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Metabolic Engineering of Cyanobacteria for Free Fatty Acid Production

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U.S. DEPARTMENT OF ENERGY

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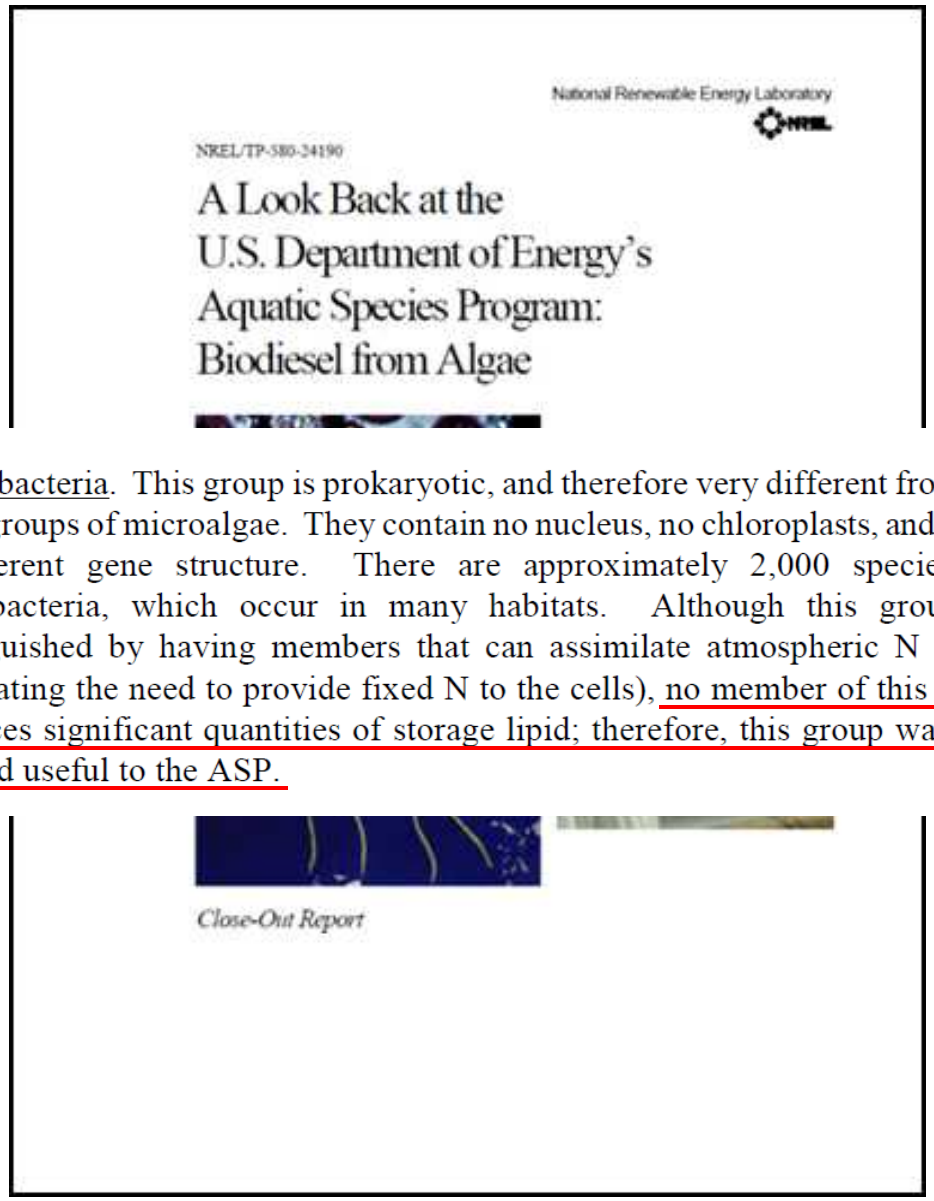


Cyanobacteria. This group is prokaryotic, and therefore very different from all other groups of microalgae. They contain no nucleus, no chloroplasts, and have a different gene structure. There are approximately 2,000 species of cyanobacteria, which occur in many habitats. Although this group is distinguished by having members that can assimilate atmospheric N (thus eliminating the need to provide fixed N to the cells), no member of this class produces significant quantities of storage lipid; therefore, this group was not deemed useful to the ASP.

1. Sheehan, J. et al. (1998). DOE Close-Out Report.

Why Cyanobacteria ?

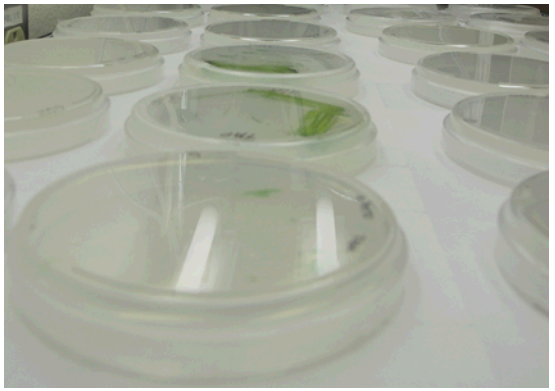
Advantages of Cyanobacteria for Fuel Production

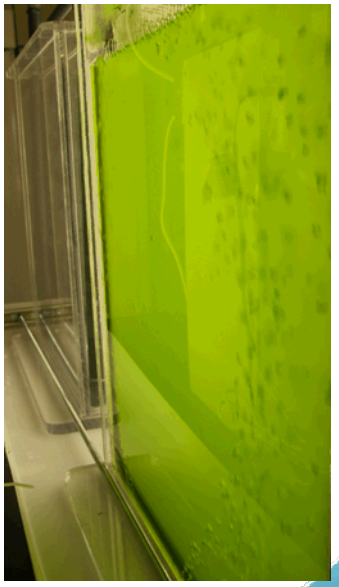
Desirable strain traits

- Easily transformed
- Homologous recombination – targeted genome integration
- Gene expression not complicated by RNAi
- Established genetic tools
- Fast growth rates and strain robustness

Process design advantages

- Product excretion enables continuous production
- No extraction process required
- Lower nutrient requirements (N&P)

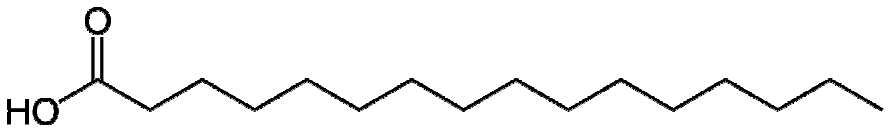




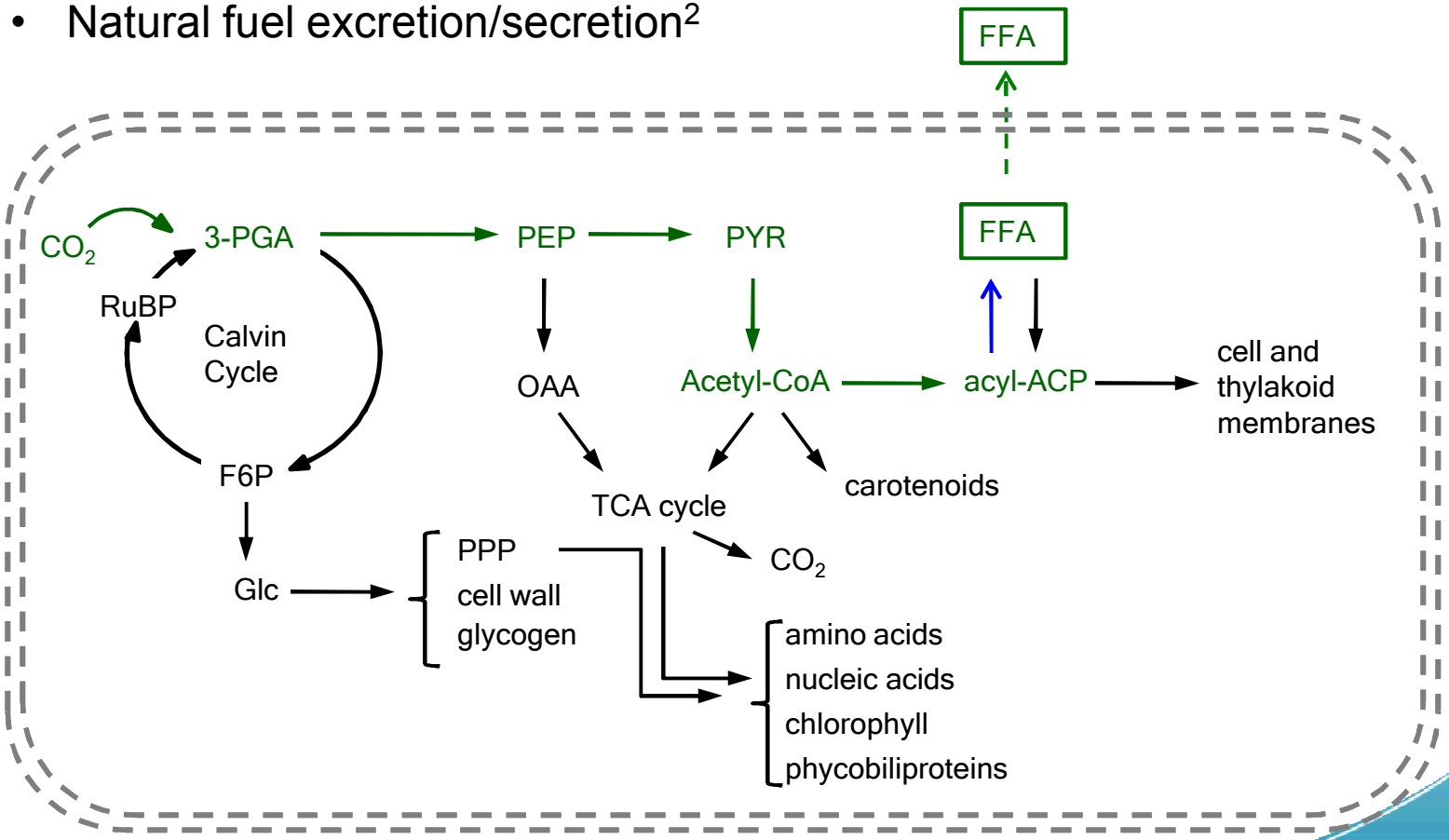
Target Fuel: Free Fatty Acids (FFA)

Desirable Product Characteristics

- Photoautotrophic growth
- Naturally produced biomolecule
- High energy density
- Natural fuel excretion/secretion[2]



FFA: hexadecanoic acid



2. Kaczmarzyk D, Fulda M. (2010) Plant Physiology.

Outline

1. Engineering a model cyanobacterium *Synechococcus elongatus* PCC 7942 for FFA production

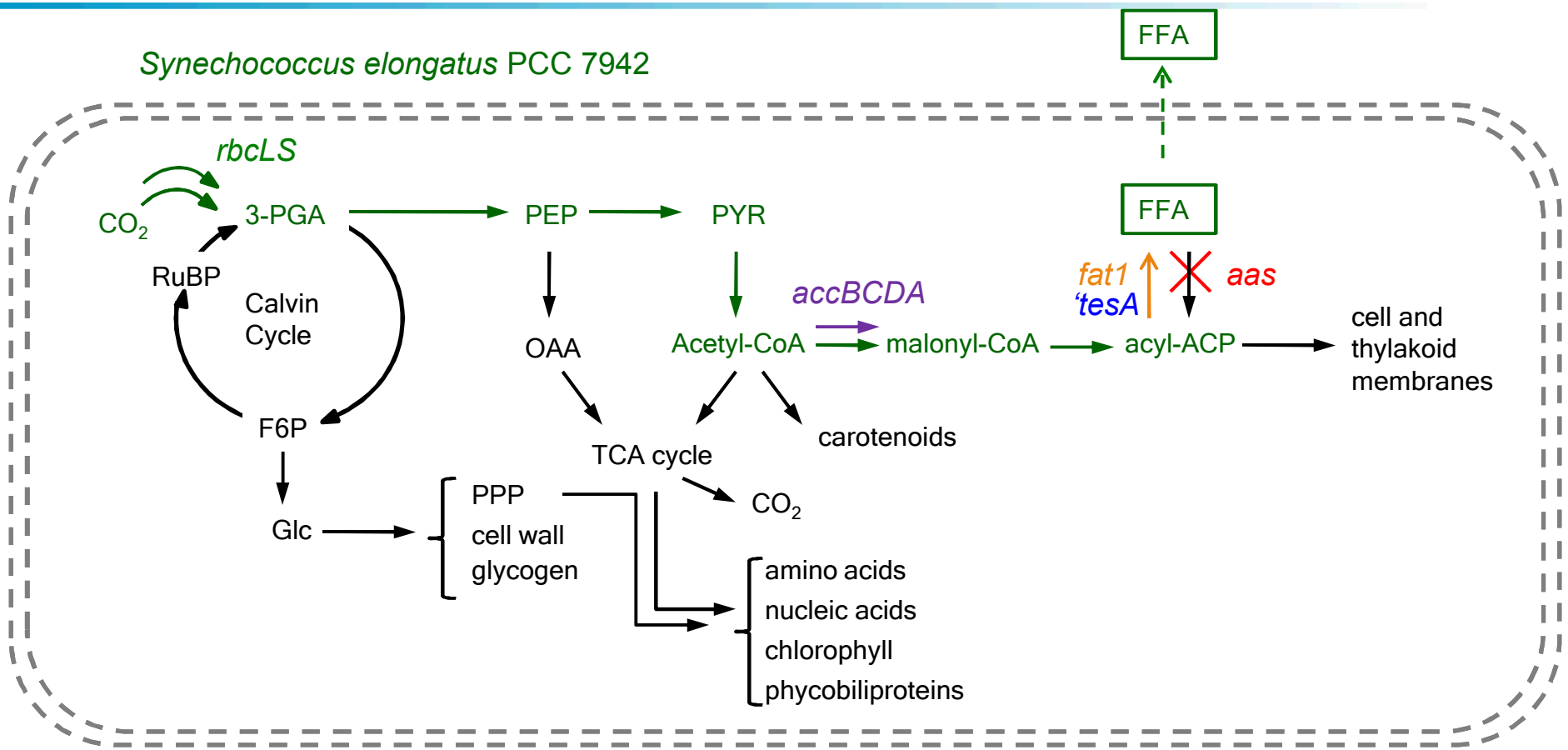
2. Physiological effects of FFA production in cyanobacteria

3. Seq-ing targets for improving FFA production

4. Is cyanobacterial host selection critical for FFA production? … *Synechococcus* sp. PCC 7002: Another model host

5. Biofuel toxicity for cyanobacteria

Genetic Engineering of Cyanobacteria to Produce FFA



7942: wild type; SE01: Δ*aas*; SE02: Δ*aas*, *'tesA*; SE03: Δ*aas*, *fat1*; SE04: Δ*aas*, *fat1*, *rbcLS*; SE05: Δ*aas*, *fat1*, *rbcLS*, *accBCDA*

aas – acyl-ACP synthetase / long-chain-fatty-acid CoA ligase

'tesA – truncated thioesterase from *Escherichia coli*

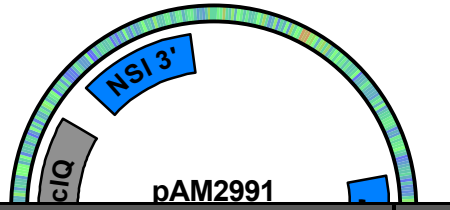
fat1 – acyl-ACP thioesterase from *Chlamydomonas reinhardtii*

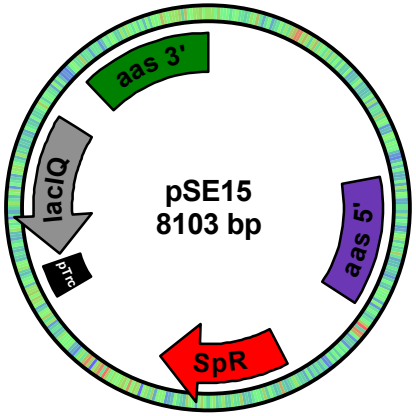
rbcLS – native RuBisCO

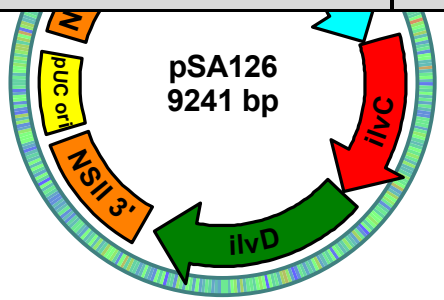
accBCDA – multi-subunit acetyl-CoA carboxylase from *C. reinhardtii* (chloroplast associated)

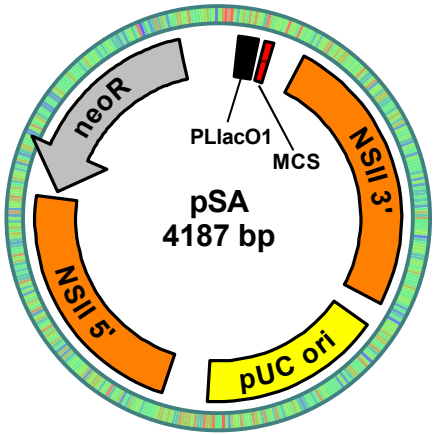
7942 Strain Construction

Plasmid	Construct	Strain (s)
pSE15	*Δaas*	SE01
pSE16	*Δaas*, Ptrc - *'tesA*	SE02
pSE17	*Δaas*, Ptrc - *fat1*	SE03
pSE18	*Δaas*, Ptrc - *fat1* - *rbcLS*	SE04, SE05
pBCDA	PLlacO1 - *accBCDA*	SE05





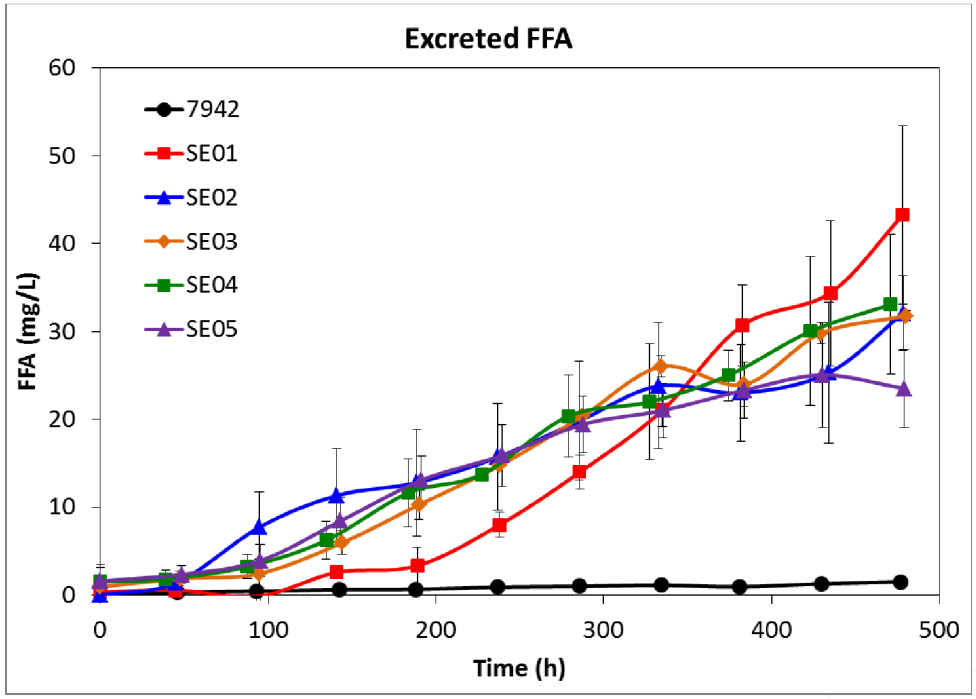




pAM2991 generously provided by Susan Golden (UCSD). pSA126 kindly provided by James Liao (UCLA).

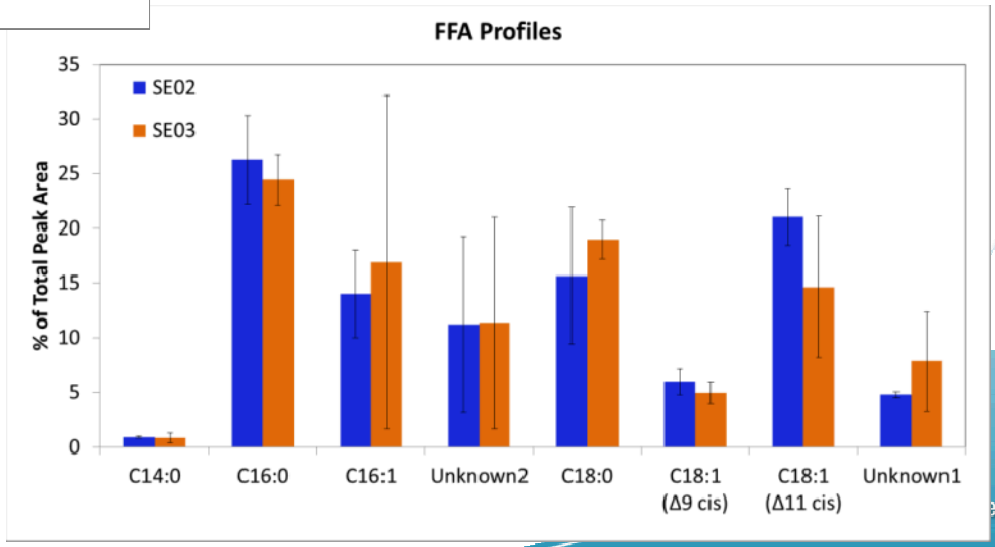
FFA Production in Engineered 7942 Strains

7942: wild type; SE01: Δ*aas*; SE02: Δ*aas, 'tesA*; SE03: Δ*aas, fat1*; SE04: Δ*aas, fat1*, *rbcLS*; SE05: Δ*aas, fat1*, *rbcLS*, *accBCDA* 1

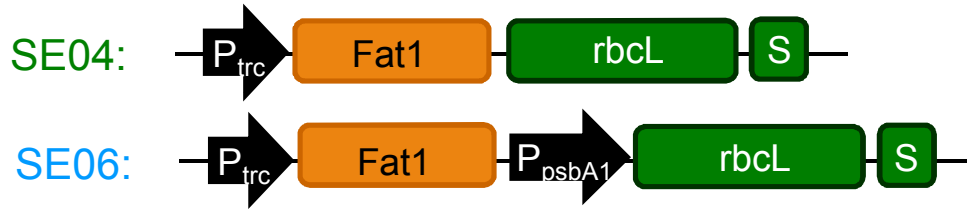


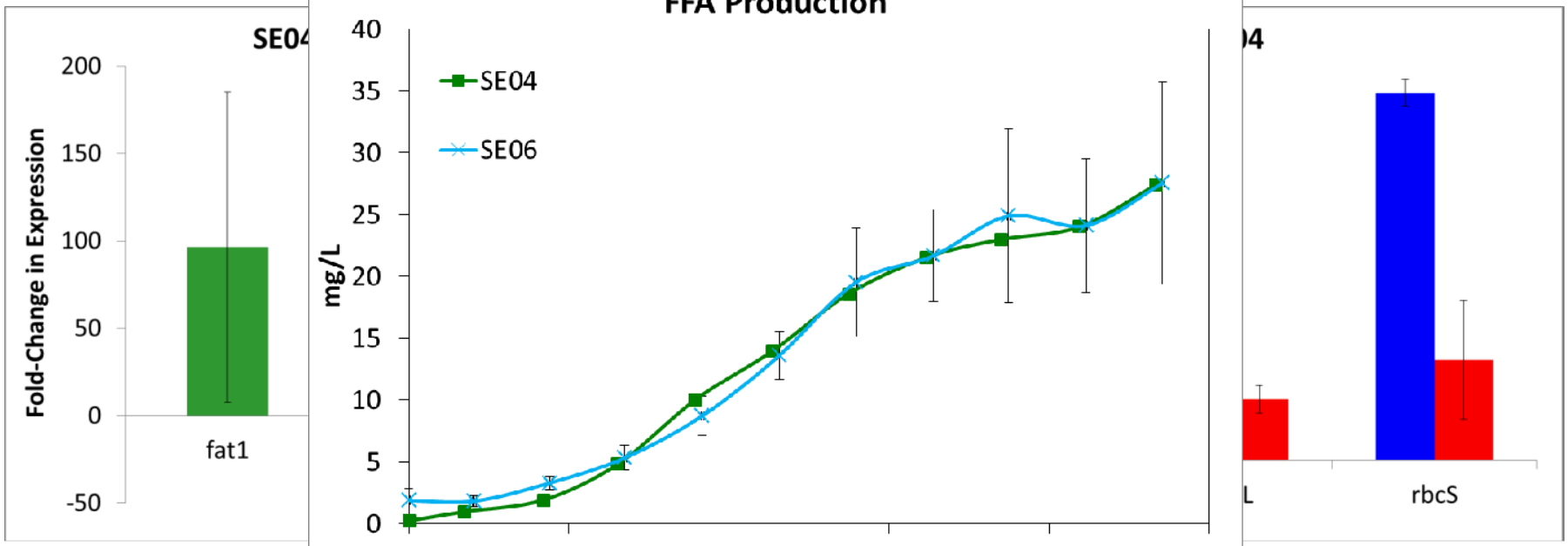
- All engineered strains produce and excrete FFA
- Without thioesterase expression, FFA only accumulate during stationary phase
- Despite targeting rate-limiting steps, the rate of FFA production is not improved

- Primarily C16 and C18 saturated and monounsaturated FFA are produced
- Both thioesterases (*E. coli* and *C. reinhardtii*) produce similar chemical profiles for excreted FFA



Does Increasing Gene Expression Improve FFA Production?





- No inducibl l promoter improves *rbcLS* expression

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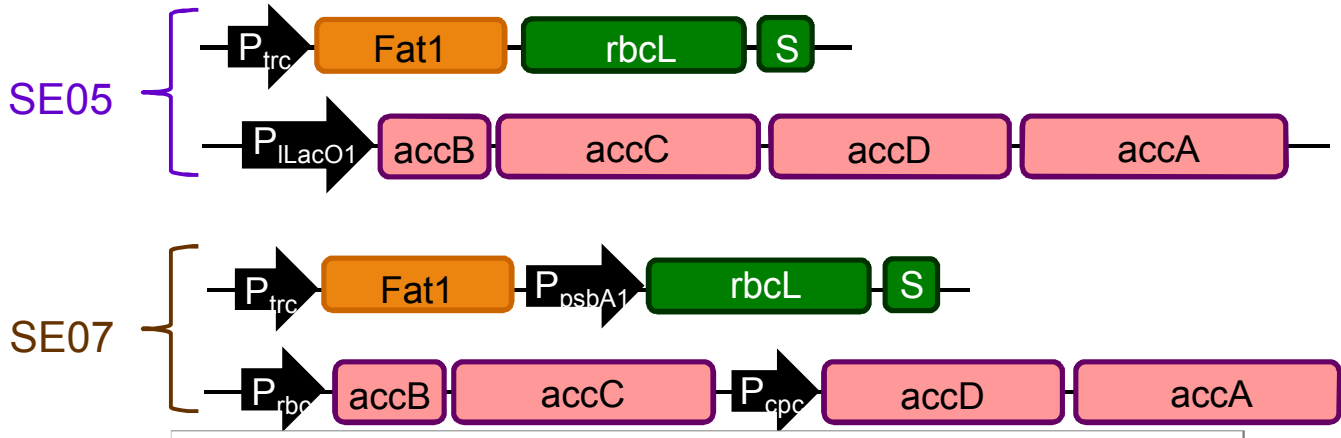

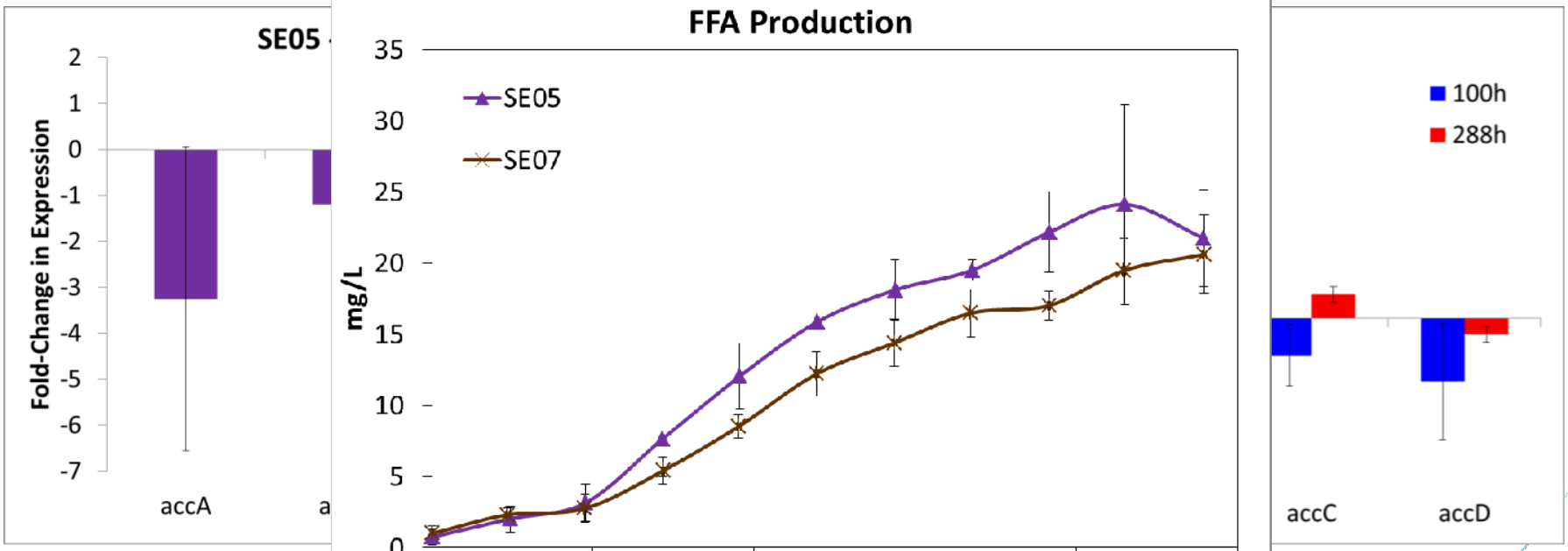

- No inducible ex c does not with PLlacO1 *CDA* expression

Ruffing AM. Borrowing Genes from *Chlamydomonas reinhardtii* for Free Fatty Acid Production in Engineered Cyanobacteria. (2012) J. Appl. Phycol. Submitted.

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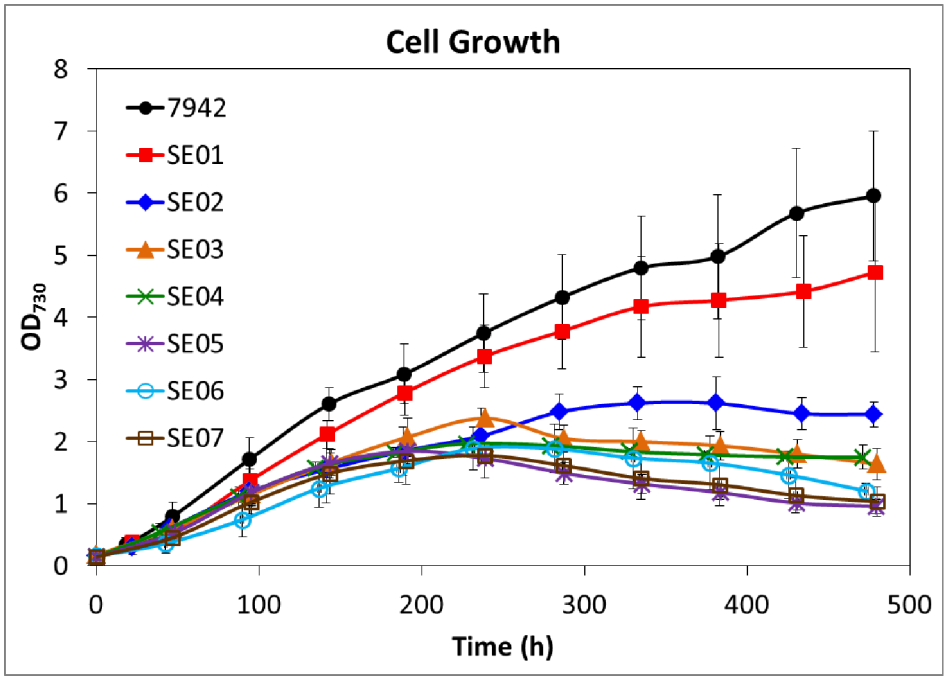
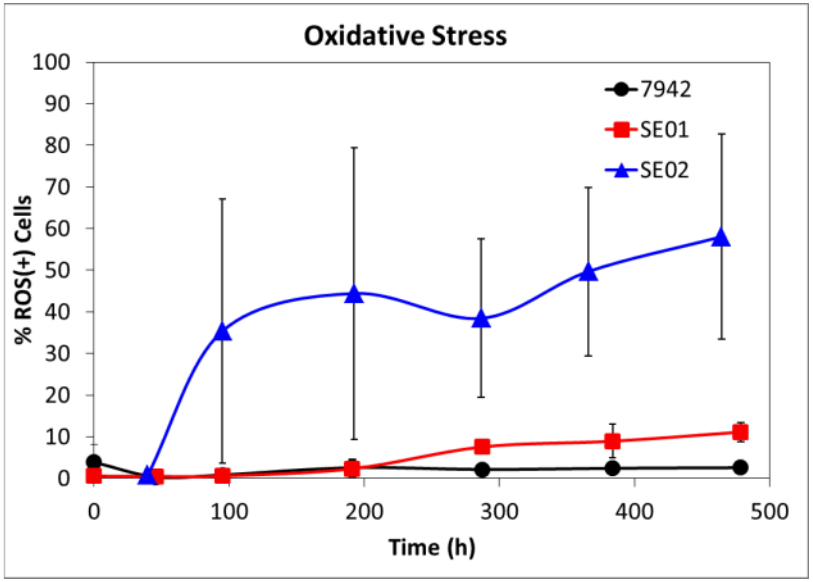
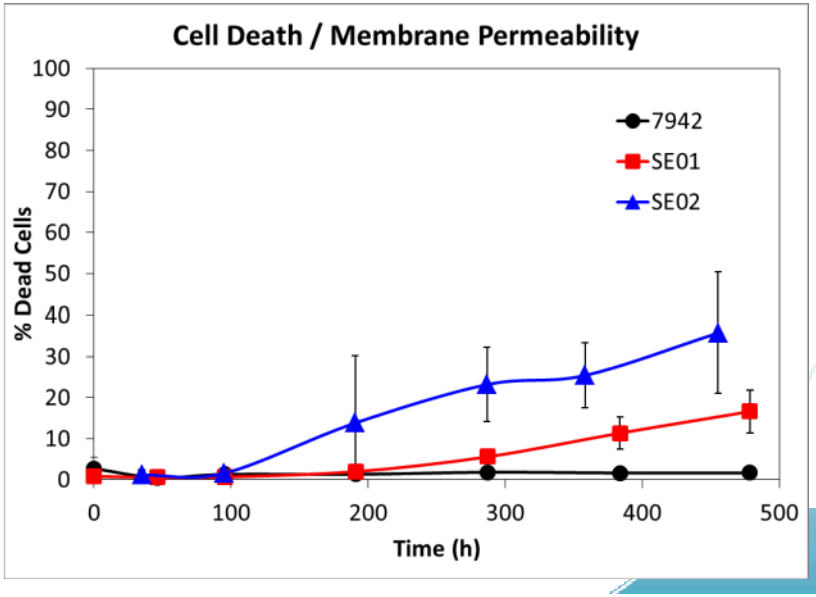
Physiological Effects: Growth, Stress, and Cell Death

- Final cell concentration reduced by more than 80% in SE05 and SE07
- FFA-producing strains have elevated levels of reactive oxygen species (ROS) and increased cell death / membrane permeability







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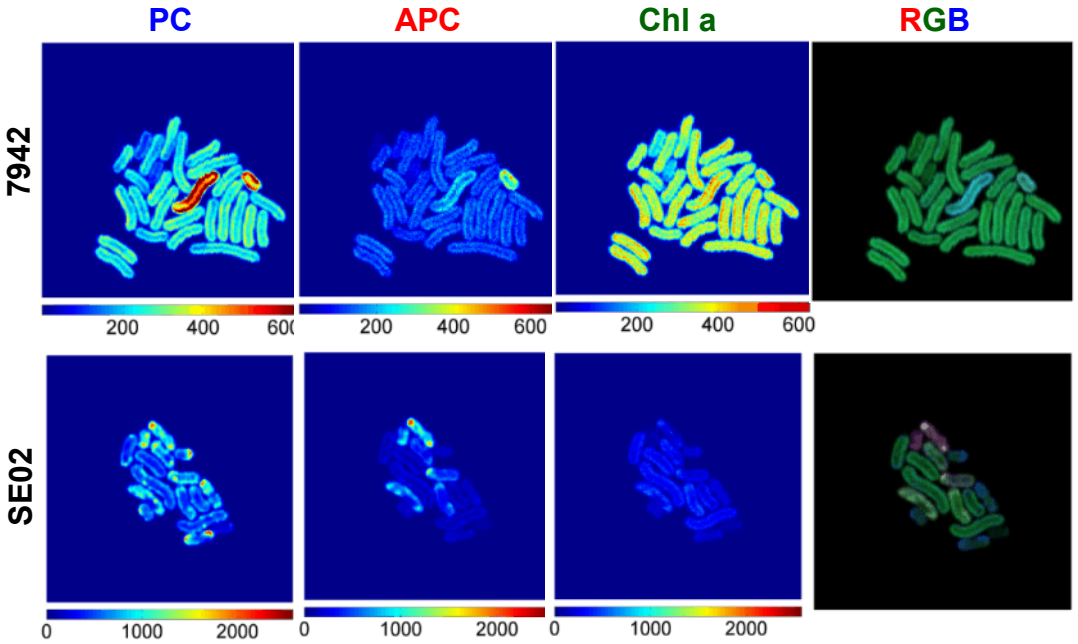
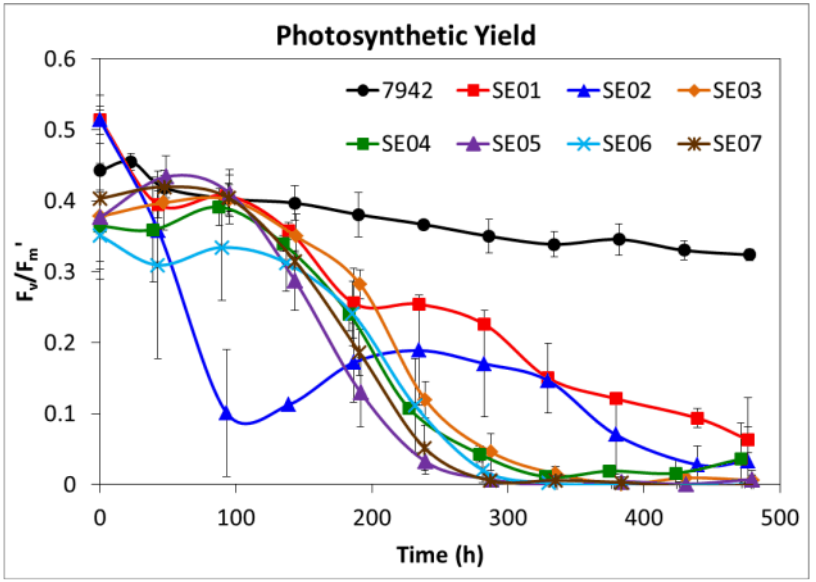
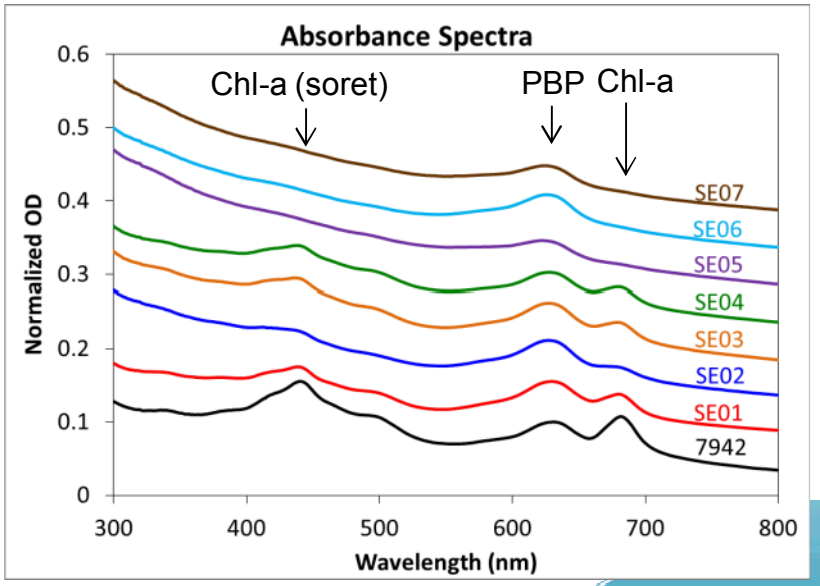
SE06: Δ*aas*, Fat1, P_{psbAI} *rbcLS*; SE07: Δ*aas*, Fat1, P_{psbAI} *rbcLS*, P_{rbc} *accBC* P_{cpc} *accDA*

Photosynthetic Effects

- Photosynthetic yield drops to zero in FFA-producing strains
- Bulk absorbance measurements indicate a selective degradation of chlorophyll-a pigment
- Hyperspectral confocal fluorescence microscopy shows photosynthetic pigments are aggregating at the cell poles in the engineered strain SE02







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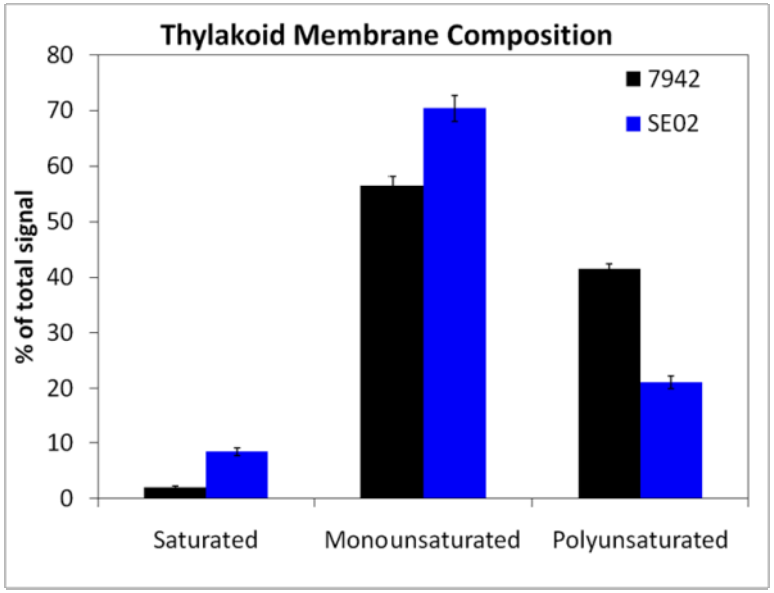
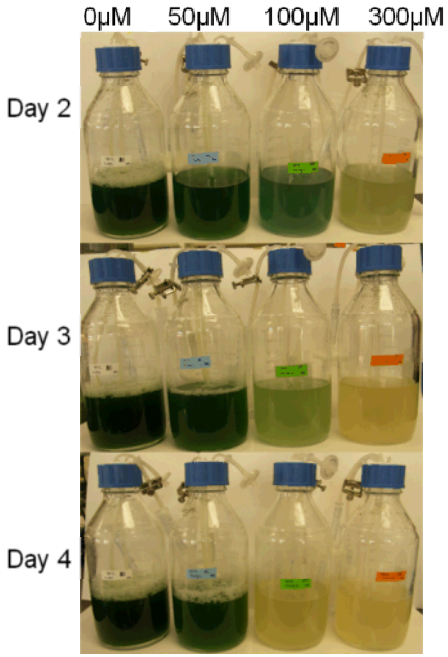
Possible Mechanisms of FFA Toxicity

Mechanism 1: Engineered strains have altered membrane composition

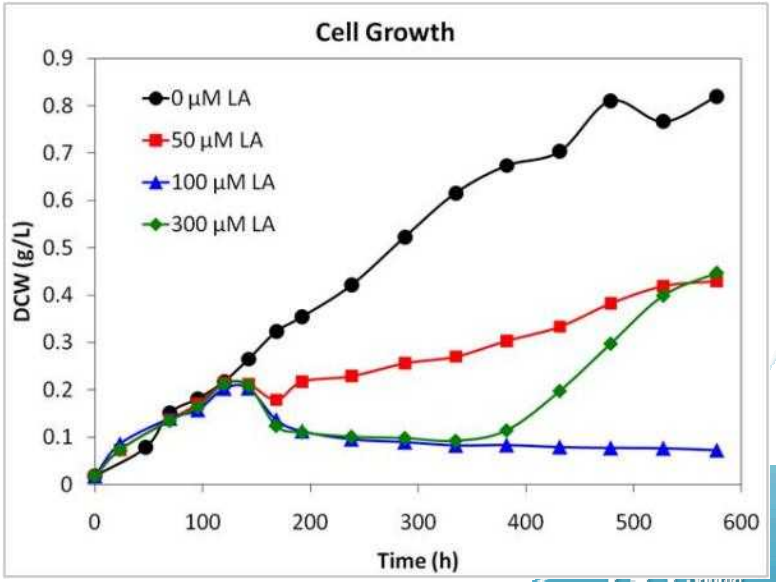
- Increased levels of saturated FA and lower levels of polyunsaturated FA in thylakoid membranes
- Leads to increased membrane viscosity and potential effect on phycobilisome attachment





Mechanism 2: FFA toxicity

- Exogenous saturated FFA has no effect on cell physiology
- Unsaturated FFA (linolenic acid - LA) oxidize into a variety of compounds, including toxic hydroperoxides



Ruffing AM, Jones HDT. Physiological effects of free fatty acid production in genetically engineered *Synechococcus elongatus* PCC 7942. (2012) Biotech. Bioeng. 109 (9): 2190-9.

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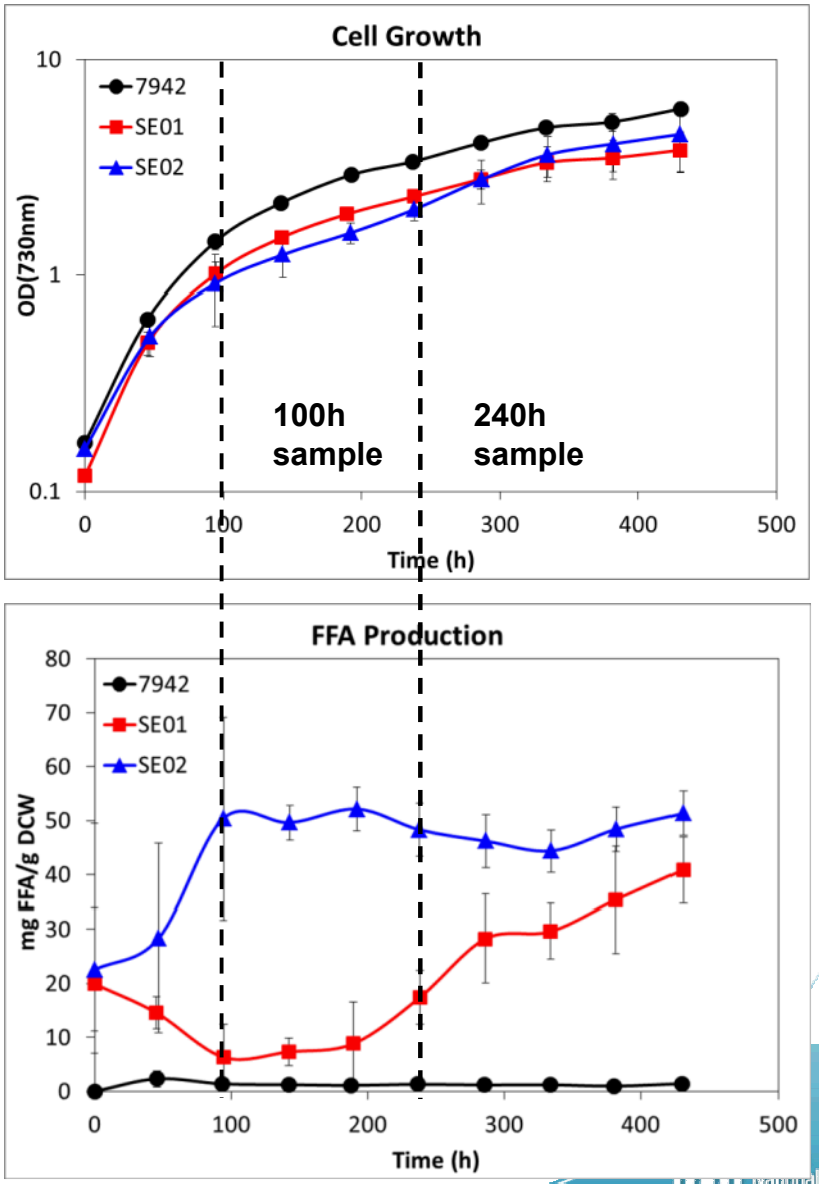
Can *S. elongatus* be engineered to overcome these effects?

RNA-seq to identify genetic response to FFA production

- 3 strains: 7942, SE01, SE02
- 2 time points: 100h, 240h
- 3 biological replicates

Differential gene expression comparisons:

Low FFA	High FFA
SE01, 100h	SE02, 100h
7942, 100h	SE02, 100h
7942, 240h	SE01, 240h
7942, 240h	SE02, 240h
SE01, 100h	SE01, 240h



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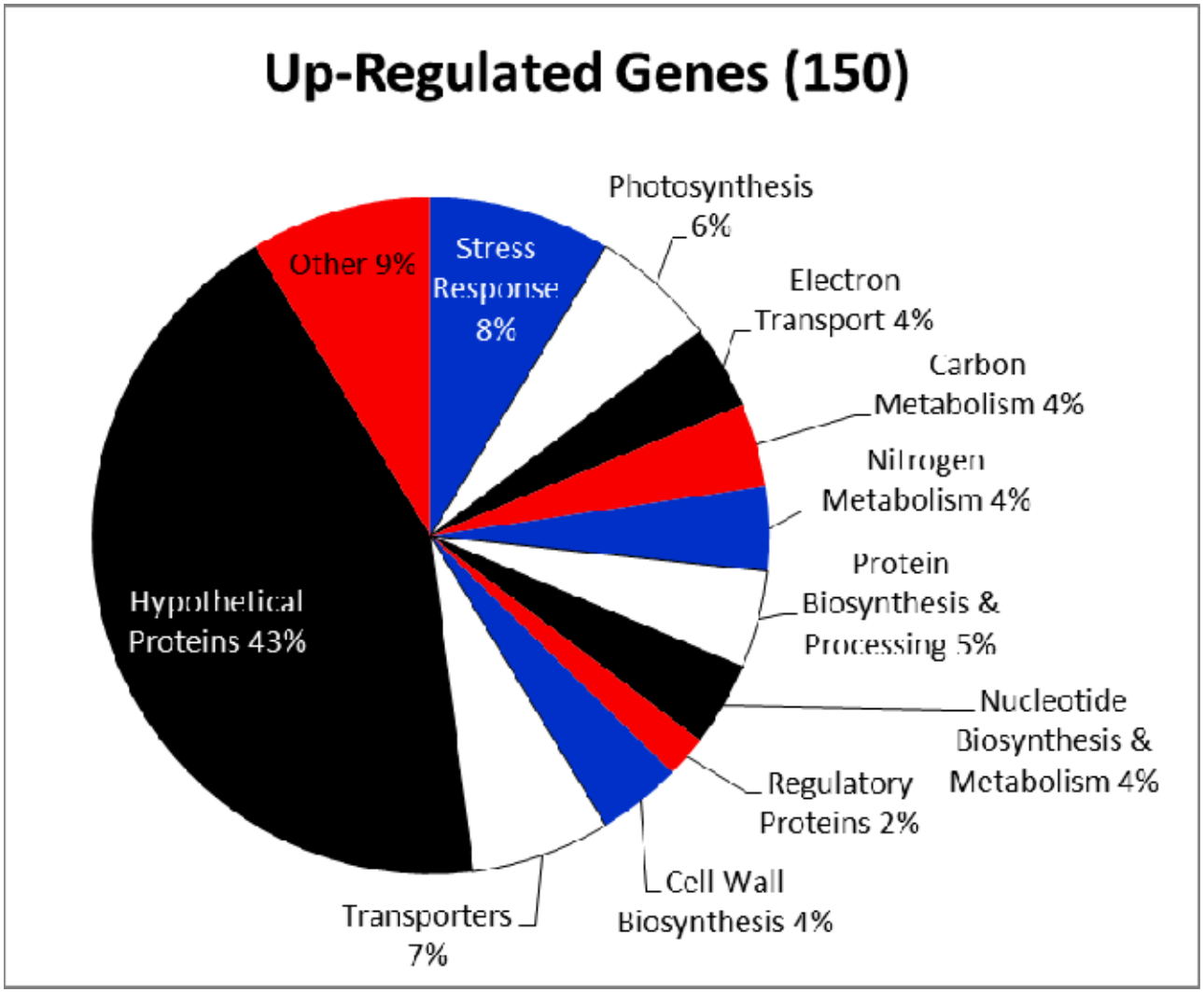
RNA-seq Analysis of FFA-Producing Cyanobacteria



Differential Gene Expression: Fold change > 2, p-value < 0.05.

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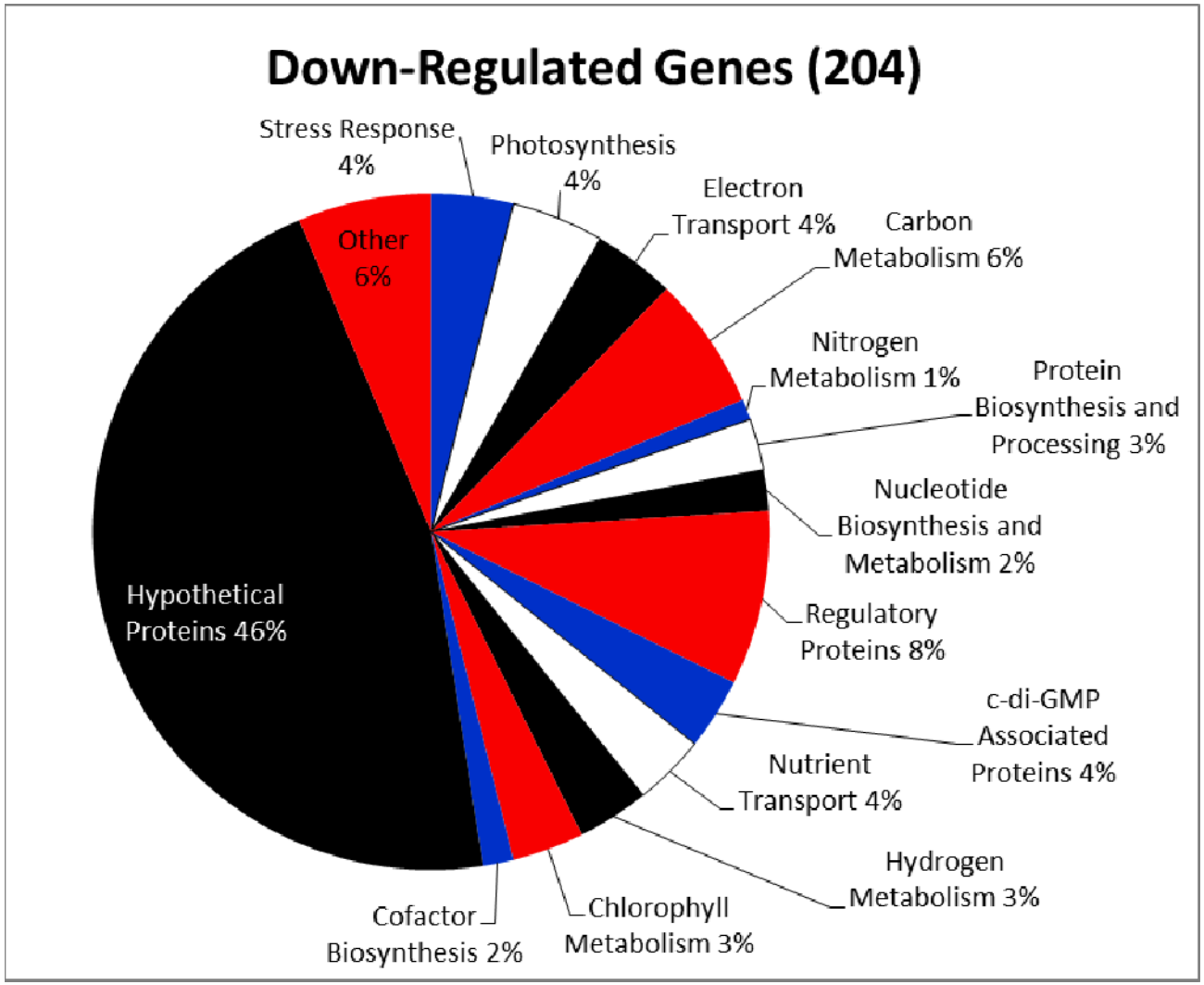


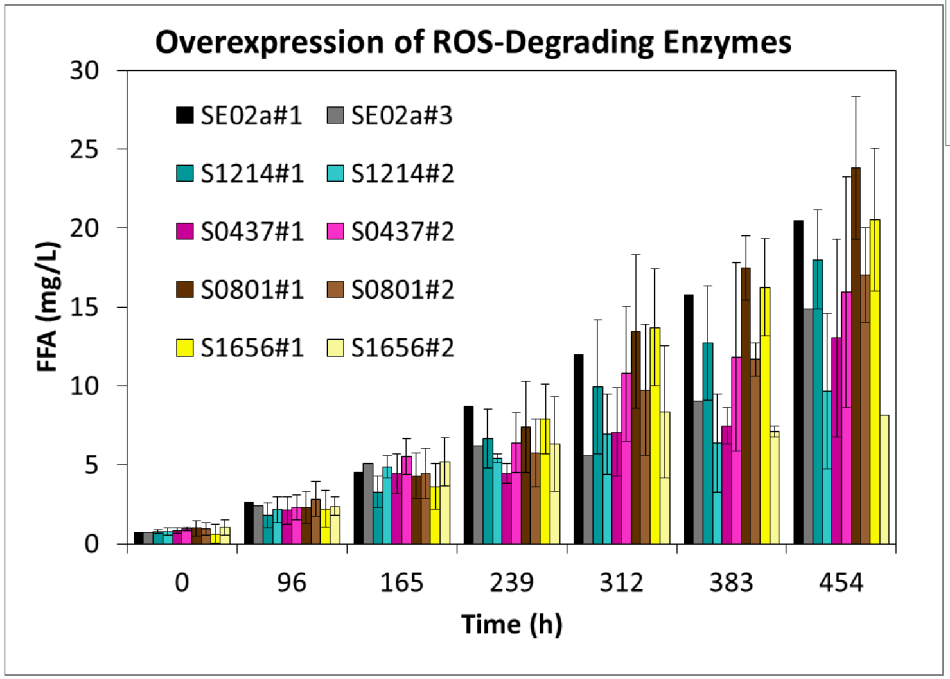
Differential Gene Expression: Fold change > 2, p-value < 0.05.

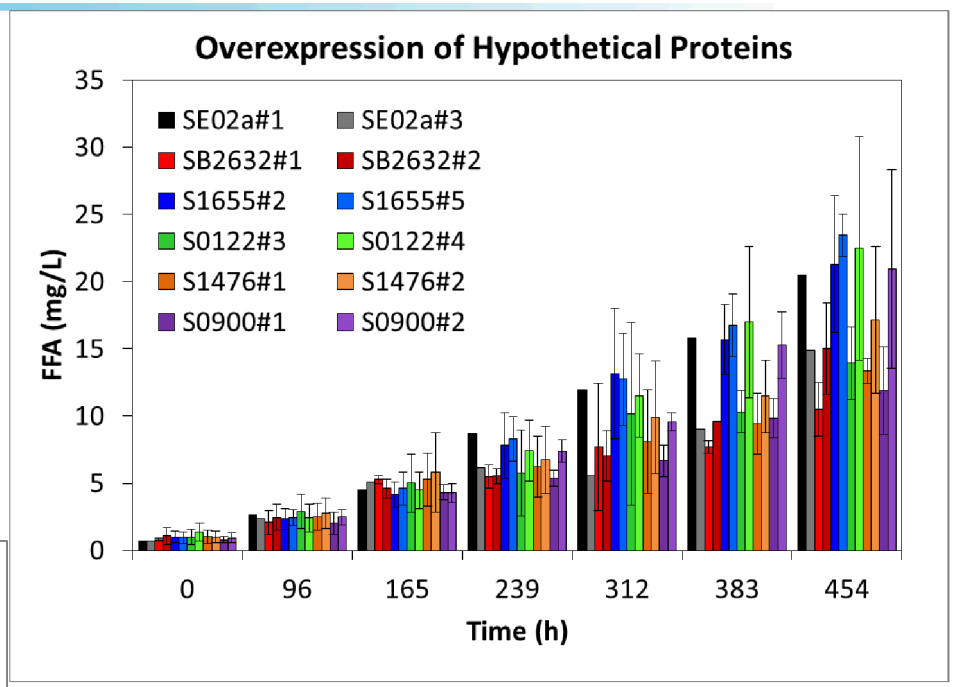
Identifying Targets for Improving FFA Production

Locus	Product	100h SE02 : SE01		100h SE02 : 7942		240h SE01 : 7942		240h SE02 : 7942		SE01 240h : 100h		Average	Overexpress or Knockout
		FC	PValue	FC	PValue	FC	PValue	FC	PValue	FC	PValue	FC	
Hypothetical Proteins													
444	hypothetical protein	3.19	3.6E-15	3.83	6.6E-20			2.01	1.6E-03	4.05	3.6E-23	3.27	Knockout
1561	hypothetical protein	2.43	1.6E-13	3.69	2.3E-12	2.14	1.2E-05	2.40	2.4E-06			2.67	Knockout
1023	hypothetical protein	2.02	1.5E-05	2.39	5.2E-07	2.11	8.2E-05	2.06	3.6E-06			2.15	Knockout
1476	hypothetical protein			-8.08	1.4E-03	-5.41	6.3E-05	-4.87	2.3E-03	-2.38	1.3E-08	-5.18	Overexpress
1655	hypothetical protein	-3.07	4.9E-07	-4.03	2.9E-09	-2.60	1.6E-08			-2.22	1.1E-06	-2.98	Overexpress
900	hypothetical protein	-2.74	5.9E-11	-4.03	1.8E-07	-2.03	4.1E-05	-2.90	4.1E-02			-2.92	Overexpress
B2632	hypothetical protein	-2.66	6.1E-12	-3.50	1.9E-10	-2.26	3.2E-09	-2.30	2.3E-05			-2.68	Overexpress
122	hypothetical protein	-2.06	3.4E-04	-3.38	5.2E-08	-2.03	5.5E-05	-2.65	2.3E-05			-2.53	Overexpress
1845	hypothetical protein			-2.34	5.4E-05	-2.14	5.1E-08	-2.64	3.0E-01	-2.01	5.2E-10	-2.28	Overexpress
ROS Degrading Proteins													
1214	glutathione peroxidase	2.04	1.3E-06	3.22	7.7E-11							2.63	Overexpress
437	glutathione peroxidase			2.83	7.5E-09			2.25	2.4E-04			2.54	Overexpress
801	superoxide dismutase			2.70	1.2E-10	2.42	7.2E-12					2.56	Overexpress
1656	catalase/peroxidase HPI			-2.38	4.6E-05							-2.38	Overexpress
Potential FFA Exporters													
2175	transport system substrate-binding			2.23	1.5E-03	3.76	3.3E-14					2.99	Knockout
1224	ABC-transporter membrane fusion	2.23	4.7E-06	3.26	6.0E-14							2.74	Knockout
1464	porin							2.28	5.1E-02			2.28	Knockout
1607	porin/major outer membrane protein					2.16	3.4E-06					2.16	Knockout

FFA Production in Overexpression Mutants

- pSA used to construct overexpression mutants (PLlacO1 expression)
- SE02 (Δ*aas, 'tesA*) is used as the host strain
- SE02a contains genome integration of the empty plasmid, pSA
- For each target gene, 2 transformants were screened for FFA production

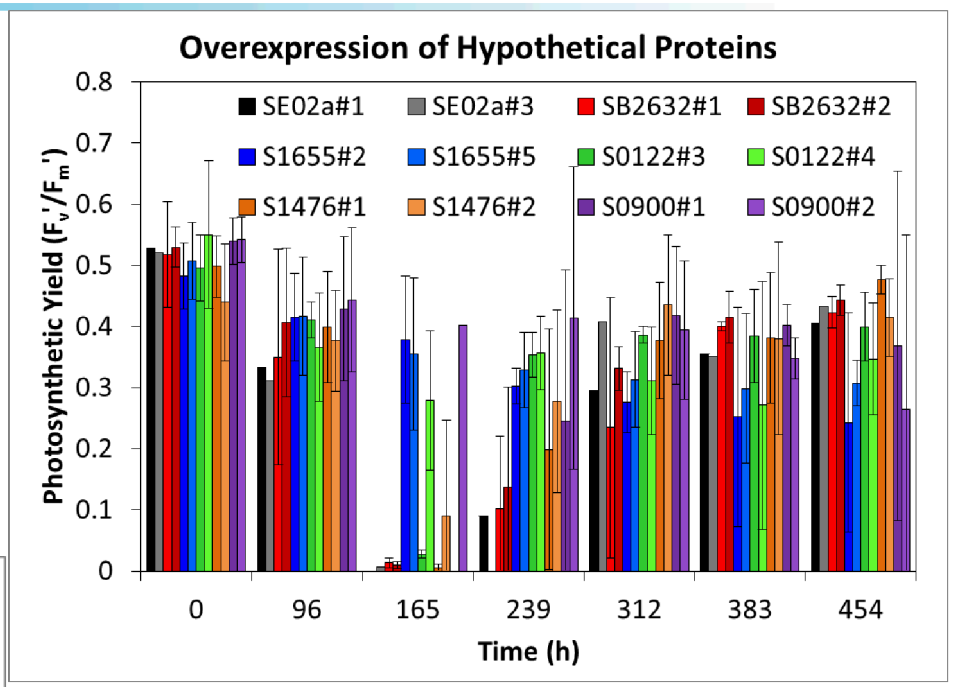


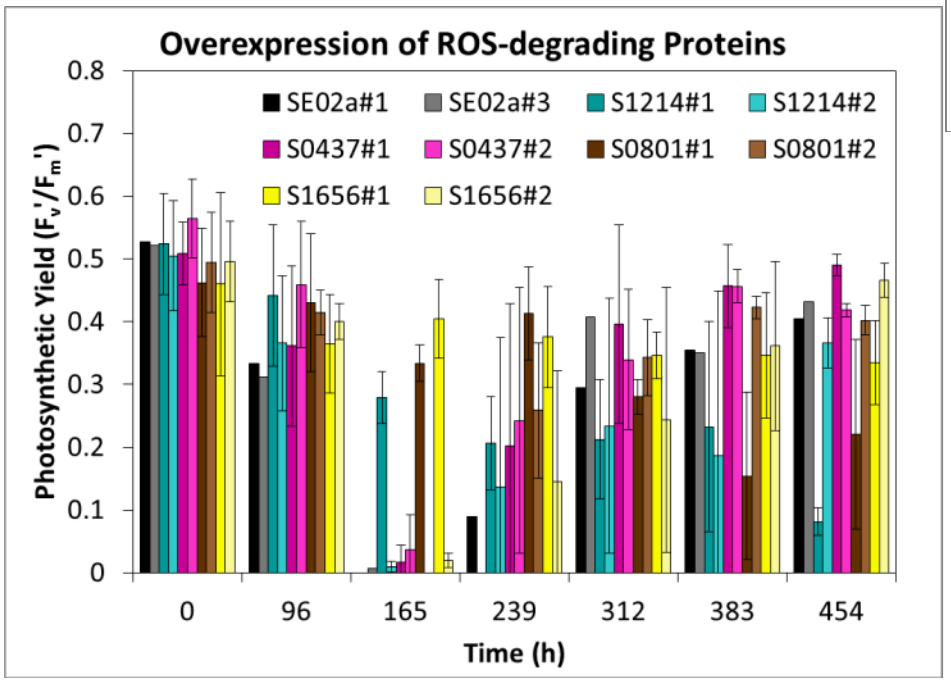


- No statistically significant change in FFA production for any of the hypothetical protein or ROS-degrading overexpression mutants
- High variability between biological replicates, particularly during late time points

Photosynthetic Yield in Overexpression Mutants

- After induction at 96h, photosynthetic yield (F_v'/F_m') drops to zero in the control strain (SE02a).
- Overexpression of several target genes prevents this drop in photosynthetic yield in the mutant strains.





Overexpression mutants with high F_v'/F_m':

- S1655 – hypothetical protein
- S0122 – hypothetical protein (putative diguanylate phosphodiesterase)
- S0900 – hypothetical protein (glutamine synthetase)
- S1214 – glutathione peroxidase
- S0801 – superoxide dismutase
- S1656 – catalase/peroxidase

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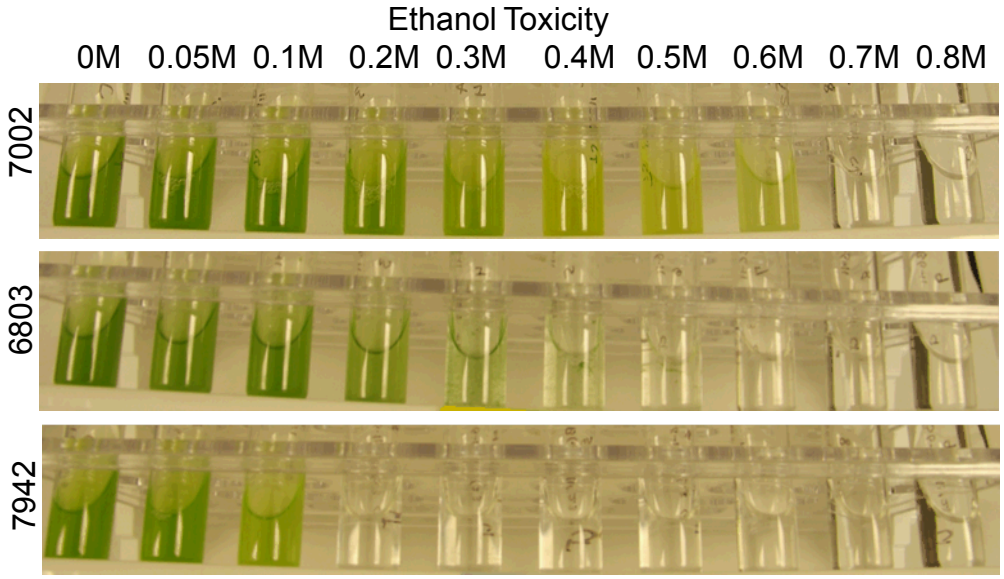
Synechococcus sp. PCC 7002: Host for FFA Production

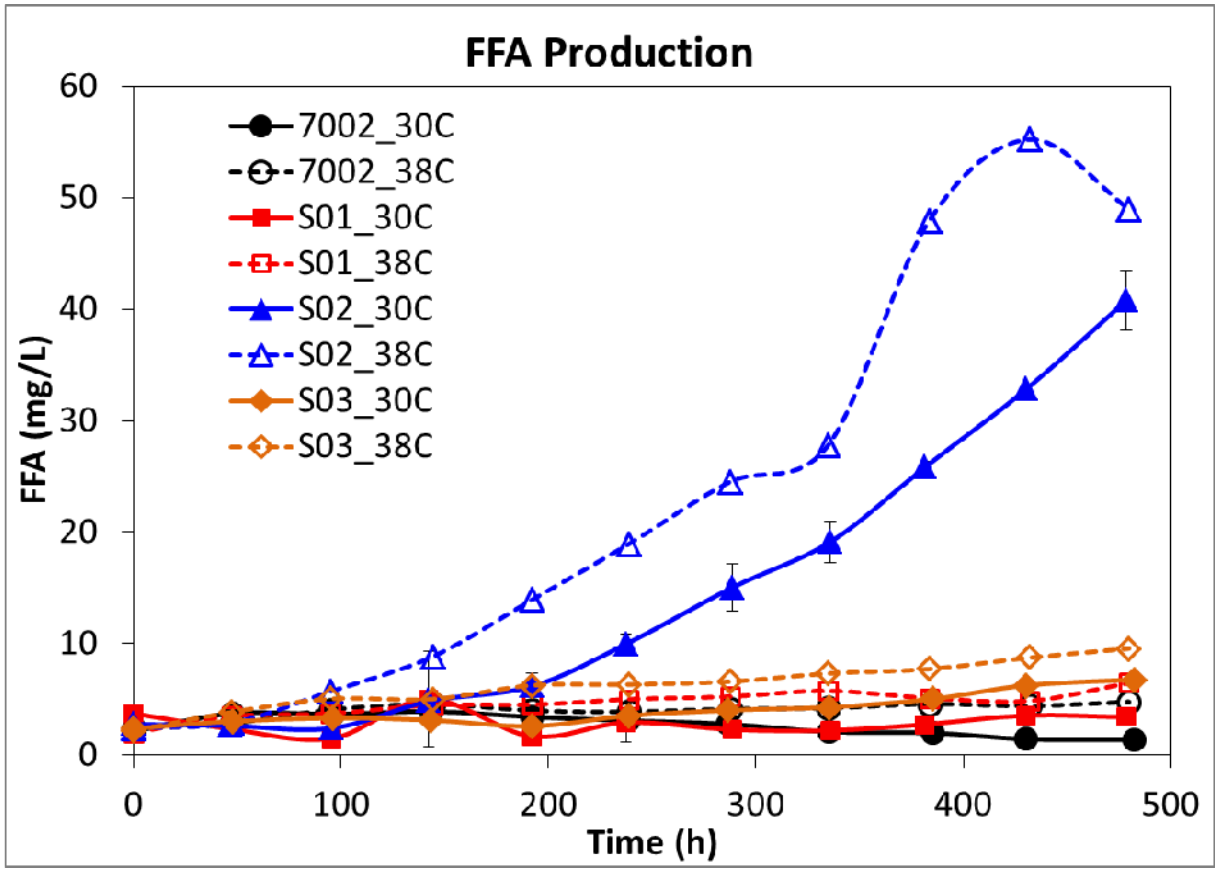
Synechococcus sp. PCC 7002

- Model cyanobacterium (genetic tools available)
- Marine strain
- High light tolerance
- Higher tolerance of biofuels compared to other model cyanobacteria



Genetic modifications	*S. elongatus* 7942	*Synechococcus* sp. 7002
Δ*aas/fadD*	SE01	S01
Δ*aas/fadD*, *'tesA*	SE02	S02
Δ*aas/fadD*, Fat1	SE03	S03
Δ*aas*, Fat1, *rbcLS*	SE04	
Δ*aas*, Fat1, *rbcLS*, *accBCDA*	SE05	
Δ*aas*, Fat1, P_{psbAI} *rbcLS*	SE06	
Δ*aas*, Fat1, P_{psbAI} *rbcLS*, P_{rbc} *accBC* P_{cpc} *accDA*	SE07	

FFA Production in 7002 Strains



7002: wild type

S01: Δ*fadD*

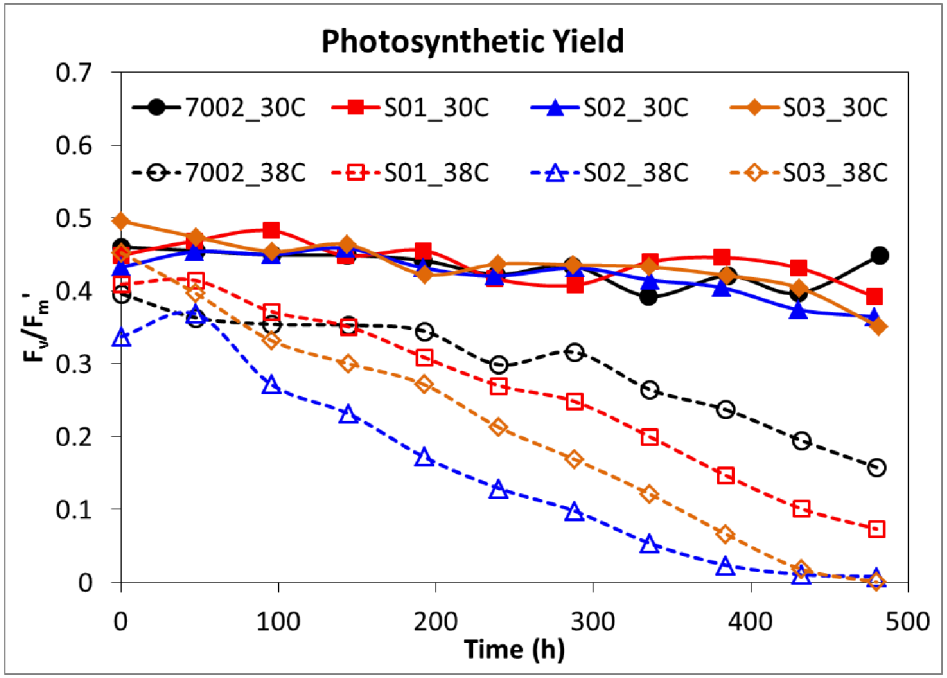
S02: Δ*fadD*, *'tesA*

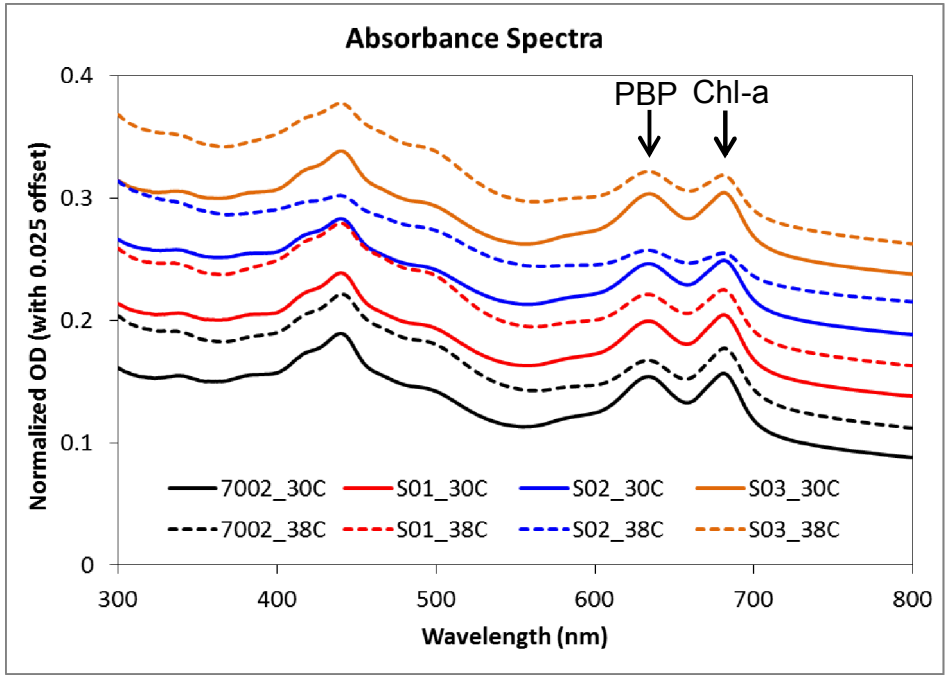
S03: Δ*fadD*, *fat1*

- FFA is produced and excreted by engineered 7002 strains
- 45–fold more FFA is produced using the *E. coli* thioesterase (*'tesA*, S02) compared to the *C. reinhardtii* acyl-ACP thioesterase (*fat1*, S03)
- The optimal growth temperature (38°C) leads to more FFA production compared to 30°C

Physiological Effects of FFA Production in 7002 Strains

- Photosynthetic yields (F_v'/F_m') remain constant at 30°C for the FFA-producing 7002 strains.
- At 38°C, there is a gradual decline in photosynthetic yield throughout FFA biosynthesis for all 7002 strains, yet this effect is most severe in the highest yielding FFA strain, S02.





- No change in photosynthetic pigments for 7002 strains at 30°C.
- S02 shows degradation of both phycobiliprotein and Chl-a pigments at 38°C.
- This response differs from that of 7942, which showed selective degradation of Chl-a.

7002: wild type; S01: Δ*fadD;* S02: Δ*fadD, 'tesA;* S03: Δ*fadD, fat1*

Outline

1. Engineering a model cyanobacterium *Synechococcus elongatus* PCC 7942 for FFA production
   - Engineered 7942 produces and excretes FFA, but the overexpression of rate-limiting steps and optimization of recombinant gene expression does not improve FFA yields.
2. Physiological effects of FFA production in cyanobacteria
   - These effects limit FFA production and must be addressed.
3. Seq-ing targets for improving FFA production
   - Several potential target genes have been identified.
4. Is cyanobacterial host selection critical for FFA production? … *Synechococcus* sp. PCC 7002: Another model host
   - Physiological effects of FFA production are minimized in 7002 at 30°C. Membrane desaturation may play an important role in FFA tolerance.
5. Biofuel toxicity for cyanobacteria

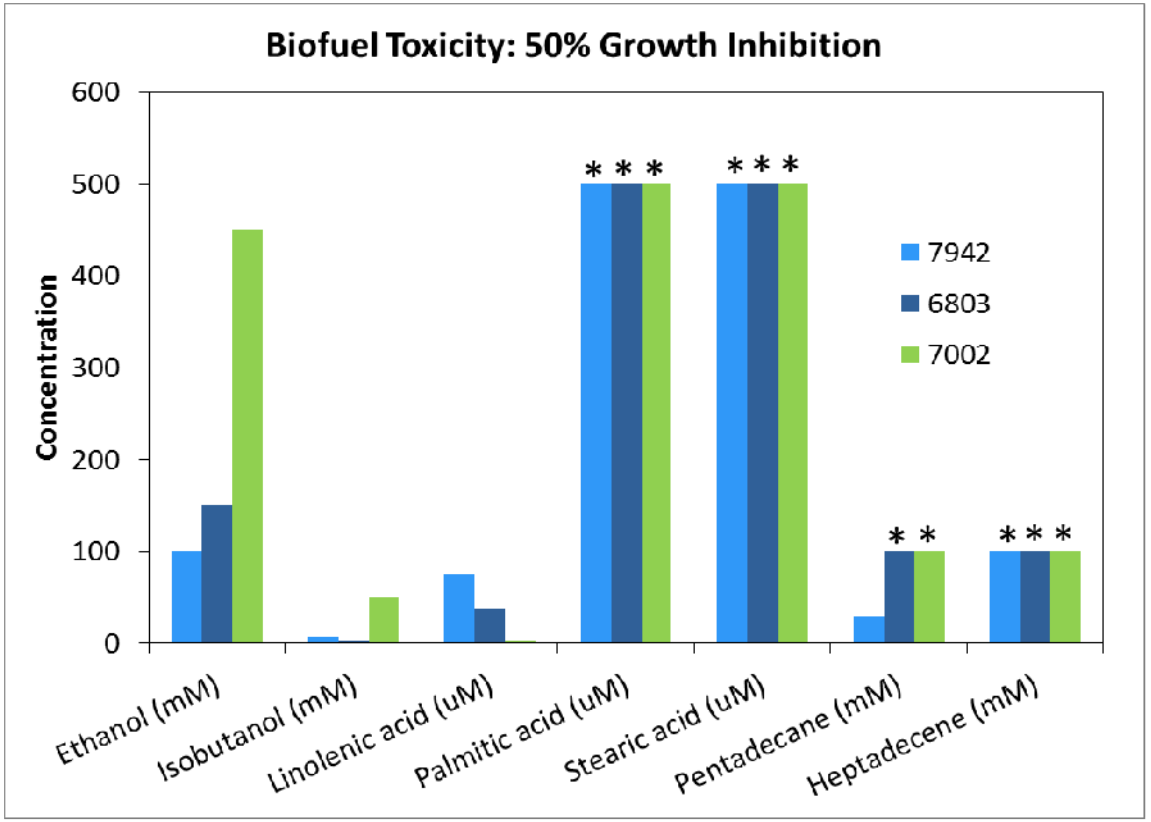
Biofuel Toxicity for Cyanobacteria

Model cyanobacteria:

- *Synechococcus elongatus* PCC 7942 (freshwater)
- *Synechocystis* sp. PCC 6803 (freshwater)
- *Synechococcus* sp. PCC 7002 (marine)

Biofuels:

- Short and long chain alcohols
- Fatty acids (saturated and unsaturated)
- Alkanes and alkenes

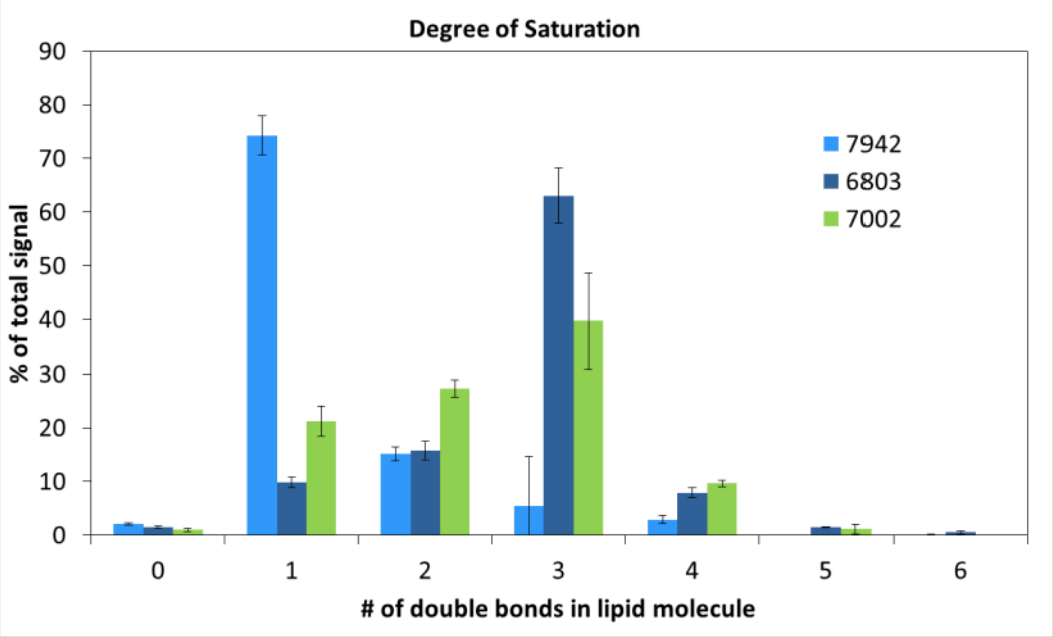


* Highest concentration tested.

- 7002 has higher tolerance of short-chain alcohols
- 7942 has higher tolerance of unsaturated fatty acids
- Saturated fatty acids and alkanes/alkenes do not appear to be toxic to cyanobacteria

Mechanisms of Biofuel Tolerance: Membrane Structure/Permeability

- ESI/MS analysis of membranes from 7942, 6803, and 7002.
  - ➢ 7002 has higher amounts of unsaturated fatty acids in its membrane.
- Construct 7002 mutants:
  - *7002ΔdesB*
  - *7002ΔdesE*
  - *7002ΔdesF*



Comparative Genomics: Desaturases

7942		6803		7002	
Locus	Description	Locus	Description	Locus	Description
2561	delta-9 acyl-phospholipid desaturase	2538	acyl-CoA desaturase, desC	A2198	delta-9 acyl-lipid desaturase, desC
		1594	fatty acid desaturase, desA	A2756	homology to SYNPCC70025_A0159, desA
		1727	delta 15 desaturase, desB	A0159	omega-3 acyl-lipid desaturase, desB
		1931	delta-6 desaturase, desD	A1989	syn-2, delta 9 acyl-lipid fatty acid desaturase, desF
				A2833	fatty acid desaturase, desE

Mechanisms of Biofuel Tolerance: Efflux Pumps

Comparative Genomics: Efflux Proteins

7942		6803		7002	
Locus	Description	Locus	Description	Locus	Description
1869	cation efflux system protein	1991	cation or drug efflux system protein	A0587	cation efflux system protein CzcA
1938	multidrug-efflux transporter	1260	quinolene resistance protein NorA	A0589	arsenite efflux pump ACR3
2032	multidrug-efflux transporter quinolene resistance protein NorA	1494	cation or drug efflux system protein, AcrB, TtgB, MexF BLAST hit	A0087	major facilitator transporter
2369	hydrophobe/amphiphile efflux-1 HAE1, AcrB, TtgB, MexF BLAST hit	2483	Probable multidrug resistance protein norM (Multidrug-efflux transporter)	A1013	hydrophobe/amphiphile efflux-1 (HAE1) family protein, AcrB, TtgB, MexF BLAST hit
1989	cation diffusion facilitator family transporter	2125	cation or drug efflux system protein	A1574	RND family efflux transporter MFP subunit
1699	MATE efflux family protein	2737	cation or drug efflux system protein	A2463	cation efflux system protein
792	multidrug efflux MFS transporter	3105	cation or drug efflux system protein	A2552	RND family efflux transporter MFP subunit
				A0585	Outer membrane efflux protein
				A0591	RND family efflux transporter MFP subunit
				A0719	multidrug efflux transporter
				A1483	RND family efflux transporter MFP subunit

- Construct 7002 mutants:
  - 7002ΔA1013
  - 7002ΔA0585
  - 7002ΔA0719

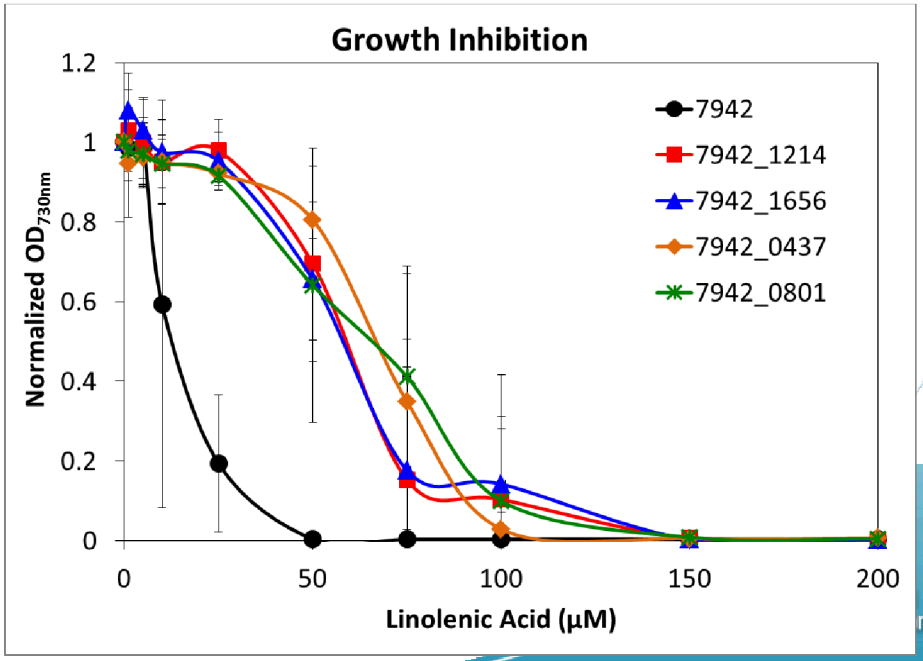
Mechanisms of Biofuel Tolerance: Stress Response

Comparative Genomics: ROS-Degrading Enzymes

7942		6803		7002	
Locus	Description	Locus	Description	Locus	Description
801	superoxide dismutase	1451	superoxide dismutase, sodB	A0242	Mn-superoxide dismutase, sodB
1214	glutathione peroxidase	1769	glutathione peroxidase	A0117	glutathione peroxidase
1656	catalase/peroxidase HPI	1399	catalase HPI, katG	A2422	catalase/peroxidase HPI, katG
1937	peptide methionine sulfoxide reductase	46	methionine sulfoxide reductase A	A0215	methionine sulfoxide reductase A
2190	methionine sulfoxide reductase B	218	methionine sulfoxide reductase B	A0672	methionine-R-sulfoxide reductase
437	glutathione peroxidase	1305	glutathione peroxidase	A0970	glutathione peroxidase
B2620	putative catalase	239	methionine sulfoxide reductase A (protects against oxidative stress)		

- 7942 overexpression mutants
  - 7942_*1214*
  - 7942_1656
  - 7942_0437
  - 7942_0801

➢ All mutants overexpressing ROS-degrading enzymes showed reduced growth inhibition with linolenic acid addition.



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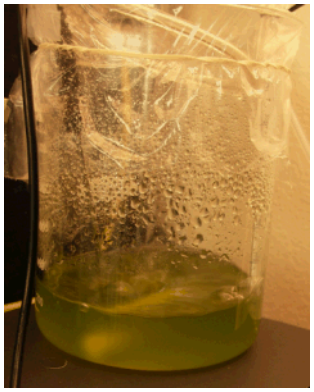
5. Biofuel toxicity for cyanobacteria
   - 7002 has high tolerance for short-chain alcohols; 7942 has tolerance for unsaturated fatty acids (UFAs).
   - ROS-degrading enzymes are important for UFA tolerance.

Conclusions and Contributions

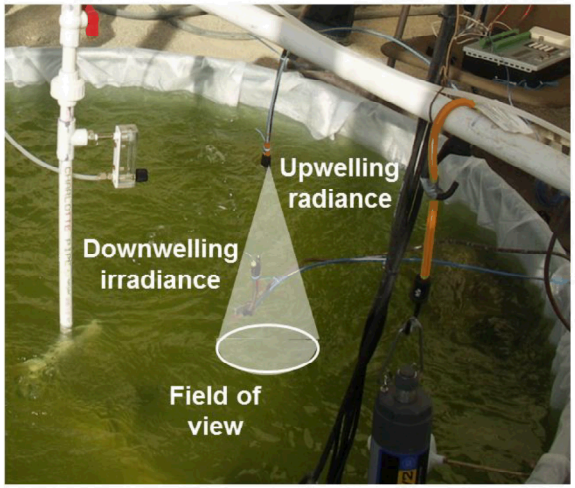
- Advancements in engineering cyanobacteria for FFA production
  - Successful FFA production and excretion in two cyanobacterial hosts
  - Cloning and expression of green algal genes for FFA synthesis
  - Investigation of inducible and native promoters for gene expression
- Characterization of the effects of FFA production in cyanobacteria
  - Physiological effects: cell growth, stress, cell death, photosynthetic yield, photosynthetic pigments
  - Identification of target genes affecting cell physiology during FFA production (RNA-seq, mutants)
- Host strain selection and characterization
  - Minimal physiological effects of FFA production in 7002 at 30°C
  - Degree of membrane saturation and ROS-degrading enzymes are important for biofuel tolerance.

Spectroscopic Signatures of Algal Pond Health

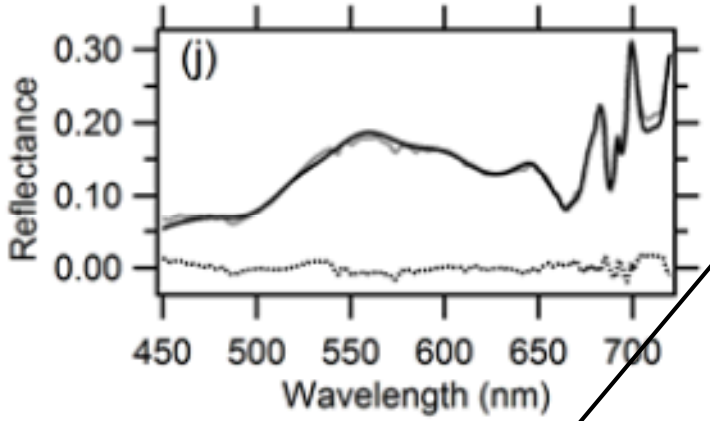
Reflectance measurements for real-time monitoring of algal ponds



Laboratory (0.3 L)



Greenhouse Pond (530 L)

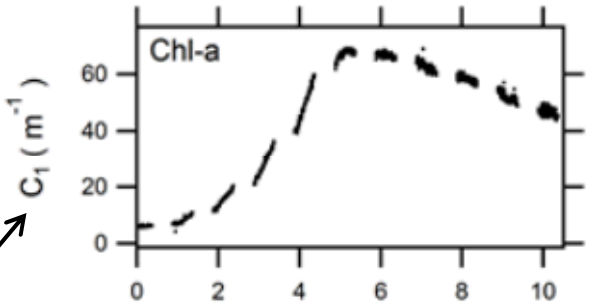


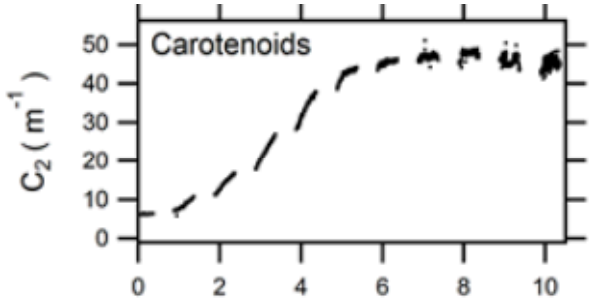
$$r_{bs} = \frac{\bar{L}_w}{\bar{E}_d} = f(u)$$

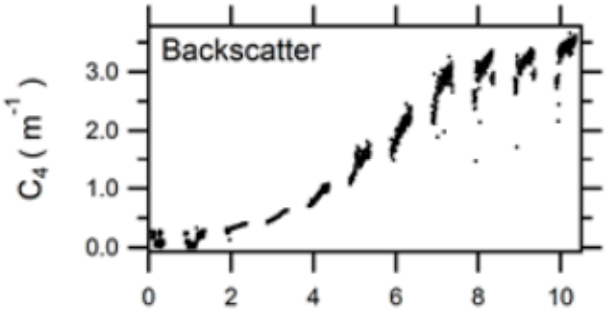
$$u = \frac{b_b}{a + b_b}$$

$$a = C_1 A_{\text{Chl-a}} + C_2 A_{\text{caroteoids}} + C_3 A_{\text{other}} + a_{\text{water}}$$

$$b_b = C_4 \bar{B}$$





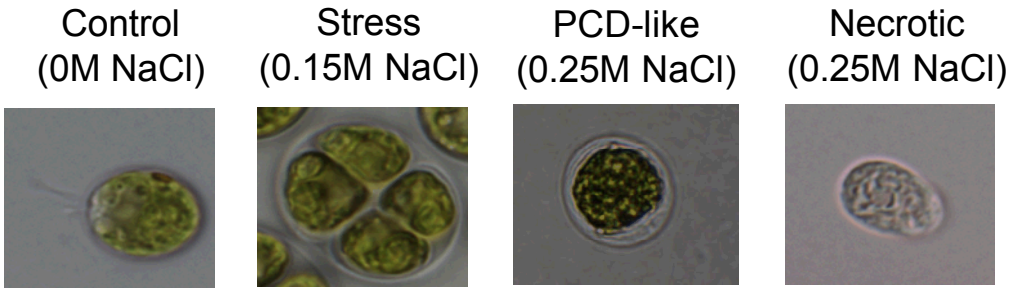


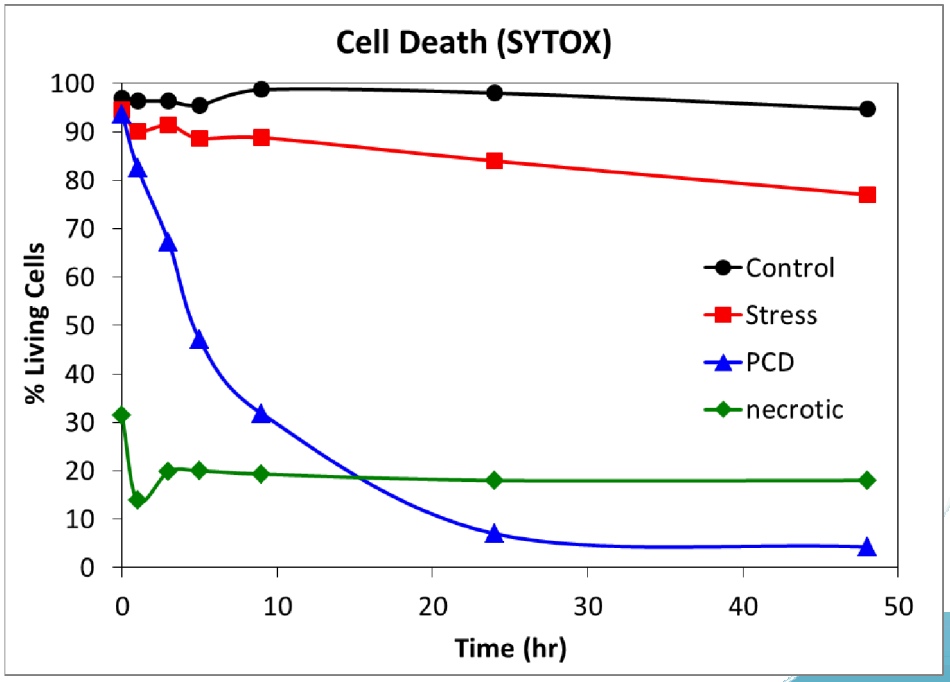
Reichardt TA, Garcia OF, Collins AM, Jones HDT, Ruffing AM, Timlin JA. Spectroradiometric monitoring of *Nannochloropsis salina* growth. (2012) Algal Research. 1 (1): 22-31.

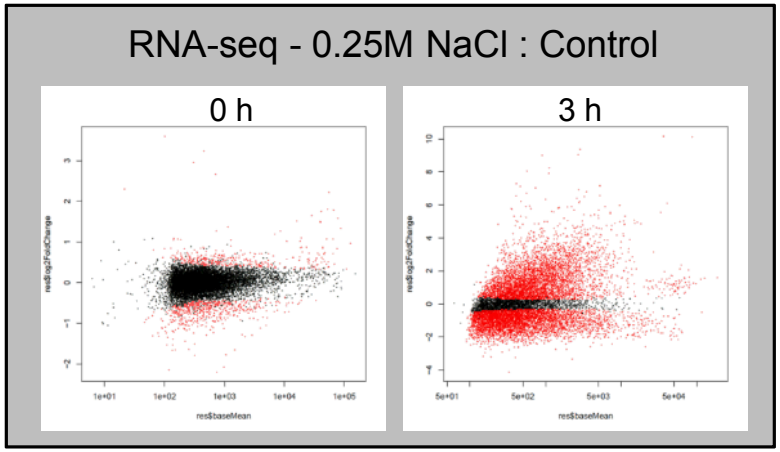
Delineating Stress and Programmed Cell Death in Green Algae

- Stress is important for high TAG biosynthesis in algae.
- However, stress can also lead to programmed cell death (PCD) and necrotic cell death.
- It is necessary to understand the boundaries between stress, PCD, and necrosis.
- qRT-PCR and RNA-seq to identify genetic markers specific to PCD
  - ➢ Metacaspase

Chlamydomonas reinhardtii CC-125

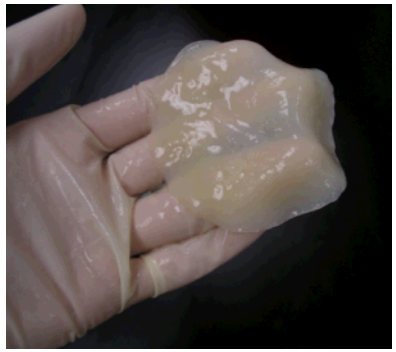


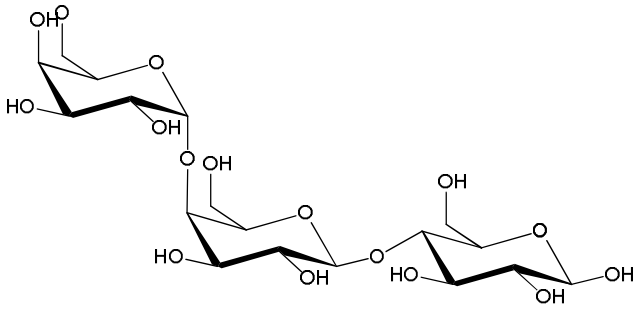




Metabolic Engineering of an *Agrobacterium* sp.

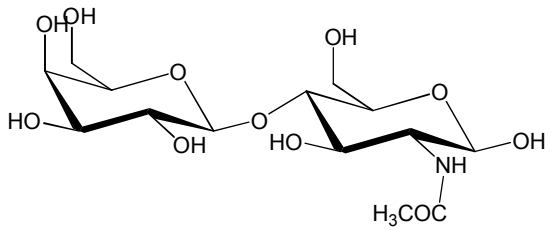
- Engineered *Agrobacterium* sp. ATCC 31749 as a whole-cell biocatalyst for production of medically-relevant oligosaccharides.
- Optimized environmental variables for maximum oligosaccharide production using metabolic flux analysis.
- Sequenced, assembled, and annotated the genome of *Agrobacterium* sp. ATCC 31749.
- Designed custom microarrays for transcriptome analysis of polysaccharide (curdlan) production.
- Generated knockout mutants to identify regulatory factors affecting polysaccharide production in this *Agrobacterium*.





α-Gal epitope

(Gal-α1,3-Gal-β1,4-Glc)



LacNAc

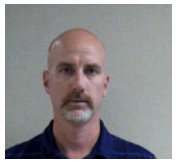
(Gal-β1,4-GlcNAc)

Georgia Institute of Technology

Sandia National Laboratories

Acknowledgements

Hyperspectral Imaging



Howland D.T. Jones

Michelle Raymer

Omar F. Garcia

Biofuel Toxicity Experiments

Christine Trahan

Project Mentors / Managers

Anthony Martino

Eric Ackerman

Jim Carney

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Lipid analysis

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Sequencing (RNA-Seq)

Los Alamos National Laboratory (LANL)

Signatures of Algal Pond Health:

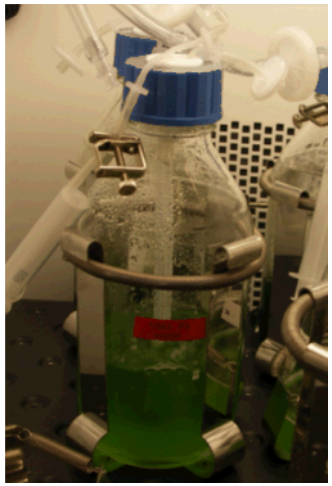
Howland Jones, Tom Reichardt, Scott James, Patricia Gharagozloo, Aaron Collins, Anne Ruffing, David Hanson, Amy Powell, Kylea Parchert, Omar Garcia, Thomas Dempster, John McGowen, Christine Trahan, Tom Turner, Brian Dwyer, Vokau Kamardjanam, Andrew August, Jeri Timlin (PI)

Funding: Laboratory Directed Research & Development (LDRD)

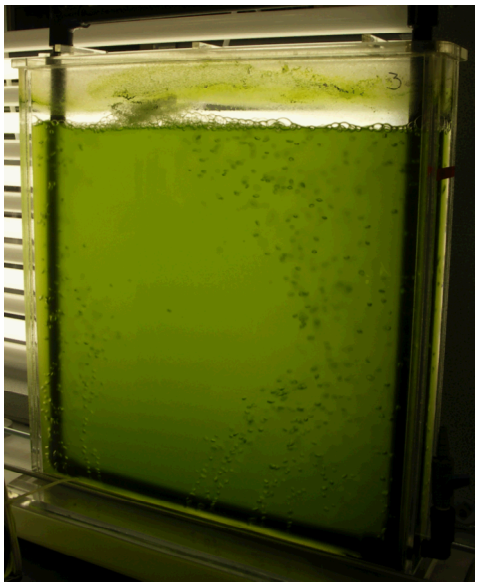
Cultivation Scales

4 mL

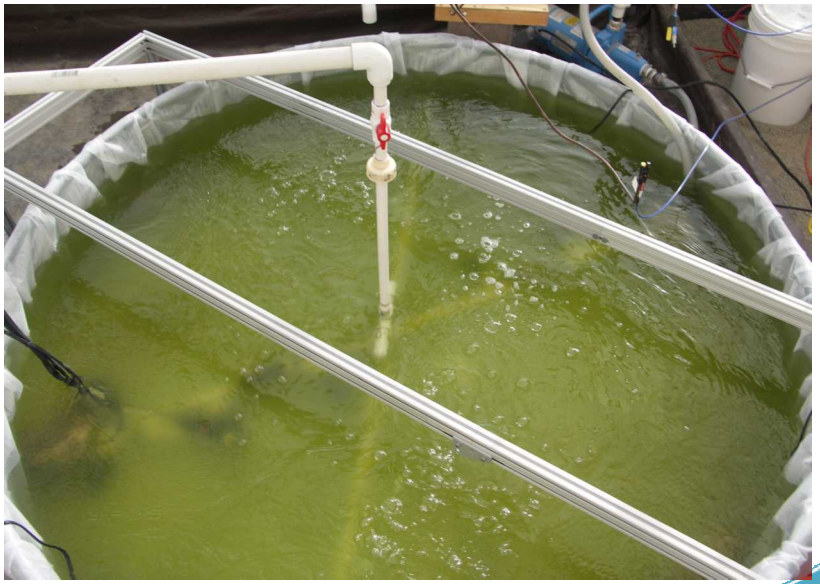
100 mL



400 mL



15 L

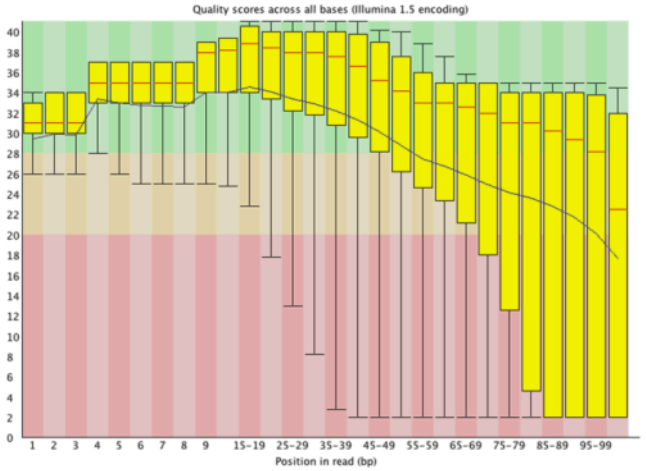


530 L

RNA-seq Protocol

RNA preparation:

- Hot-phenol extraction of RNA
- Quant-iT Ribogreen for RNA quantitation
- RNA Nano 6000 kit with Agilent 2100 Bioanalyzer
- RiboZero rRNA Removal kit for Gram-negative bacteria



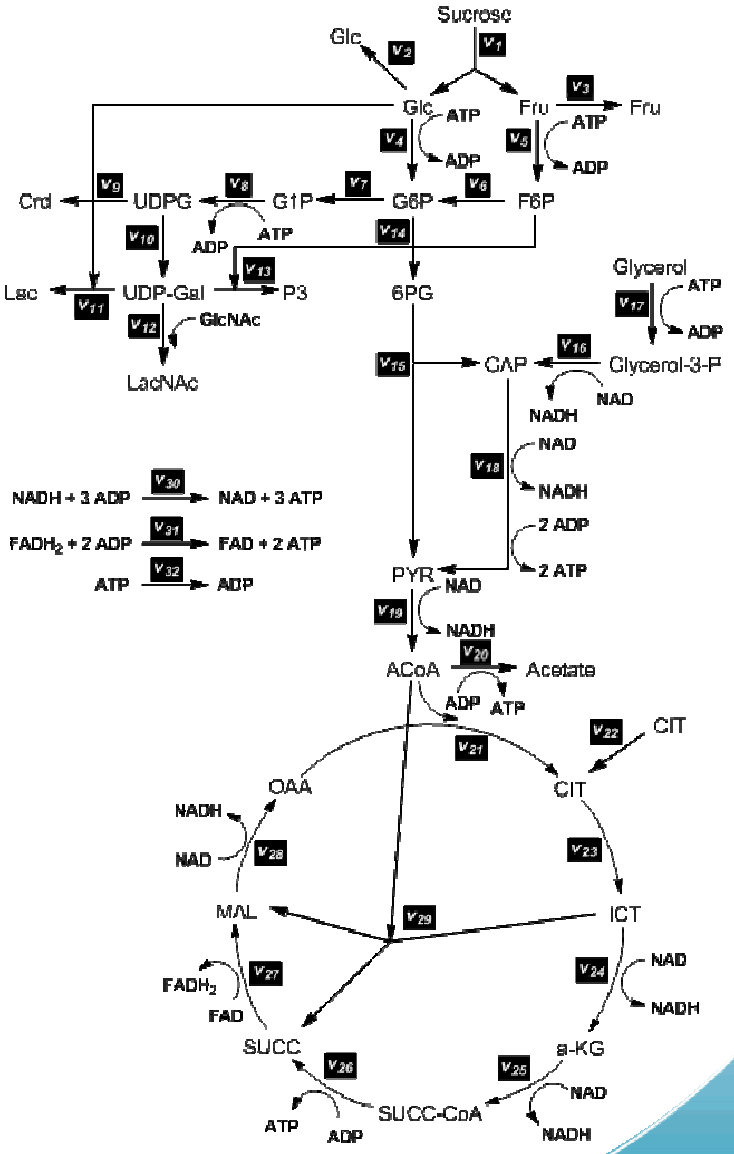
Data Analysis

- FastQC for assessing read quality
- Cutadapt to remove adapter sequences and low quality nt calls (phred < 20)
- Prinseq to remove polyA/T ends and reads < 20 nt
- Bowtie for read alignment, best alignment selected
- HTSeq used to obtain read hit counts from Bowtie alignments
- EdgeR for differential gene expression analysis and significance testing
  - Normalization methods: trimmed mean of M-values (TMM), relative log expression (RLE), and upperquartile normalization (UQ)
  - Fisher's exact test to compute p-values
  - Fold changes > 2-fold with p-values < 0.05 were considered to be differentially expressed.

Metabolic Flux Analysis (MFA)

Flux	Enzyme(s)	Citrate-free	5 g/L sodium citrate
v_1*	sucrose hydrolase	19	100
v_2*	glucose accumulation	3.3	24
v_3*	fructose accumulation	4.3	8.8
v_4	glucokinase	16	73
v_5	fructokinase	15	91
v_6	phosphoglucose isomerase	15	90
v_7	phosphoglucomutase	-0.08	9.0
v_8	UTP glucose-1-phosphate uridylyltransferase	-0.08	9.0
v_9*	curdlan synthase	0.39	0.44
v_{10}	UDP-galactose 4'-epimerase	-0.47	8.6
v_{11}*	β1,4-galactosyltransferase (glucose as substrate)	-0.23	3.6
v_{12}*	β1,4-galactosyltransferase (GlcNAc as substrate)	-0.17	3.3
v_{13}*	β1,4-galactosyltransferase (mannose as substrate)	-0.07	1.6
v_{14}	glucose-6-phosphate dehydrogenase; 6-phospho-gluconolactonase	31	153
v_{15}	6-phosphogluconate hydrolase; 2-keto-3-deoxy-gluconate aldolase	31	153
v_{16}	glycerol-3-phosphate dehydrogenase	-3.5	24
v_{17}*	glycerol kinase	-3.5	24
v_{18}	glyceraldehyde-3-phosphate dehydrogenase; phosphoglycerate kinase; phosphoglycerate mutase; pyruvate kinase	27	177
v_{19}	pyruvate dehydrogenase	58	330
v_{20}*	acetyl-CoA synthetase	17	25
v_{21}	citrate synthase	41	308
v_{22}*	citrate uptake	0	2.8
v_{23}	aconitase	41	310
v_{24}	isocitrate dehydrogenase	41	314
v_{25}	α-ketoglutarate dehydrogenase complex	41	314
v_{26}	succinyl-CoA synthetase	41	314
v_{27}	succinate dehydrogenase; fumarase	41	311
v_{28}	malate dehydrogenase	41	308
v_{29}	isocitrate lyase; malate synthase	0	-2.8
v_{30}	oxidative phosphorylation (NAD/NADH)	205	1466
v_{31}	oxidative phosphorylation (FAD/$FADH_2$)	41	311
v_{32}	maintenance energy (ATP consumption)	784	5516

2. Values normalized based on sucrose consumption in the 5 g/L sodium citrate reaction.
* Measured flux



Sandia National Laboratories

Publications

Research Articles

- Anne M Ruffing. Borrowing genes from *Chlamydomonas reinhardtii* for free fatty acid production in cyanobacteria. *Journal of Applied Phycology.* 2012. Submitted.
- Anne M Ruffing and Howland DT Jones. Physiological effects of free fatty acid production in genetically engineered *Synechococcus elongatus* PCC 7942. *Biotechnology and Bioengineering*. 2012. 100(9): 2190-2199. (Cover, Spotlight)
- Anne M Ruffing and Rachel R Chen. Transcriptome profiling of a curdlan-producing *Agrobacterium* reveals conserved regulatory mechanisms of exopolysaccharide biosynthesis. *Microbial Cell Factories*. 2012. 11:17.
- Thomas A Reichardt, Omar F Garcia, Aaron M Collins, Howland DT Jones, Anne M Ruffing, and Jerilyn A Timlin. Spectroradiometric monitoring of *Nannochloropsis salina* growth. *Algal Research*. 2012. 1(1): 22-31.
- Anne M Ruffing, Marlene Castro-Melchor, Wei-Shou Hu, and Rachel R Chen. Genome sequencing of the curdlan-producing *Agrobacterium* sp. ATCC 31749. *Journal of Bacteriology*. 2011. 193(16): 4294-4295.
- Anne M Ruffing and Rachel R Chen. Citrate stimulates oligosaccharide synthesis in metabolically engineered *Agrobacterium* sp. *Applied Biochemistry and Biotechnology*. 2011. 164(6): 851-866.
- Anne M Ruffing and Rachel R Chen. Metabolic engineering of *Agrobacterium* sp. strain ATCC 31749 for production of an α-Gal epitope. *Microbial Cell Factories*. 2010. 9(1).
- Anne Ruffing, Zichao Mao, and Rachel Ruizhen Chen. Metabolic engineering of *Agrobacterium* sp. for UDP-galactose regeneration and oligosaccharide synthesis. *Metabolic Engineering*. 2006. 8(5): 465-473.

Review Articles

- Anne M Ruffing. Engineered Cyanobacteria: Teaching an old bug new tricks. *Bioengineered Bugs*. 2011. 2(3).
- Anne Ruffing and Rachel Ruizhen Chen. Metabolic engineering of microbes for oligosaccharide and polysaccharide synthesis. *Microbial Cell Factories*. 2006. 5: 25-33.

Book Chapters

- Anne M Ruffing. Metabolic Engineering of Hydrocarbon Biosynthesis for Biofuel Production. Book chapter in *Biofuels*. Intech, Rijeka, Croatia. 2012. Accepted.
- Anne Ruffing and Rachel Ruizhen Chen. Metabolic Engineering of Microorganisms for Oligosaccharide and Polysaccharide Production. Book chapter in *Microbial Production of Biopolymers and Polymer Precursors: Applications and Perspectives*. Horizon Bioscience, Wymondham, UK. 2009. p197-228.
- Anne Ruffing and Rachel Ruizhen Chen. Metabolic Engineering and Other Methods of Strain Improvement. Book chapter in *Advances in Fermentation Technology*. Asiatech Publishers, Inc. New Delhi. 2008. p119-144.