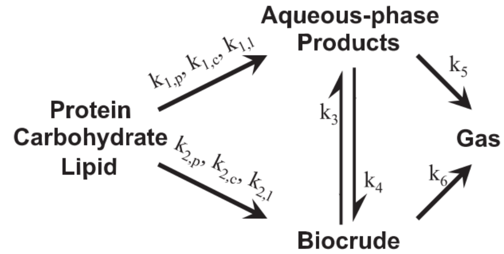
Presentations:

- Lieve M. L. Laurens, G. Peers, M. C. Posewitz, E. P. Knoshaug, K. F. Reardon, P. T. Pienkos, J. McGowen, N. Nagle, C. Behnke. Improving Biofuel and Bioproduct in Algae through Rewiring Algal Carbon Energetics for Renewables (RACER), a Consortium Overview. Algal Biomass Summit, oral presentation, October 15th 2018.
- Eric P. Knoshaug, A. Fedders, N. Sweeney, D. Douchi, B. Wang, A. Politis, S. Van Wychen, and L. Laurens. Manipulating transitory carbohydrate synthesis and degradation rates in Desmodesmus armatus to drive biomass productivity and carbon assimilation potential. Algal Biomass Summit, oral presentation, October 15th 2018.
- Ware, M. and Peers, G., Characterization of photosynthetic energy loss processes in *Desmodesmus armatus*. *The 8th International Conference on Algal Biomass, Biofuels and Bioproducts*. Seattle, WA. 2018

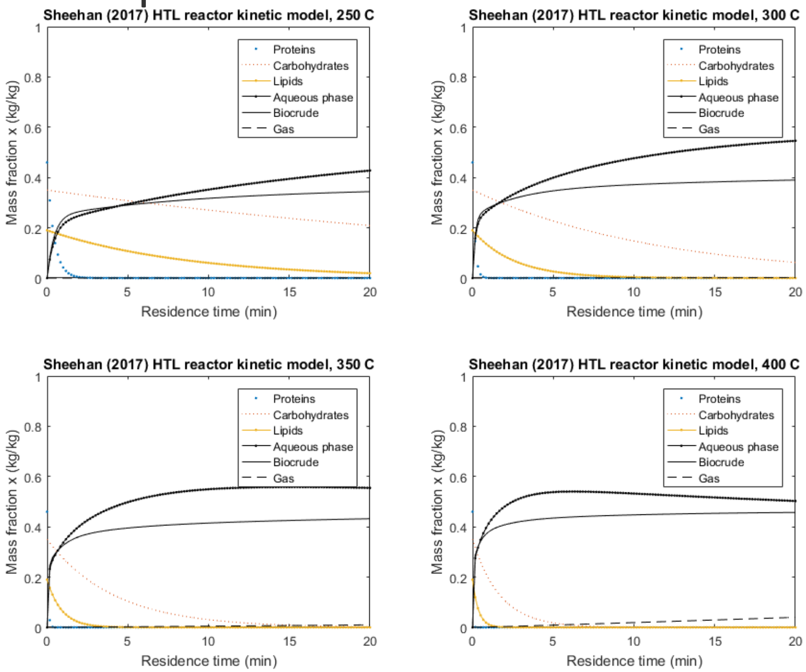
Poster presentations:

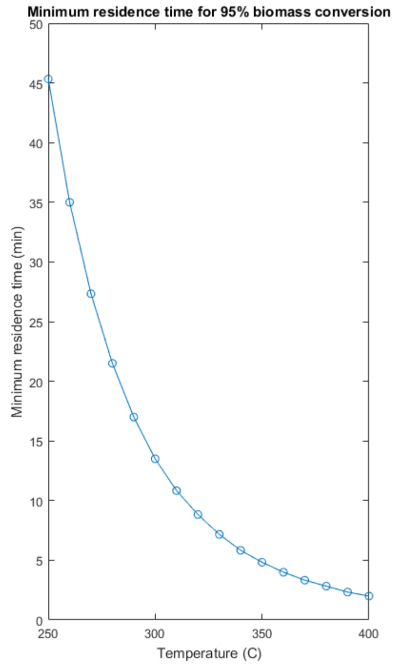
- Xing-Feng Huang, Nicholas J. Nagle, Wylie P. Borden, Zachary M. Dwyer, Lieve Laurens, and Kenneth F. Reardon, "High Productivity Conversion of Algal Biomass Hydrolysate using Immobilized Cell Technology". Poster presentation at ABO annual meeting, Houston, TX, October 15th 2018.
- Damien Douchi, E. P. Knoshaug, J. Yu, and L. Laurens. Engineering the central carbon metabolism for the optimization of energy harvest and utilization. Algal Biomass Summit, poster presentation, October 15th 2018.
- Venegas, J., Chen, P., Rowland, S., Laurens, L., Remediation of Aqueous Phase from Hydrothermal Liquefaction as Nutrient Source for Algae Cultivation, Poster presentation at ABO annual meeting, Houston, TX, October 15th 2018
- Ware, M., and Peers, G., 2018, Photophysiology of *Desmodesmus armatus*, an emerging algal biofuel platform. ISPR Conference on Microbial Photosynthesis. Vancouver, BC, Canada. 2018

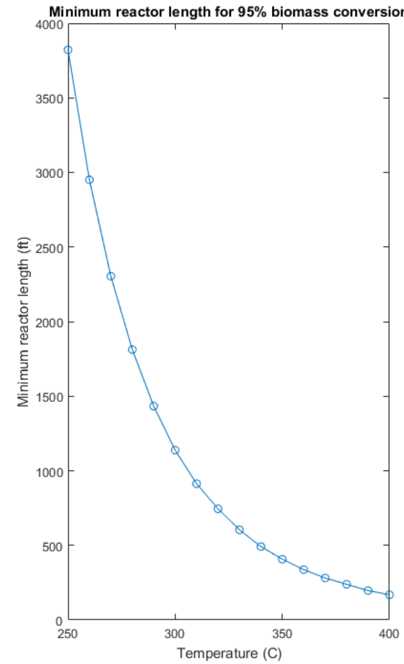
TEA/LCA – HTL Modeling



- HTL kinetic model (Valdez et al., 2014; Sheehan et al., 2017) to be integrated
- Key outputs (biocrude/aqueous phase/gas) dynamic with protein/carbohydrate/lipid feed composition and temperature
- Determine reactor length and capital cost impact based on yield data and temperature







Future Work

- Evaluate yield/TEA/LCA tradeoffs for BDO fermentation vs EtOH
- Incorporate partner's conversion approaches (immobilized cell fermentation, POS extraction technologies) to evaluate design/cost benefits
- Optimize overall process/heat integration between CAP, HTL

TERA: ASU Site Schematic and Containment Plan

- Secondary containment outdoor cultivation established for GM support
- RACER TERA will be submitted to EPA in Feb 2019 for at least 2 strains outdoor deployment

mCherry Fluorescence variable in cell population

Transformant C6