Microalgal Biodiesel: Analysis of the "Starvation Trigger" for Algal Oil Synthesis

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Pamela Lane[1], Frank Zendejas[2], Eizadora Yu[1], Sara Gaucher[3], Blake Simmons[2], and Todd W. Lane[1]
[1]Biosystems Research Department,[2] Energy Systems Department, Sandia National Laboratories, Livermore, CA 94551
[3]currently at Amyris Biotechnologies Inc, Emeryville, CA 94608

OVERVIEW

PURPOSE

• Characterize effect of nutrient limitation on growth and triacylglyceride (TAG) accumulation and speciation in model microalgae

METHODS

• Microalgae was grown in either silica- or nitrate-deficient media

• TAGs were extracted from biomass and analyzed by TLC, GC-MS

• TAG speciation and FA composition were characterized by ESI-MS and MS[2]

RESULTS

• Establishment of Tp and Pt as model systems for the study of TAG formation.

• Difference in TAG accumulation rate and speciation with starvation triggers.

MICROALGAE AS BIODIESEL FEEDSTOCK

• **Higher oil yield** (up to 60% cellular mass as a TAG feedstock

• **Shorter growth rates** (3-8 growth cycles a day)

• **Reduced demand for land & clean water use** (grow in non-potable water supply)

• **Low impact on food market**

• Added benefit: diatoms are great at **carbon sequestration**

Challenges in efficient biodiesel production from algae lie in TAG production, conversions, and scalability

To become economically viable, there is a need to improve algal feedstock by metabolic engineering.

BIODIESEL MOTIVATION

Reduce national dependence on fossil fuels (20.7M barrels of il/day)

Sustainable biomass-derived transportation fuels

Current biodiesel feedstocks: soybeans, corn, waste cooking oil
Meeting 50% of existing U.S. fuel dependence would require:

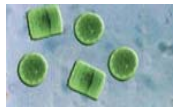
Crop	Oil Yield (L/acre*)	Land Area Needed (M acre)	% of existing US crop area
Soybeans	180	240	326
Corn	70	623	846
Oil palm	2408	18	24
Microalgae‡	**23,755**	**1.82**	**2.5**

*1 acre = 4046.8 m^2

‡ Based on 30% oil (by wt) in biomass (Experimentally demonstrated in photobioreactors)

Y. Chisti, "Biotechnology Advances" Vol. 25, (2007)

ALGAL MODEL SYSTEMS



Thalassiosira pseudonana

Centric, Marine
~ 3- 6 um diameter
Complete genome sequence
31.3 Mbp
11390 gene models
3000 additional transcripts
Biolistic transfection

Phaeodactylum tricornutum

Pennate, marine
Cell dimension : ~ 27 x 3 um
Lightly silicified
High lipid strains
Complete genome sequenced
26.1 Mbp
10010 gene models
Biolistic transfection

MATERIALS AND METHODS

Cell Culture

Cultures of *T.pseudonana* (T.p.) and *P.tricornutum* (P.t.) were cultivated in artificial seawater media (ESAW)[1] with constant light and aeration. Small scale cultures with varying reduced concentrations of nitrate and silica were grown to optimize cell growth and TAG accumulation. For the detailed time-point analysis, 8L cultures were cultivated and 0.5-1L samples were taken daily starting from mid-log phase. Cell counts were measured using a Beckman Coulter Analyzer and TAG accumulation was monitored by Nile Red staining and fluorimetry. One-liter Cells were harvested either by filtration or centrifugation (depending on cell density) and lyophilized.

Lipid extraction from algal biomass

Lipids were extracted from biomass by hexane solvent extraction. The lyophilized biomass was combined with 3ml of hexane in a scintillation vial. A 1-minute sonication step was necessary to break-up larger pieces of biomass. The samples were stirred at 500RPM for 60 minutes at a temperature of 60ºC. The supernatant hexane extracts were filtered through a 20nm PTFE inline syringe filter. The supernatant was then dried down and the vials weighed to determine the amount of TAG recovery. TLC and GC-MS were performed to rapidly analyze the TAG content of lipid extracts.

ESI-MS of Algal Biomass Lipid Extracts

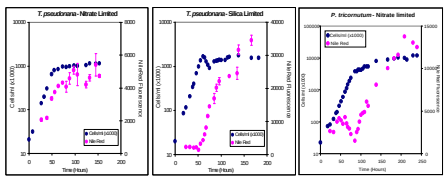
Dry algal biomass was redissolved in 100 µL of chloroform. Appropriate dilutions of analyte solutions were prepared in ES buffer (1:4 (v/v) chloroform:methanol). Lithium acetate was added to achieve a final [Li+] of 2 mM. Analyte solutions were infused directly into the source via syringe pump at a flow-rate of 1 µL/min. All mass spectrometric determinations were performed on a Waters Q-TOF Ultima MS equipped with a nanospray assembly. The mass spectrometer was operated in positive mode and typical MS settings are as follows: 3.5 kV electrospray voltage, 200 V cone voltage, 220ºC capillary temperature. Spectra were acquired over a 3-minute period of signal averaging for each sample/extract. TAG molecular species were quantitated according to the method reported by Han and Gross.[2] Triheptadecenoin (TAG 51:3) was added to each sample and used as an internal standard.

Analysis of low-energy CAD tandem mass spectra of lithiated TAG ions provide information on fatty acid (FA) composition of TAG species obtained from microalgal oils.[3] For ion isolation, the LM/HM was set to 15/15. Ions subjected to 30-35 eV collision energy were sufficient to achieve optimal fragmentation.

MS^2 data analysis was performed with the aid of an on-line tool in the LipidMaps[3] website:
(http://www.lipidmaps.org/tools/ms/triacylglycerols.php)

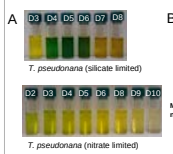
RESULTS

Silica-starved *T. Pseudonana* produced the most lipid/cell count



Effect of nutrient (silica or nitrate) depletion on algal cell growth and lipid accumulation. Cell growth was tracked as cell counts (cells/ml). Changes in lipid content was followed by Nile Red fluorescence. Nile Red fluorescence readings were normalized to cell counts (xM cells).

Hexane extraction yielded 5~15% TAGs

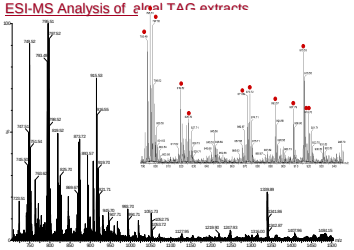


C

	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
P. tricornutum (nitrate limited)	NH	4.82	1.67	4.30	5.71	7.45	6.31	NH	NH
T. pseudonana (nitrate limited)	1.60	4.91	6.27	10.07	8.88	NH	13.04	15.10	14.92
T. pseudonana (silicate limited)	NH	9.14	5.62	11.00	16.55	15.49	16.19	NH	NH

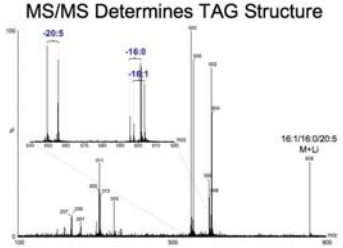
Summary of Lipid extraction from Algal Biomass. (A) Hexane extracts recovered from nutrient limited diatom cultures. (B) TAG content of hexane extracts by TLC. Highlighted boxes (in C) indicate samples compared in TLC plate. (C) Table of lipid yields obtained from hexane extraction. Percentages are based on dry weights of recovered lipids vs initial algal biomass. Algal biomass was recovered from 1L cultures except for the Nitrate-limited *T.pseudonana* samples (0.5L). NH: Not harvested

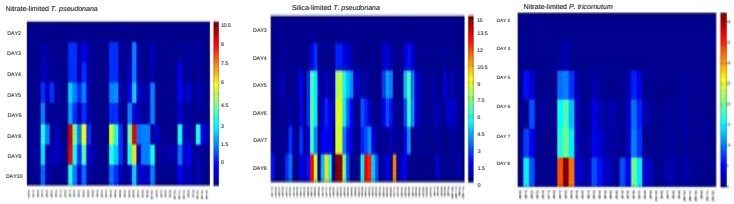
ESI-MS Analysis of algal TAG extracts



ESI-MS of TAGs extracted from *T.pseudonana*. Zoom inset of the region spanning the 790-950 m/z range shows TAG species verified by MS/MS (red dots).

MS/MS Determines TAG Structure





Heatmaps showing TAG speciation during nutrient limitation. Ion peak intensities of individual TAG species were compared with that of the standard TAG (51:3) after correction for C13 isotope and sensitivity effects.[2]

FATTY ACID COMPOSITION OF ALGAL TAGs

TAG/FA	14:0	14:1	16:0	16:1	16:2	18:2	18:3	18:4	20:4	20:5	22:6
(46:4)	■							■			
(46:3)	■	■			■						
(46:2)	■				■						
(48:5)	■			■	■					■	
(48:3)				■	■						
(48:2)				■	■						
(50:6)	■			■	■					■	
(52:7)a				■	■	■				■	
(52:6)				■	■					■	
(54:10)					■		■			■	
(56:12)							■	■		■	
(56:11)					■					■	
(56:10)				■	■				■	■	
(58:12)					■	■	■			■	■

Partial TAG profile of *T.pseudonana*. Fatty acid composition of TAGs were confirmed by tandem mass spectrometry. * Order of branching is unknown, position of unsaturation is unknown.

SUMMARY

For P. tricornutum

• At mid-log, identified 4 TAG species (46:1, 48:1, 48:2, 48:3) comprised of fatty acids 14:0, 16:0, 16:1.
• Identified more TAG species (~38) from DAY 5-8 (stationary phase). P.t. started packaging longer and more unsaturated fatty acids: 18:1, 18:2, 18:3, 18:4, 20:5, and 22:6.
• Some TAG species appear to have isomers (varying FA compositions) based on MS^2 analysis.
• TAG species 48:1, 48:2, 48:3 were predominant throughout the cell growth.

For T. Pseudonana

• At mid-log, abundant TAG species were 48:1, 48:2 comprised of fatty acids 16:0, 16:1
• Identified more TAG species as cells go from mid-log to stationary phase. T.p. started packaging longer and more unsaturated fatty acids: 16:2, 16:3, 18:3, 18:4, 20:5, 22:6
• TAG species 48:1, 48:2, 48:3 were predominant throughout the cell growth.
•Identified more diverse set of TAG species in silica-limited versus nitrate-limited cells.

CONCLUSIONS

• Nitrate-starved P.t. reached the highest cell density measurements, however, silica-starved T.p. produced the most TAG/cell count.

• Preliminary hexane extraction methods yielded 5 – 15% TAGs based on the dry weight of algal biomass.

• We observed a time delay between entering stationary phase and onset of accumulation of TAGs. This may indicate that there is some sort of regulatory process going on and it is not just increase in [TAG] per cell due to a cessation of cell division.

• We observed faster accumulation of TAG in silica-versus nitrate-starved T.p.

• In both diatoms, the TAG species were 48:1, 48:2, 48:3 consisting mostly of fatty acids 16:0 and 16:1 were predominant throughout the cell growth.

• Nutrient limitation also resulted in packaging of longer and more unsaturated fatty acids in both diatoms

FUTURE DIRECTIONS

• Explore other starvation triggers (light cycle, temperature, other nutrients) to optimize oil synthesis and accumulation
• Explore other lipid extraction techniques to increase yield
• Parallel transcriptomics and proteomics analysis underway to characterize TAG synthesis pathway and regulation
• Perform transesterification reactions of algal feedstock
• Assess suitability of TAG mixture for fuel.

REFERENCES AND ACKNOWLEDGEMENTS

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3. Schmelzer, K., Fahy, E., Deems, R., Subramaniam, S., and Dennis, E. A. (2007) *Meth. Enzymol*., Academic Press, 432, 169-181.

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