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Engineering Cyanobacteria for Biofuel Production:

New Host, New Challenges

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U.S. DEPARTMENT OF ENERGY

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Outline

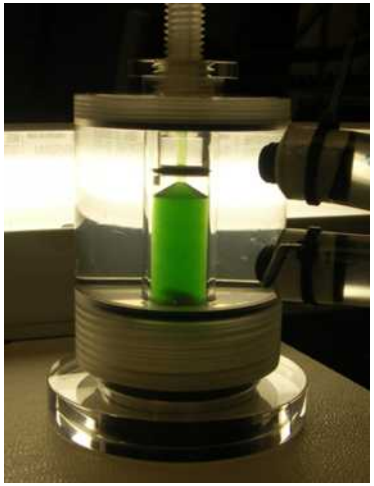
Background | Cyanobacterial Fuels | New Challenges & Potential Solutions | Conclusions

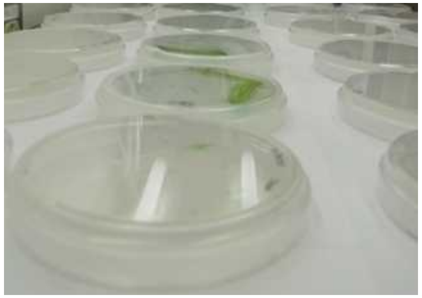
- Algal Biofuels
- Cyanobacteria

- Review of past and current work in the field
- Truman project: Engineering *Synechococcus elongatus* PCC 7942 for FFA production

- Physiological effects of FFA production
- Product toxicity
- Contamination

- Advantages
- Disadvantages





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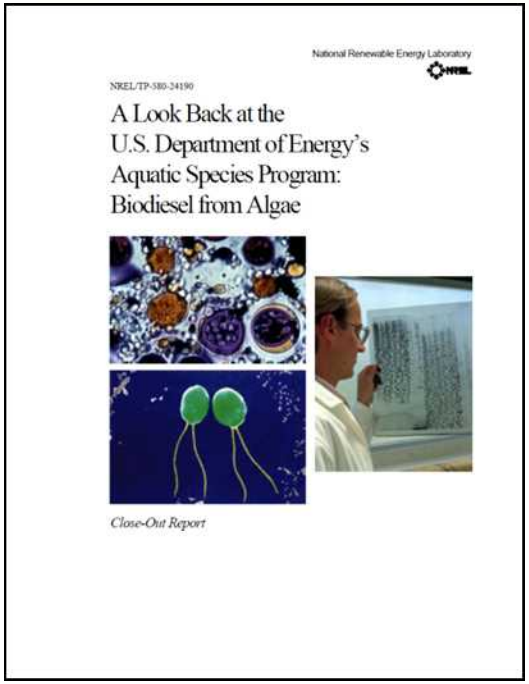
History of Algal Biofuels

DOE’s Aquatic Species Program (1978 – 1996)

- Bioprospecting - focused on eukaryotic algae

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.

- Genetic engineering – only reported for *Chlamydomonas reinhardtii* (model green alga) and diatoms
  - ➢ Successful expression of acetyl-CoA carboxylase (ACCase) in *Cyclotella cryptica* and *Navicula saprophila*
  - ➢ No improvement in lipid production



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

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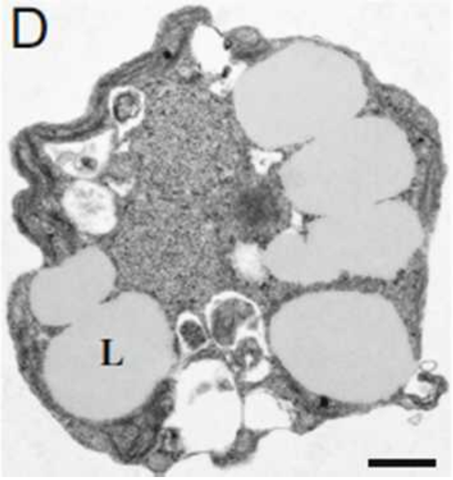
Challenges in Algal Biofuels

- Productivity
- Fuel extraction
- Raw materials
  - Water
  - Nitrogen
  - Phosphorus
- Scale-up / Cultivation
  - Crop protection
  - Temperature

Algal Species	Doubling time (h)	Oil content (% dry wt)
Chlamydomonas reinhardtii	8	15-25
Chlorella vulgaris	8	28-35
Dunaliella salina	10-20	16-44
Nannochloropsis salina	14-23	31-68
Botryococcus braunii	72-96	25-75



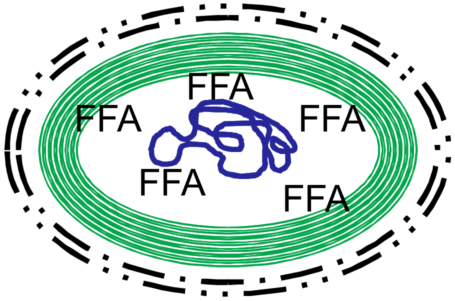
Li, Y. et al. (2010) Met. Eng.12: 387--91.

Seambiotic

Challenges in Algal Biofuels
… Cyanobacteria: A Potential Solution

- Productivity ✓
- Fuel extraction ✓
- Raw materials
  - Water
  - Nitrogen ✓
  - Phosphorus ✓
- Scale-up / Cultivation
  - Crop protection ✓
  - Temperature ✓

Cyanobacterial-based fuel production



- Cyanobacteria secrete fuel precursors (i.e. free fatty acids)
- Continuous production during stationary phase
  - No destruction of biocatalyst
  - Decouples growth and fuel production
- Easy to genetically engineer
- Growth at high temperatures

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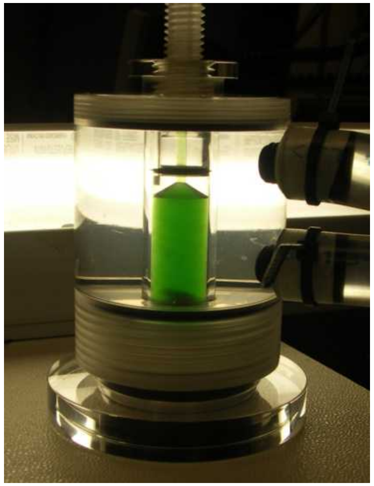

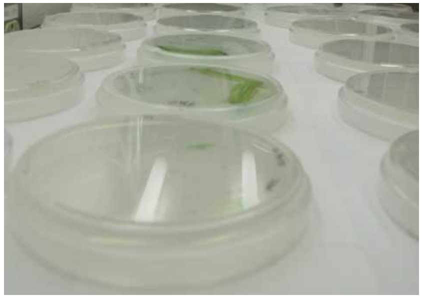

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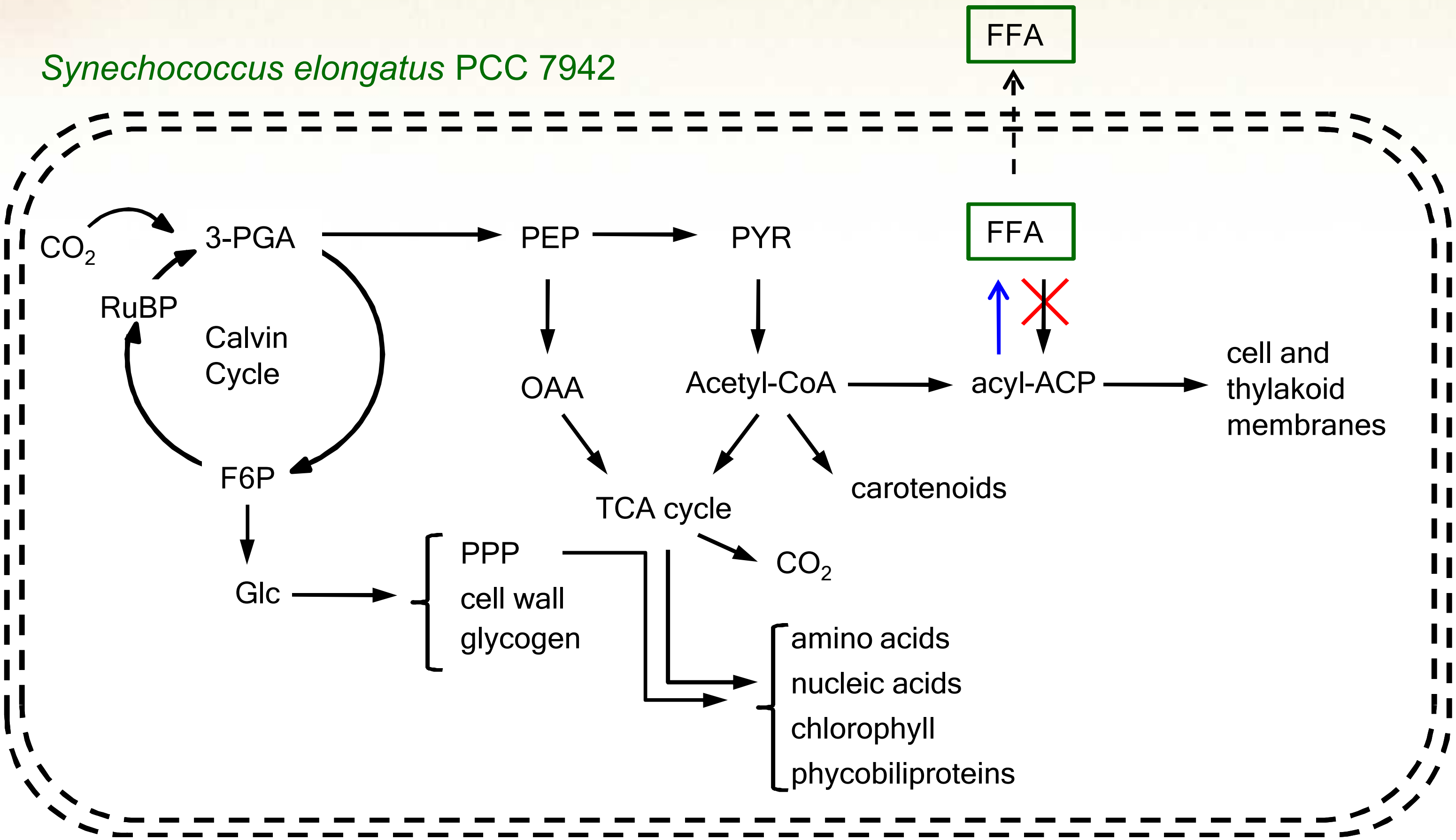
Engineering Cyanobacteria for Biofuel Production

Fuel Product	Amount (g/L)	Cyanobacterium	Reference
Ethanol	0.23	Synechococcus elongatus PCC 7942	Deng, 1999
	0.55	Synechocystis sp. PCC 6803	Dexter, 2009
	~ 2.5	Synechocystis sp. PCC 6803; Anabaena sp. PCC 7120	Algenol (2010 Patent)
Ethylene	0.016	Synechococcus elongatus PCC 7942	Sakai, 1997
	~ 5	Synechococcus elongatus PCC 7942	Takahama, 2003
Isobutyraldehyde / Isobutanol	1.1 / 0.45	Synechococcus elongatus PCC 7942	Atsumi, 2009
Isoprene	~ 0.0005	Synechocystis sp. PCC 6803	Lindberg, 2010
Free fatty acids	0.007	Synechocystis sp. PCC 6803; Synechococcus elongatus PCC 7942	Kaczmarzyk, 2010
	0.141	Synechocystis sp. PCC 6803; Synechococcus elongatus PCC 7942	Synthetic Genomics (2009 Patent)
	0.197	Synechocystis sp. PCC 6803	Liu, 2011
N-alkanes	~0.05	Synechococcus sp. PCC 7002; Thermosynechococcus elongatus BP-1	Joule Unlimited (2010 Patent)
Fatty alcohols	0.0002	Synechocystis sp. PCC 6803	Tan, 2011

Genetic Engineering of Cyanobacteria to Produce FFA

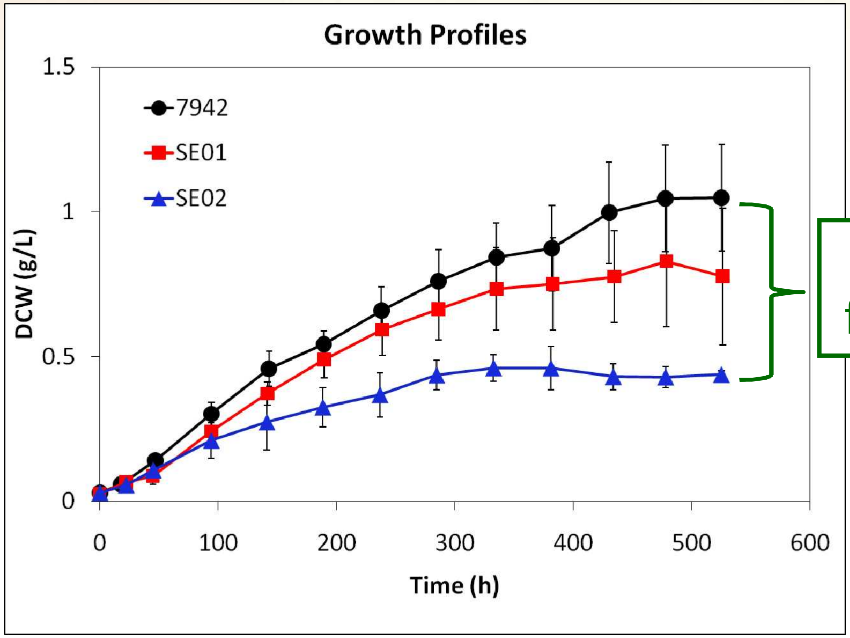


7942: Wild type

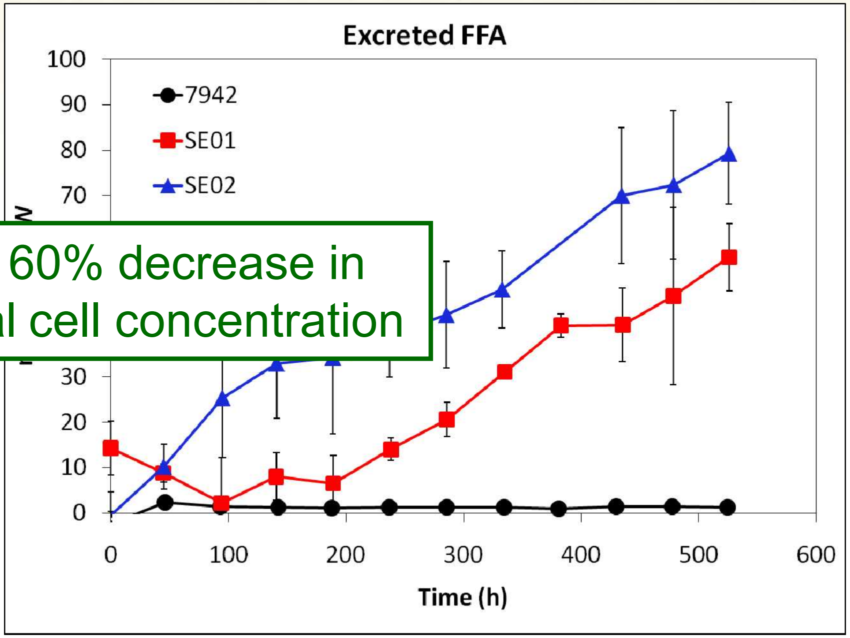
SE01: Knockout of acyl-ACP synthetase

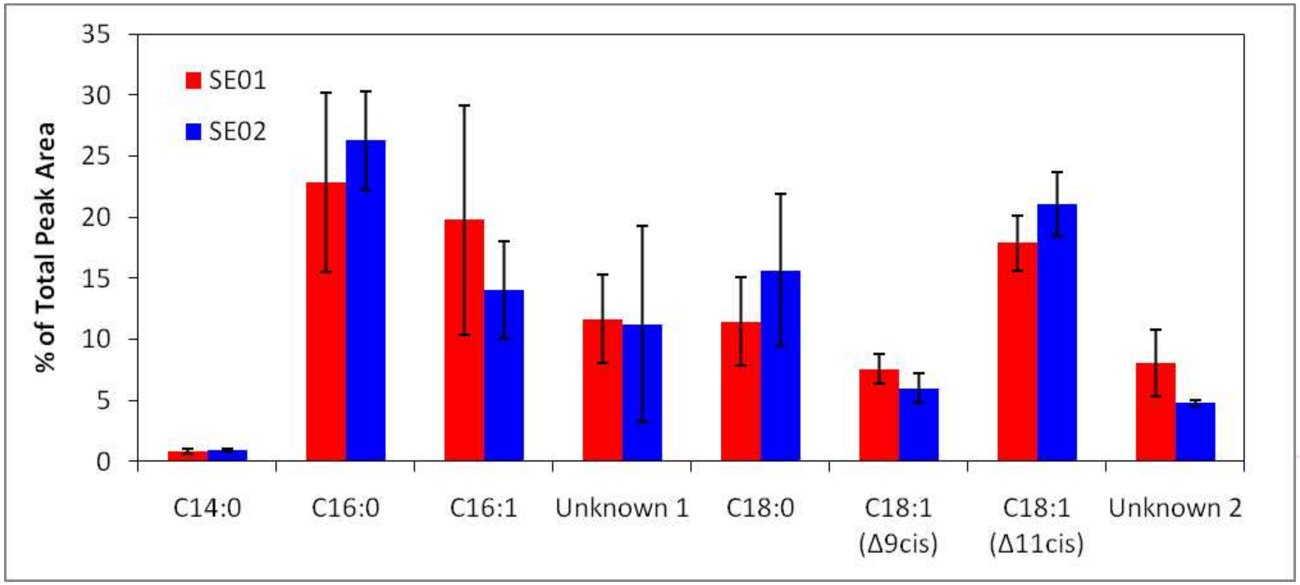
SE02: Knockout of acyl-ACP synthetase; expression of thioesterase ('*tesA* from *E. coli*)

Growth and FFA Production



~ 60% decrease in final cell concentration





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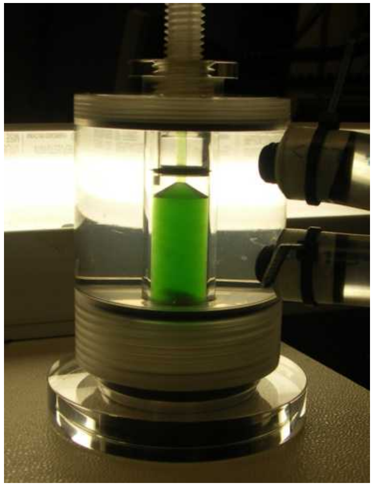

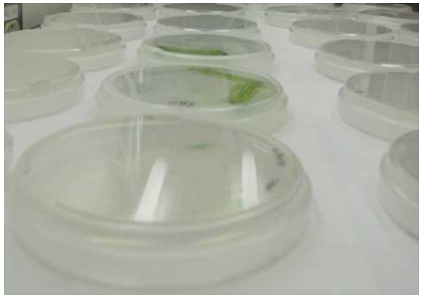

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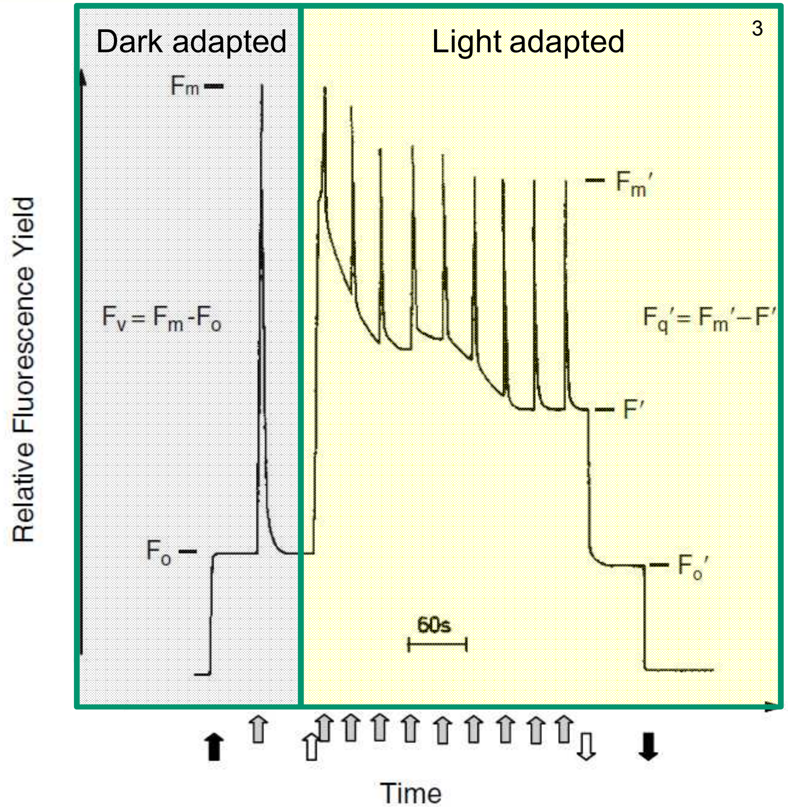
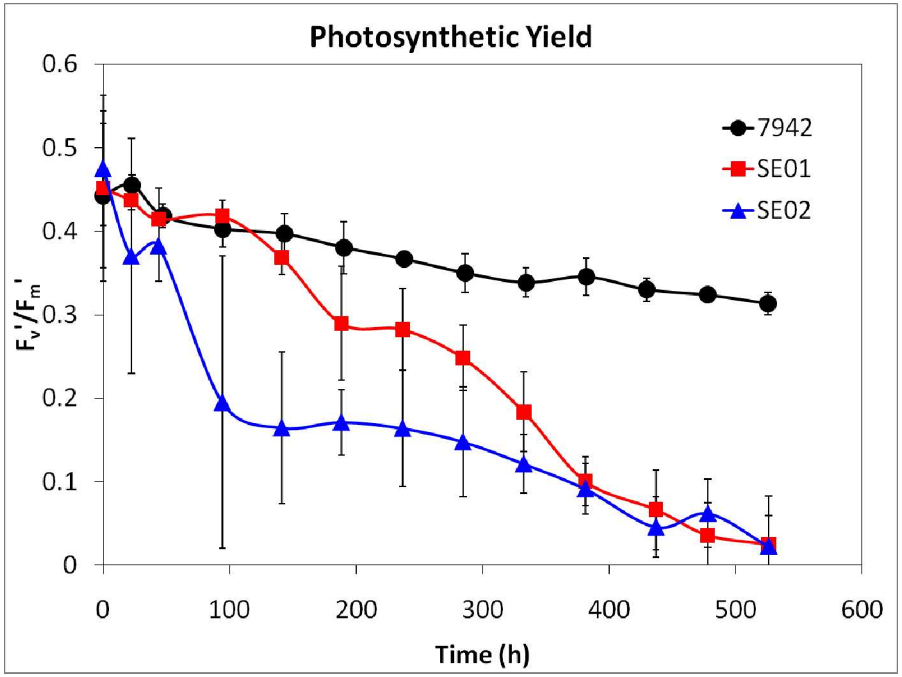
Effect on Photosynthesis

Pulse Amplitude Modulation (PAM) Fluorescence



Energy (light) → Photochemistry, Heat Dissipation, Fluorescence

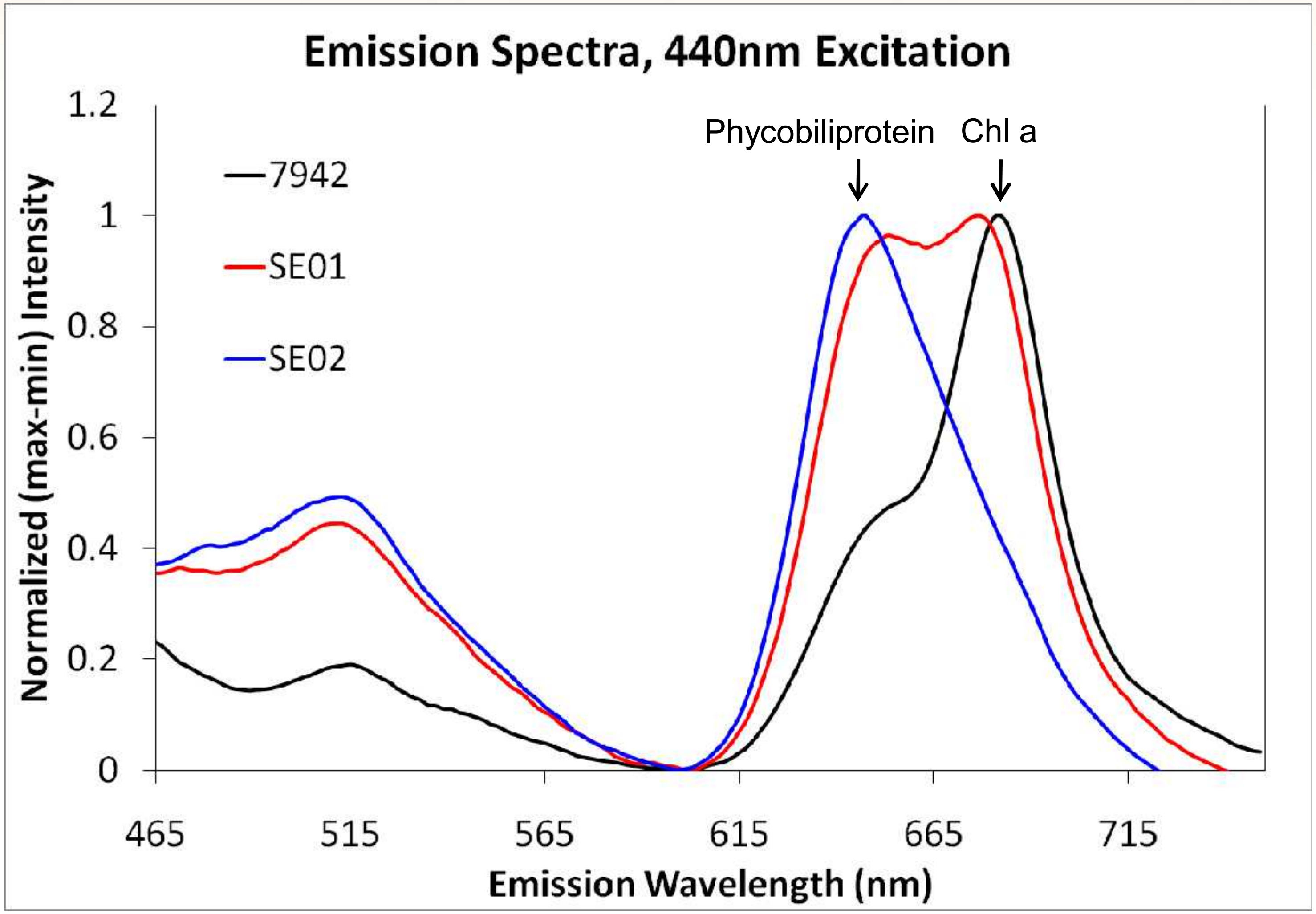


$$\frac{F_v'}{F_m'} = \frac{F_m' - F_0'}{F_m'}$$

Maximum PSII photochemical efficiency or quantum yield in actinic light

3. Cosgrove, J. and M.A. Borowitzka (2010) Chlorophyll a Fluorescence in Aquatic Science. Chapter 1. p1-17.

Effect on Photosynthetic Pigments



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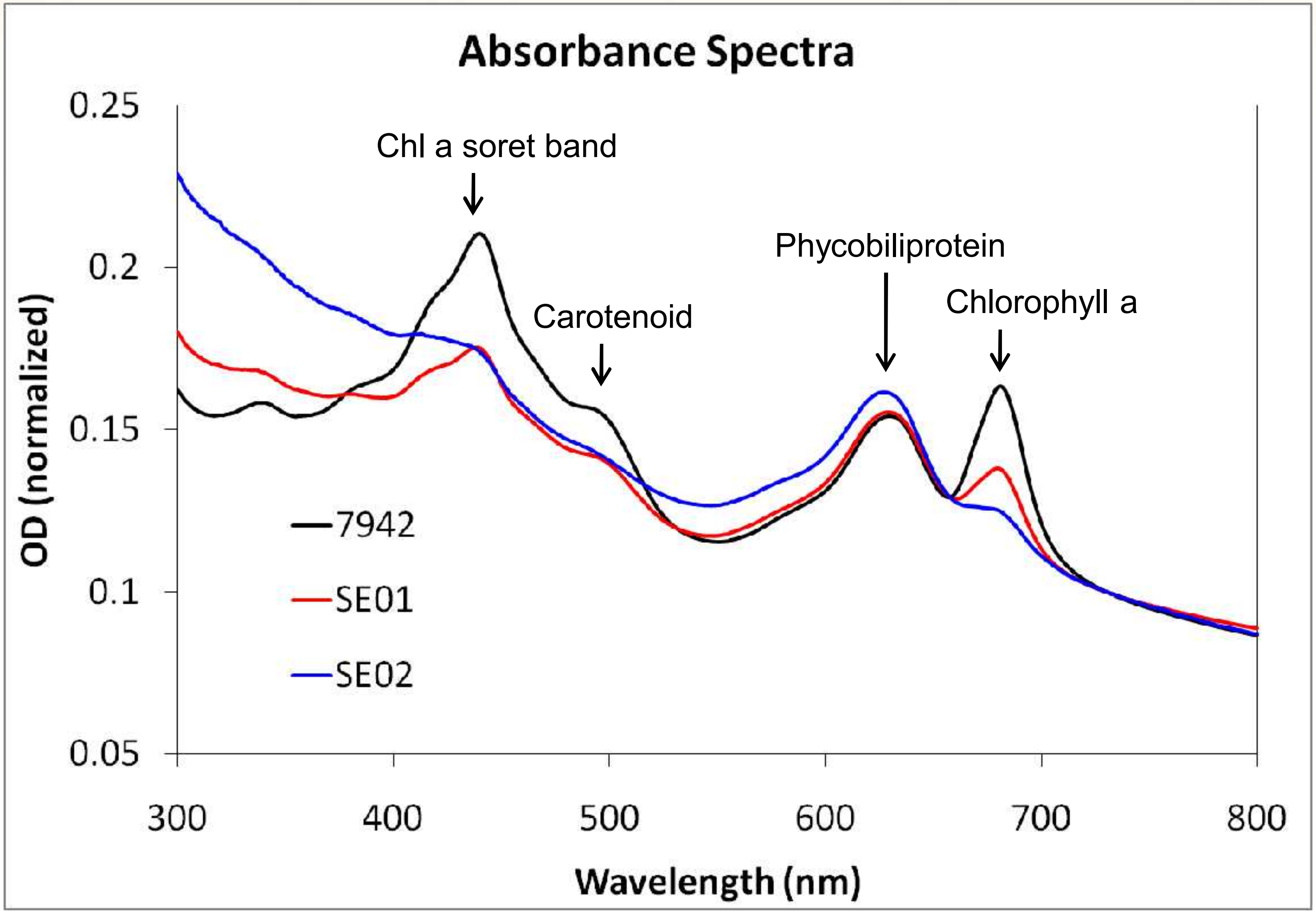

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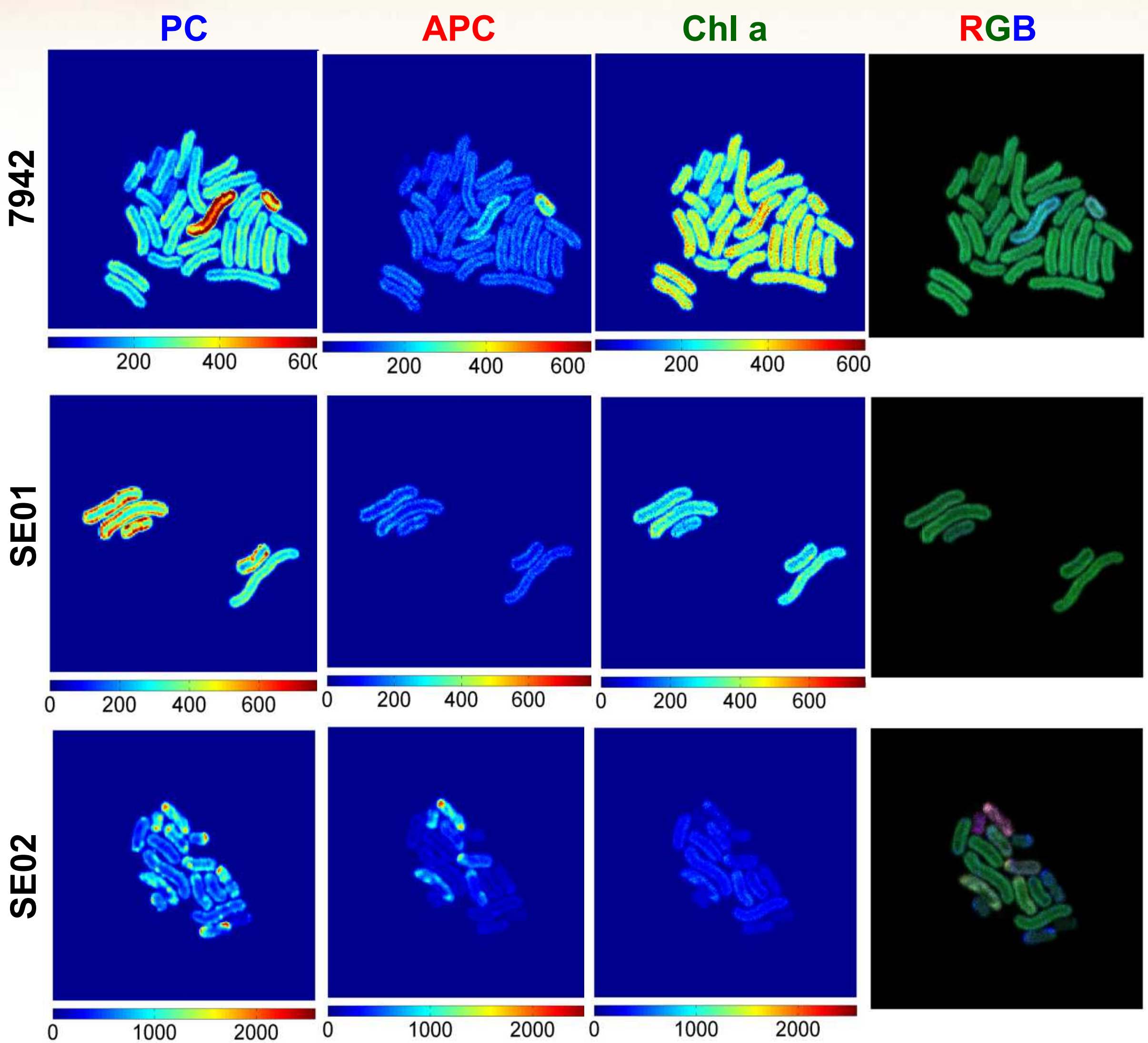

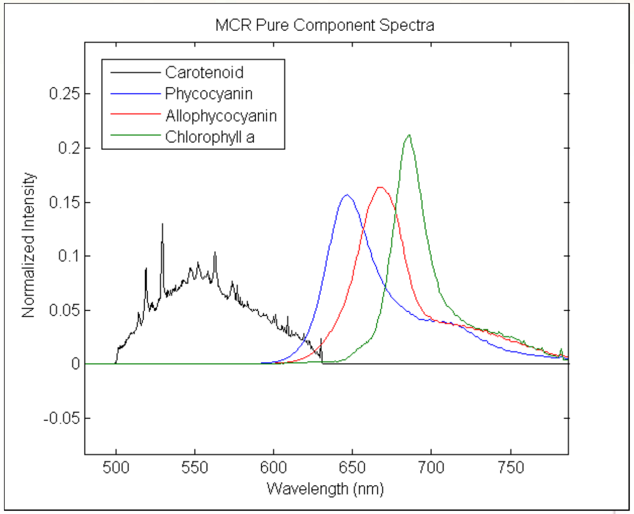

- Reduced Chl-*a* in SE02
- Aggregation of phycobiliproteins at the cell poles in SE02

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FFA Toxicity

Table 1 Selected bioactivites of various saturated and unsaturated FFAs

Activity	Fatty acid(s)	Key reference
Antimicrobial		
Anti-algal	C8:0, C10:0, C12:0	McGrattan et al. (1976)
	C18:4n-3	Kakisawa et al. (1988)
	C18:1, C18:2, C18:4, C18:5, C20:4, C20:5, C22:6	Arzul et al. (1995)
	C18:2n-6, C18:3n-3	Ikawa et al. (1997)
	C16:0, C18:0, C18:1n-9, C18:2, C18:3n-3, C20:5n-3, C22:6n-3	Wu et al. (2006)
	C16:0, C16:1n-7, C16:1n-7*t*, C16:4n-3, C18:0, C18:1n-9, C18:2n-6, C18:3n-3, C18:4n-3, C20:0, C20:1n-9, C20:4n-6, C20:5n-3, C22:0, C22:1n-9, C22:6n-3	Alamsjah et al. (2008)
Antibacterial (Gram-negative)	C20:4n-6	Knapp and Melly (1986)
	C10:0, C12:0	Bergsson et al. (1998)
	C10:0, C12:0, C14:0, C16:1	Bergsson et al. (1999)
	C15:0, C16:0, C17:0, C18:0, C18:1, C18:4, C20:4, C20:5, C22:0, C22:4, C22:5	Benkendorff et al. (2005)
Antibacterial (Gram-positive)	C8:0, C10:0, C12:0, C14:0, C16:0, C18:0, C18:1, C18:2, C18:3	Galbraith et al. (1971)
	C10:0, C12:0, C14:0, C14:1, C16:0, C16:1, C18:1, C18:2, C18:3	Kabara et al. (1972)
	C8:0, C9:0, C10:0, C11:0, C12:0, C13:0, C14:0, C14:1n-5, C16:1n-7, C16:1n-7*t*, C18:2n-6, C18:3n-3, C18:3n-6, C20:1n-9, C20:3n-6, C20:3n-3, C20:4n-6, C22:2n-6, C22:3n-3, C20:4n-6, C22:6n-3	Feldlaufer et al. (1993)
	C16:1n-10	Wille and Kydonieus (2003)
	C15:0, C18:1, C18:4, C20:4, C20:5, C22:0, C22:4, C22:5	Benkendorff et al. (2005)

Desbois AP and Smith VJ. (2010*) Appl. Microbiol. Biotechnol.*. 85: 1629-1642.

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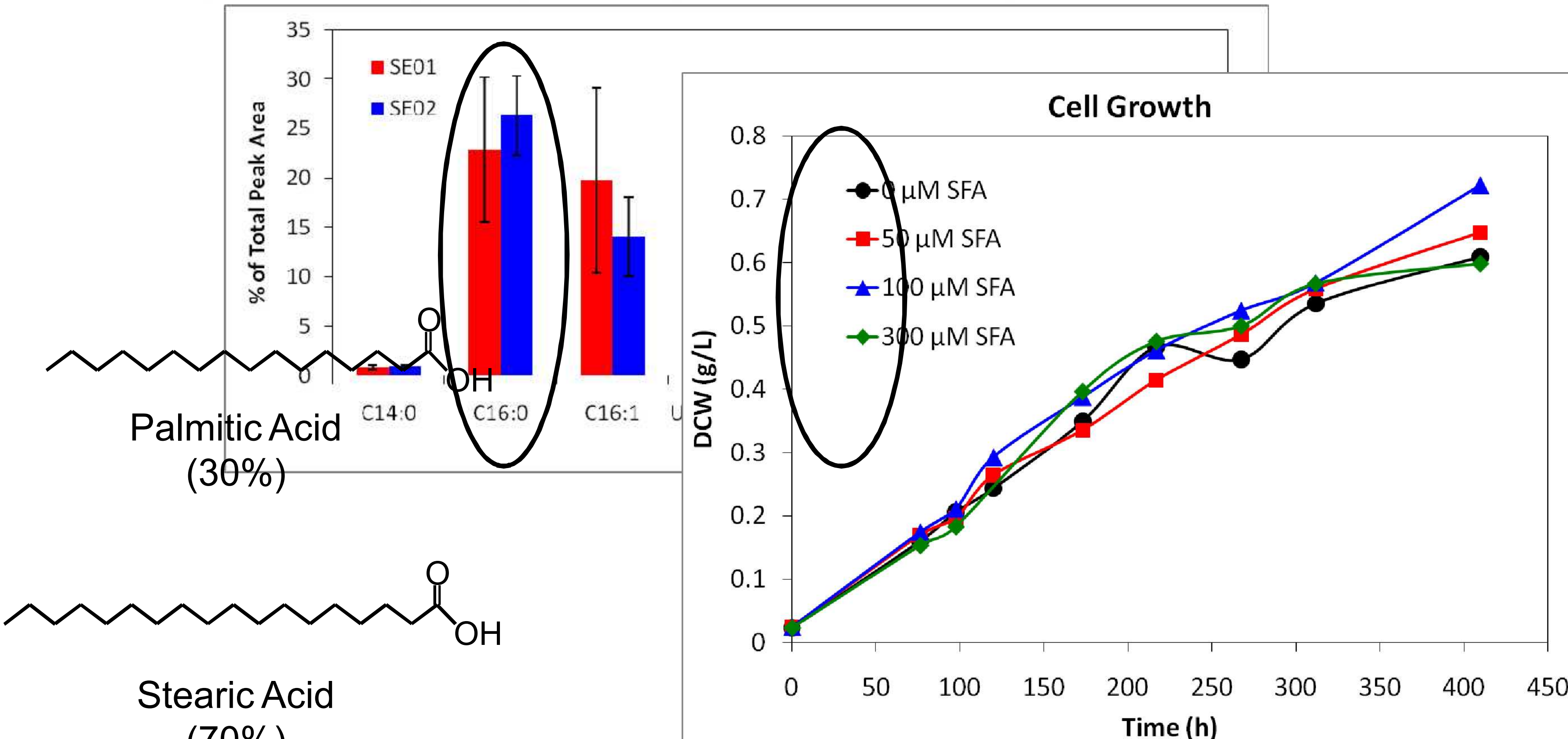
Effect of Saturated FFA



Palmitic Acid
(30%)

Stearic Acid
(70%)

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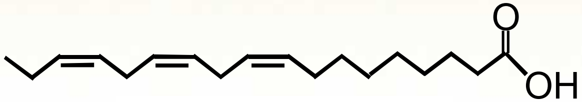
Effect of Unsaturated FFA

0μM 50μM 100μM 300μM

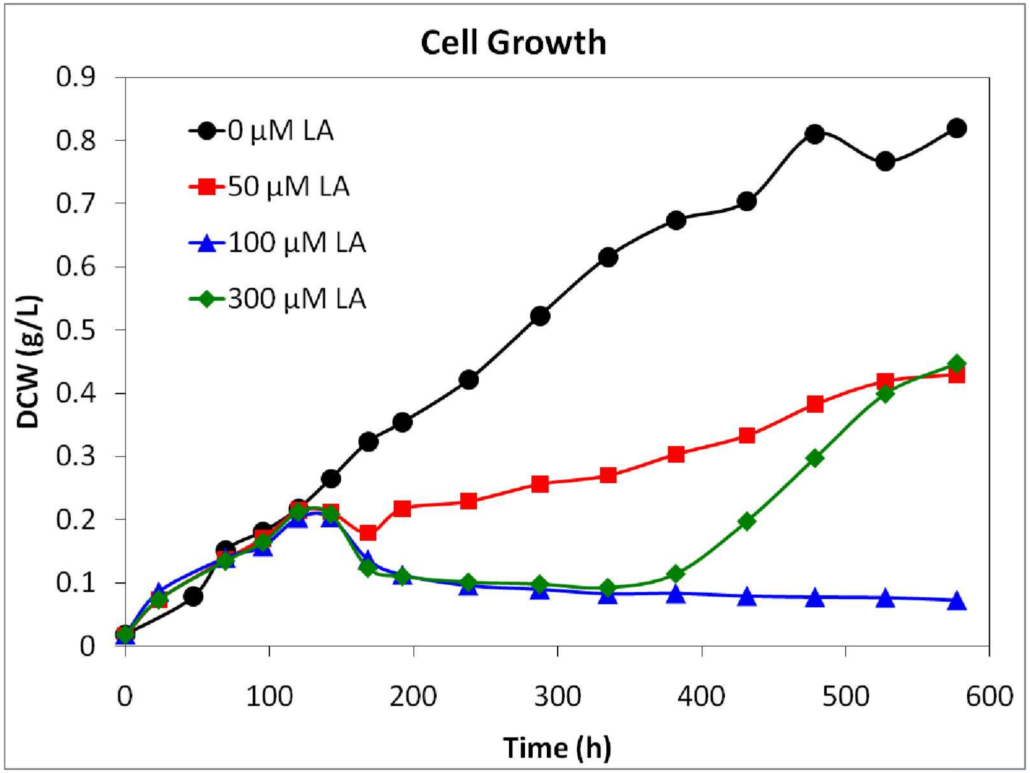
Day 2

Day 3

Day 4



Linolenic Acid



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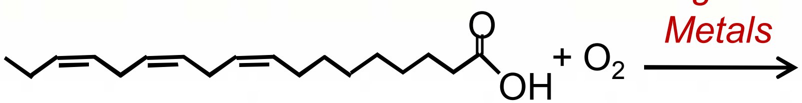
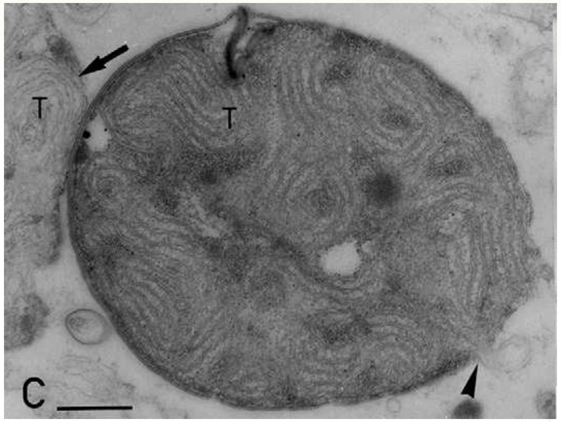
Oxidation of Unsaturated FFA



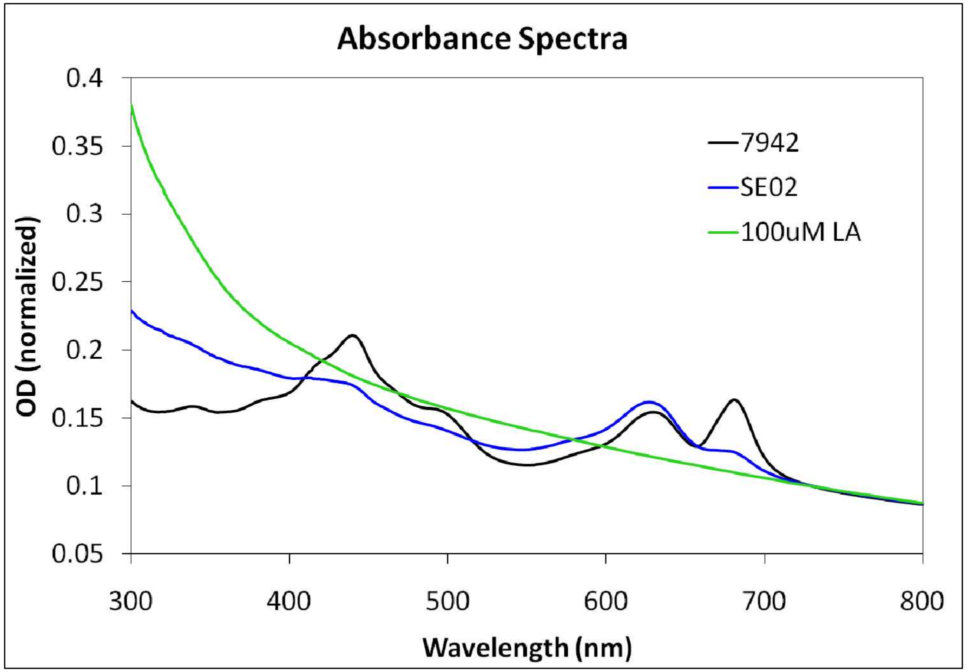
Unsaturated FFA
(linolenic acid)

- Hydroperoxides
- Aldehydes
- Acids
- High MW compounds
- Keto compounds
- Epoxy compounds
- Di and trihydroxy compounds
- Hydroperoxy epidioxides

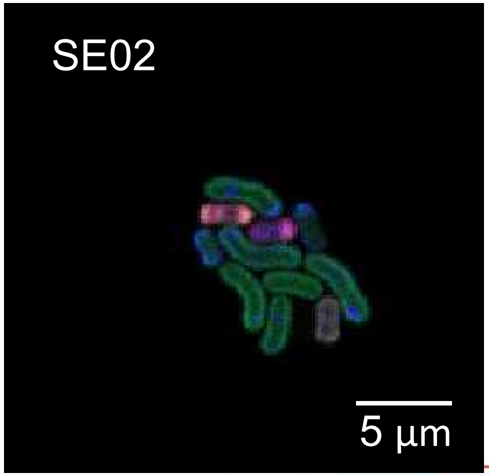
Cyanobacterium: *Anabaena* P9 [2]



0.178 μmol/L α-linolenic acid

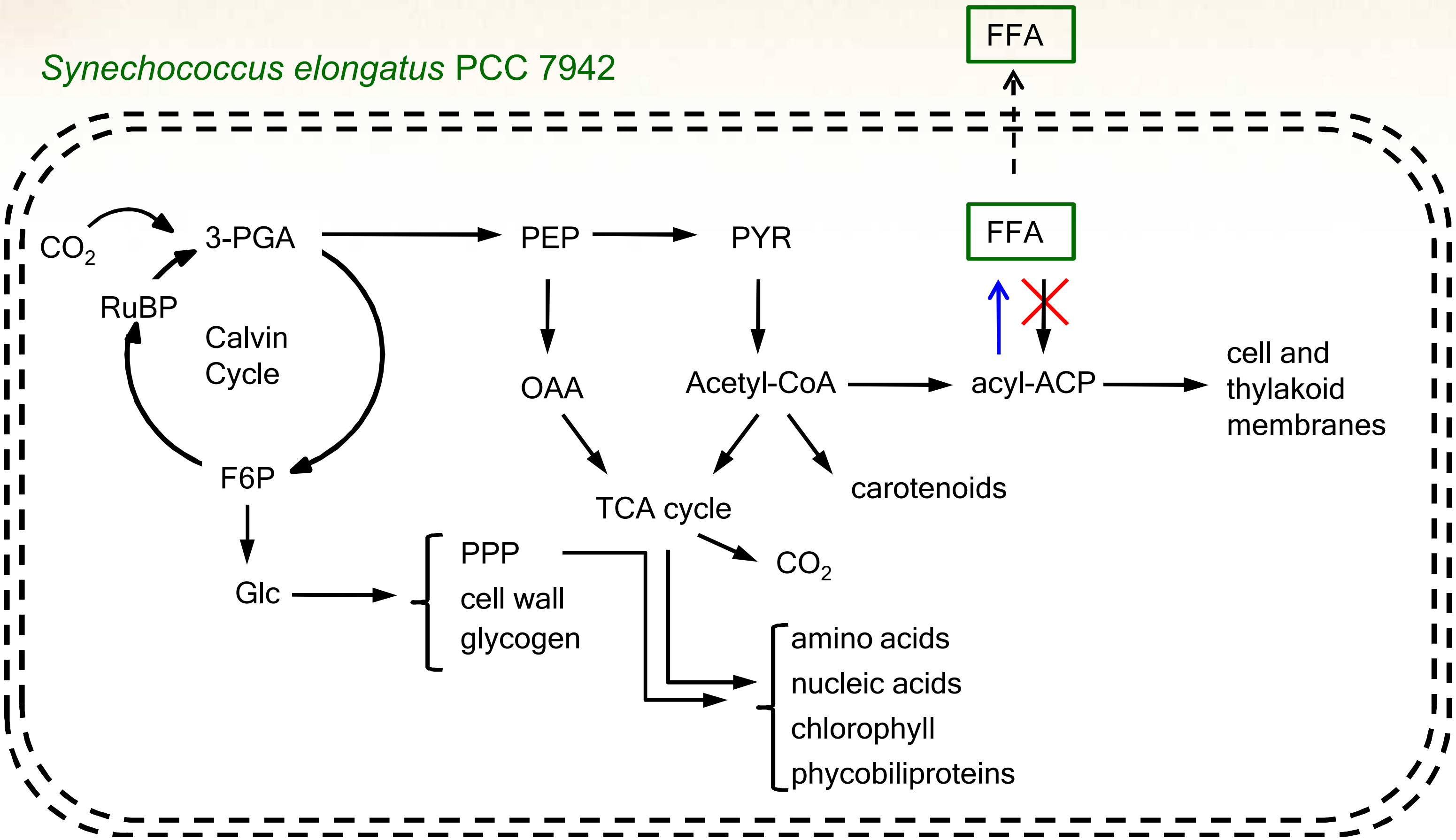


Engineered *S. elongatus* PCC 7942



2. Wu J-T, Chiang Y-R, Huang W-Y, and Jane W-N. (2006) *Aquatic Toxicology*. 80: 338-345.

Physiological Effects Caused by Genetic Engineering?



7942: Wild type

SE01: Knockout of acyl-ACP synthetase

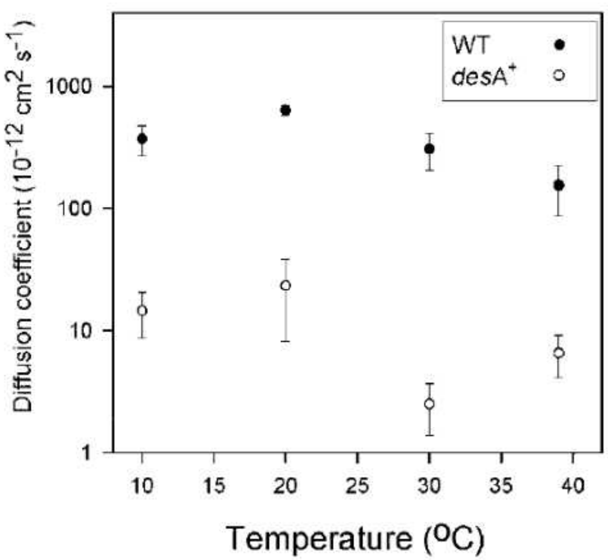
SE02: Knockout of acyl-ACP synthetase; expression of thioesterase ('*tesA* from *E. coli*)

Changes in FA Composition of Membranes

Fatty Acid Side-Chains of Membrane Lipids*

Strain	30:0	30:1	30:2	32:0	32:1	32:2	34:0	34:1	34:2	36:1	36:2	SFA	MUFA	DUFA
7942	0.20 ± 0.04	1.95 ± 0.47	0.42 ± 0.15	2.10 ± 1.29	35.3 ± 8.70	15.6 ± 1.83	0.00 ± 0.00	14.6 ± 1.69	18.5 ± 3.53	0.70 ± 0.28	5.30 ± 2.08	2.30 ± 1.34	52.5 ± 11.1	39.9 ± 7.59
SE01	0.89 ± 0.65	4.43 ± 2.47	0.65 ± 0.51	4.92 ± 1.84	30.1 ± 9.11	15.4 ± 5.3	0.08 ± 0.04	15.9 ± 5.01	16.3 ± 3.91	1.74 ± 0.91	4.78 ± 1.59	5.90 ± 2.53	52.2 ± 17.5	37.1 ± 11.3
SE02	1.44 ± 0.16	3.56 ± 3.25	0.39 ± 0.47	14.9 ± 8.84	30.9 ± 16.5	9.18 ± 6.46	0.67 ± 0.66	17.8 ± 7.21	9.09 ± 2.72	3.74 ± 2.96	3.75 ± 2.40	17.0 ± 9.67	56.0 ± 29.9	22.4 ± 12.0

* Values listed are the % of total signal from ESI-MS



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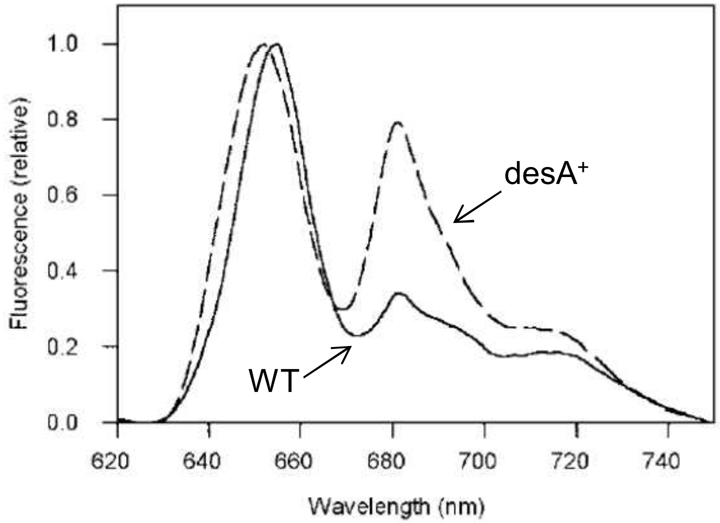
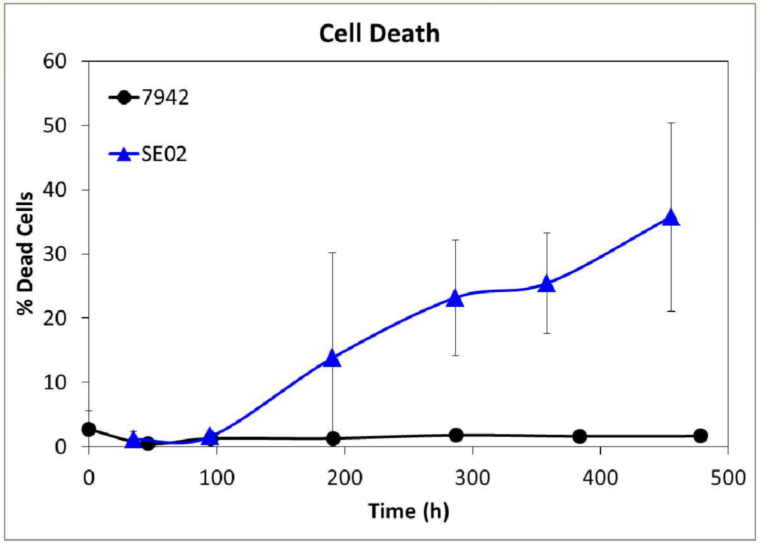


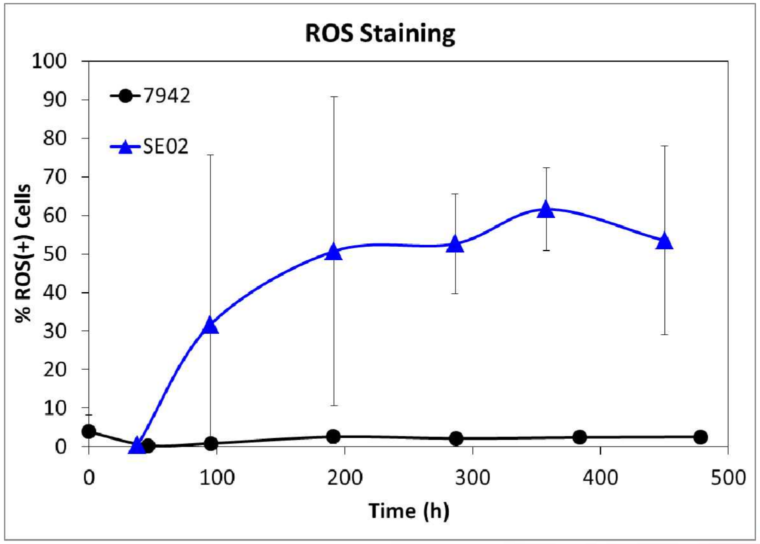
3. Sarcina M, Tobin MJ, Mullineaux CW. (2001) *J. Biol. Chem.* 276(50): 46830-4.

Potential Solution: Metabolic Engineering

Metabolic engineering targets:

- ROS-scavenging enzymes (SOD, catalase)
- RNA-seq to identify other targets
  - 7942 vs SE02
  - Exponential phase, low FFA (100h)
  - Stationary phase, high FFA (240h)





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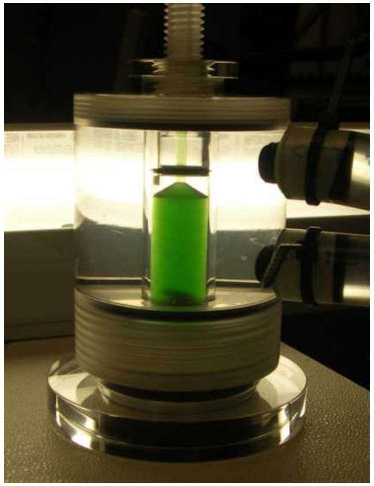
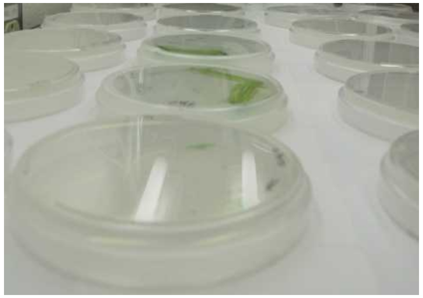
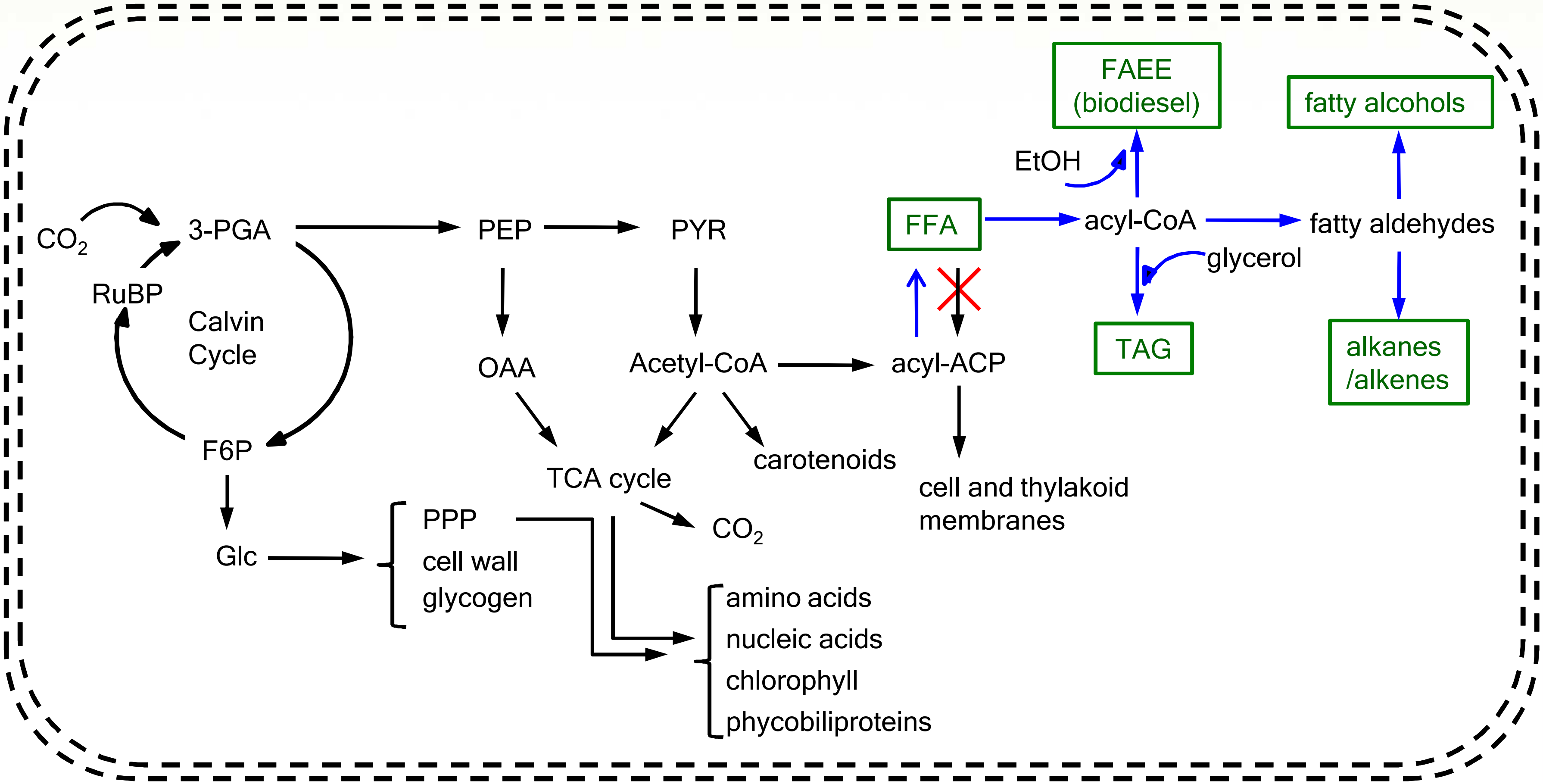




Potential Solution: Selection of Biofuel Product



Potential Solution: Host Selection

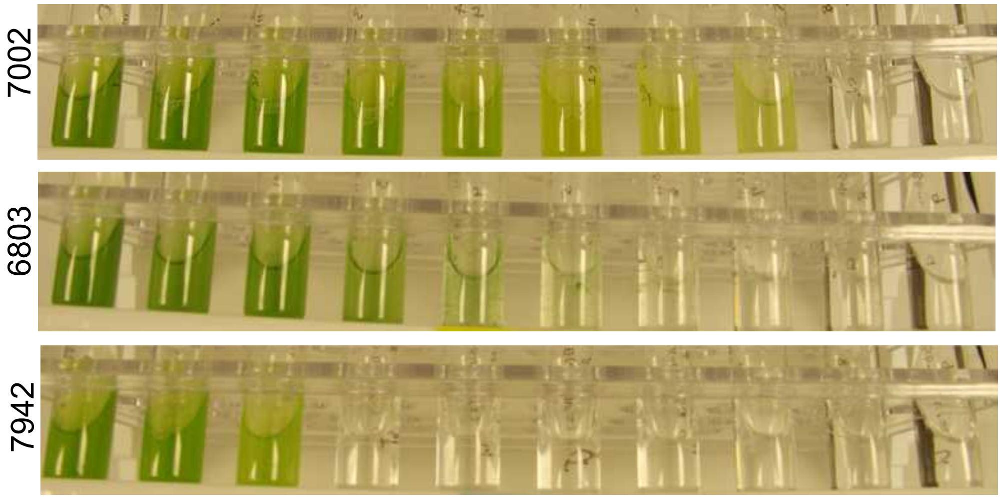
Model Cyanobacteria	Habitat
Synechococcus elongatus PCC 7942	freshwater
Synechocystis sp. PCC 6803	freshwater
Synechococcus sp. PCC 7002	marine

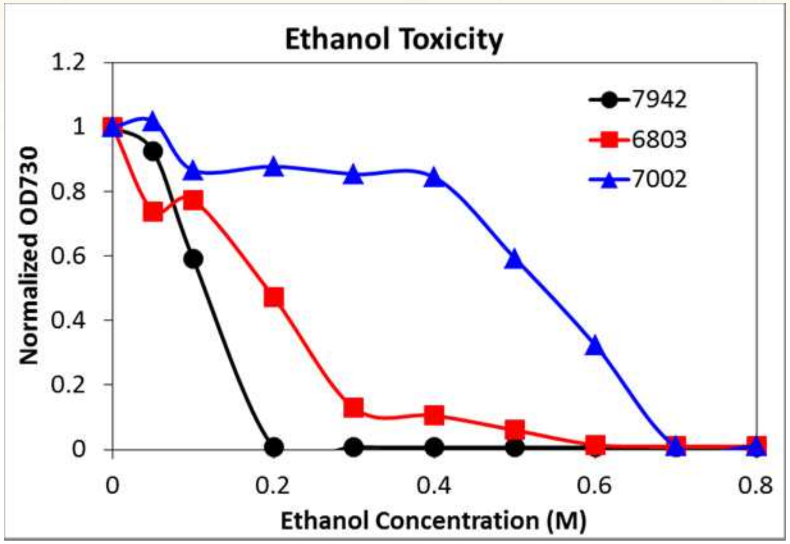
Biofuel Products:

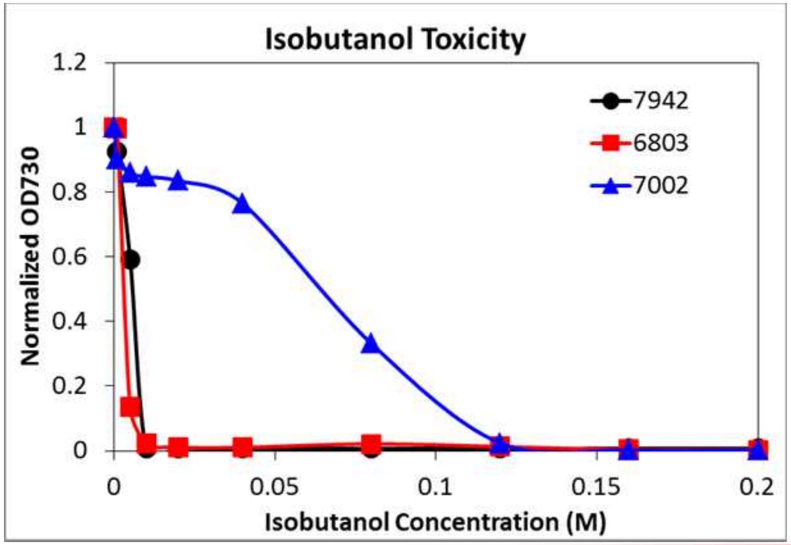
- Ethanol (C2)
- Isobutanol (C4)
- Hexadecanol (C16:0)
- Palmitic acid (C16:0)
- Stearic acid (C18:0)
- Linolenic acid (C18:3)
- Pentadecane (C15:0)
- Heptadecene (C17:1)

Ethanol Toxicity

0M 0.05M 0.1M 0.2M 0.3M 0.4M 0.5M 0.6M 0.7M 0.8M







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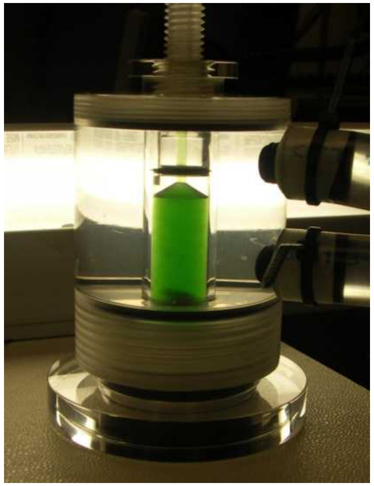

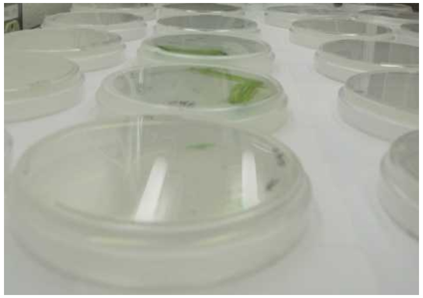

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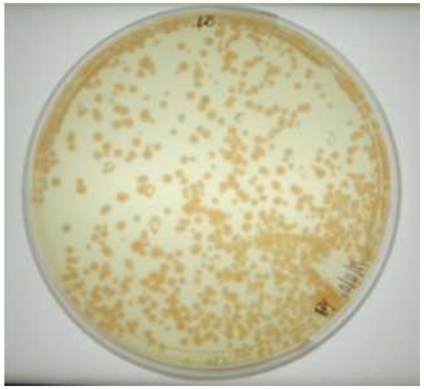
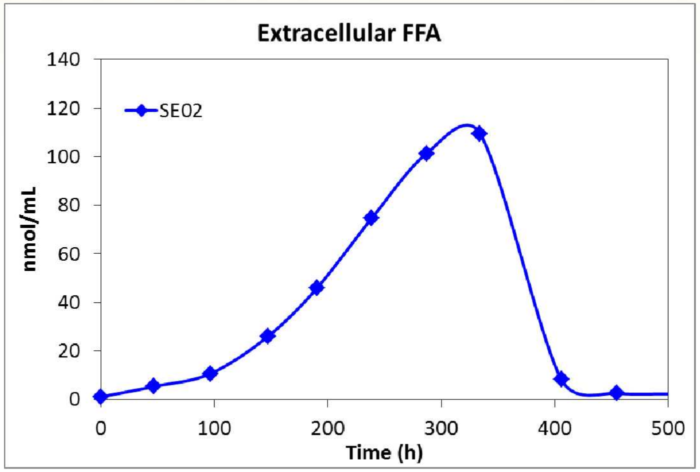
Contamination of FFA-Producing Cultures

Contaminant 1

Contaminant 2



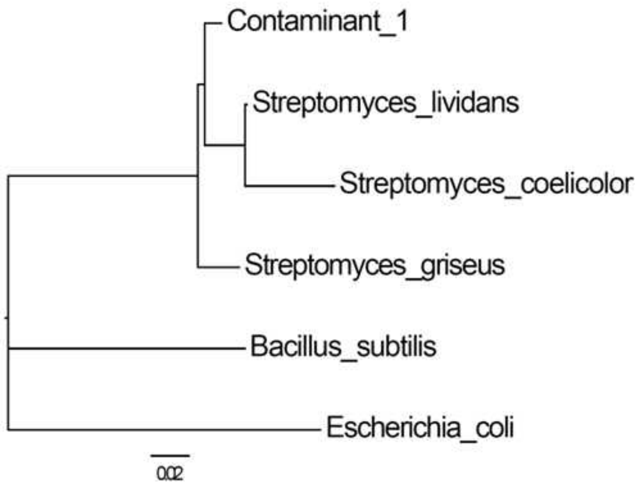


Contaminant 1

- FFA-consuming
- Cell aggregates (mycelia)
- Rigid colonies
- Universal primers for bacterial 16S rRNA
    - ➢ Streptomyces

Contaminant 2

- No FFA consumption
- Pink/orange colored colonies
- Rotten egg odor (sulfite-reducing)
- Universal primers for bacterial 16S rRNA
    - ➢ No amplification



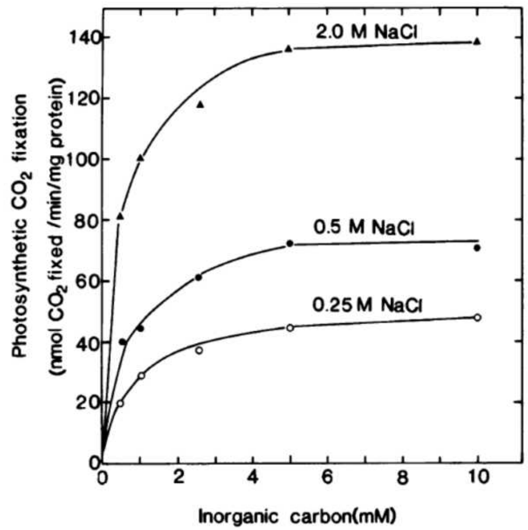
Potential Solutions

1. Co-culture
   - FFA production by *S. elongatus*
   - TAG production by *Streptomyces* sp.
2. Host selection
   - *Synechococcus* sp. PCC 7002 – marine cyanobacterium
     - 4% NaCl (0.7M)
   - *Aphanothece halophytica* - halotolerant alkaliphilic cyanobacterium
     - 3M NaCl
     - pH 11
3. Metabolic engineering

Microbiology (1994), 140, 931–943 Printed in Great Britain

Importance of stored triacylglycerols in *Streptomyces*: possible carbon source for antibiotics

Ekundayo R. Olukoshi and Neville M. Packter



Takabe T, et al. (1988) *Plant Physiol.* 88: 1120-4.

Outline

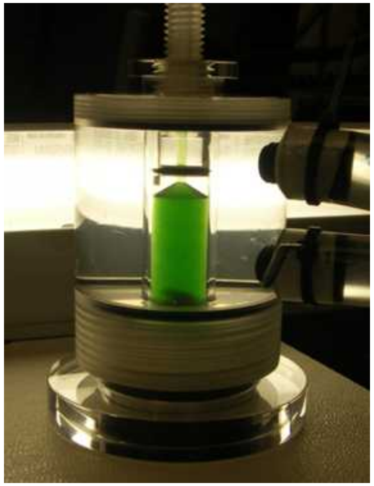
Background | Cyanobacterial Fuels | New Challenges & Potential Solutions | Conclusions

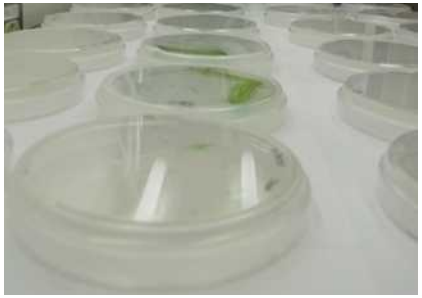
Background
- Algal Biofuels
- Cyanobacteria

Cyanobacterial Fuels
- Review of past and current work in the field
- Truman project: Engineering *Synechococcus elongatus* PCC 7942 for FFA production

New Challenges & Potential Solutions
- Physiological effects of FFA production
- Product toxicity
- Contamination

Conclusions
- Advantages
- Disadvantages





Conclusions

Biofuel production from cyanobacteria can provide solutions to some of the obstacles plaguing algal biofuels …

- Lower N and P requirement (no destruction of biocatalyst)
- Temperature (host-dependent)
- Metabolic engineering may be able to address issues with productivity and crop protection

… But, cyanobacterial fuel production also introduces new challenges

- Physiological effects
- Product toxicity
- Contamination

The bright side: Investigation into cyanobacterial fuel production began only in the past few years. Additional research efforts may overcome these limitations.

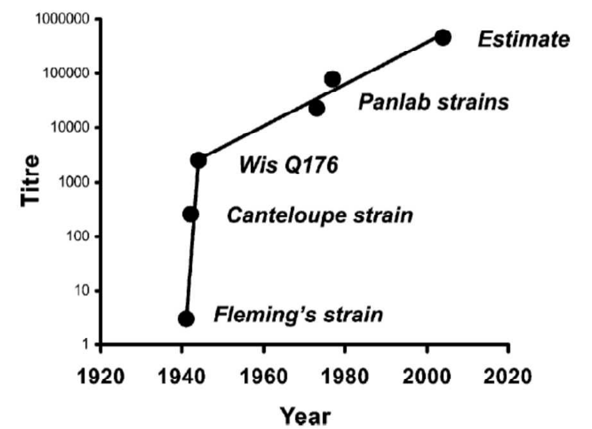


Fig. 1 The development in penicillin production titre over time.

Rokem JS et al. (2007) *Natural Product Reports.* 24: 1262-87.

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