SABC

Sustainable Algal Biofuels Consortium

Cultivating Energy Solutions

Sustainable Algal Biofuels Consortium

Thursday May 21, 2013

9.5.1.5, 9.5.1.7, 9.5.1.8

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NREL

ASU NREL National Renewable Energy Laboratory Sandia National Laboratories GT Valicor Renewables School of Mines Colorado 1874 Emerging Fuels Technology

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The primary goals were to evaluate biochemical conversion as a potentially viable strategy for converting all the components of algal biomass into biofuels and evaluate the *fit-for-use* properties of those algal derived fuels and fuel intermediates.

Objectives

- Develop a feedstock matrix of algal biomass based on species and growth/process conditions
- Determine and characterize biochemical composition of selected strains
- Explore multiple biochemical routes to hydrolyze and convert untreated or pretreated whole algal biomass, oil extracts, and algal residuals
- Determine the acceptability of algal biofuels as replacements for petroleum-based fuels

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Quad Chart Overview

Timeline

Project Start Date: 11/2010 (contract finalized 2/2011)
Project End Date: 3/2013
Percent complete: 100%

Barriers Addressed

- **Ft-J Biomass Material Properties:** Evaluation of the quality of different algal strains grown under different conditions for biochemical conversion
- **Ft-N Algal Feedstock Processing:** Identify best enzymes for saccharification and for biochemical lipid conversion with whole algal biomass
- **Bt-A Biomass Fractionation:** Identify value-added biofuel production processes for protein and carbohydrate fractions of algal biomass

Budget

Total project funding $7.5M
- DOE share: $6.0M
- Contractor share: $1.5M

Funding received in FY13: $0.7M
Funding for FY12: $3.3M
Funding for FY11: $2.0M
ARRA Funding: $0.00

Partners

Arizona State University (Lead)
National Renewable Energy Lab
Sandia National Labs
Valicor Renewables
Georgia Institute of Technology
Colorado School of Mines
Emerging Fuels Technology

Overall Technical Approach

Task 1 Biochemical Conversion of Microalgal-Derived Biomass:

Subtask 1.1 Feedstock Development and Production: Produce selected algae and algae fractions

Subtask 1.2 Characterization: Develop a fundamental understanding of algal chemical composition and structure

Subtask 1.3 Investigation of Biochemical Conversion Routes for Whole Algae and Algae Residues: Identify and test a variety of pretreatment options and hydrolytic enzyme preparations to facilitate release of fermentable sugars and other biofuel feedstocks and demonstrate conversion of these materials into fuel intermediates/products

Task 2 Product Performance of Algal-Derived Hydrocarbon Fuels and Blend Components:

Produce samples of those fuels (both lipid and carbohydrate based) and perform fuel testing to determine if those fuels are *fit-for-purpose*

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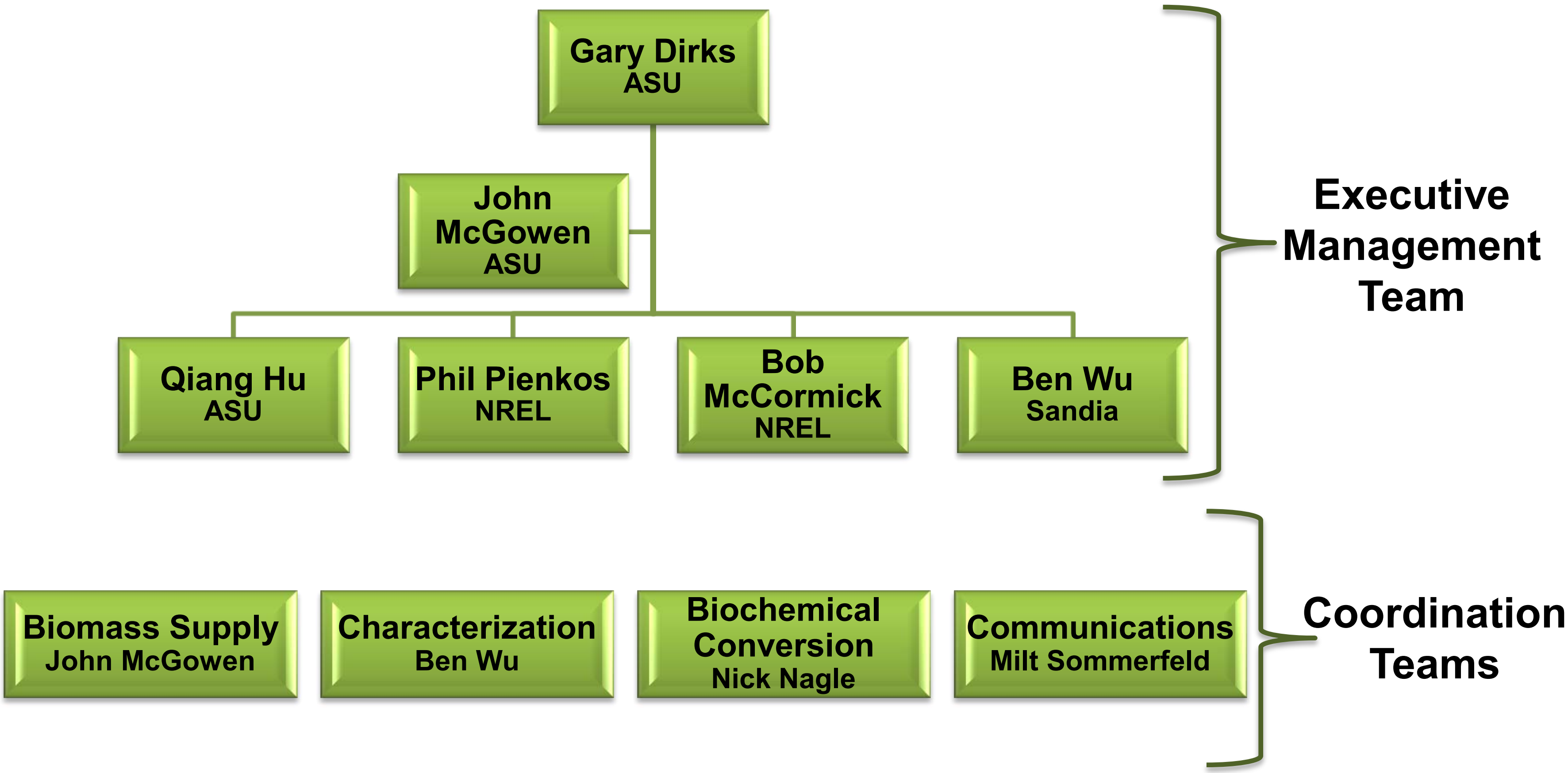
Management Structure and Approach



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Task 1 Biochemical Conversion of Microalgal-Derived Biomass

Outcomes:

Subtask 1.1: Identify robust versatile outdoor production strains/processes

Subtask 1.2: Develop standardized accurate and reproducible analytical methods

Subtask 1.3: Develop biochemical conversion process options to reduce conversion costs and improve biofuel yields

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Task 2 Product Performance of Fuels and Blend Components

Outcomes:

Effect of oil quality (detailed oil characterization/compositional analysis) on hydro-processing

Detailed chemical analysis and basic characterization of the impurities present in the fuels produced from algal biomass generated in Task 1.

Assessed compliance with ASTM specifications for chemical composition, performance, and stability requirements algal derived biofuels

Provided a conceptual process flow model, material/heat balance, and projected unit costs for an algal oil to fuels plant

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Top Level Milestones

Milestone A.1.ML.1: Produce sufficient amounts of lipid-rich and carbohydrate-rich algal biomass of selected production strains (e.g., *Scenedesmus* sp. *Chlorella* sp., and *Nannochloropsis* sp.) for the identified pretreatment, enzymatic hydrolysis, fermentation, oil extraction, fuel production and fuel characterization tasks from gram to kilogram quantities. **Throughout Project – Completed**

Milestone A.2.1.ML.2: Establish compositional analysis of algal biomass and a compositional library as a function of species and growth conditions **Completed – Milestone Report Submitted October 31, 2011**

Milestone A.3.ML.1: Evaluate multiple routes for pretreatment/enzymatic hydrolysis and selected the most promising routes for further study and integration **Completed – Milestone Report Submitted Jan 4, 2012**

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Top Level Milestones Cont.

Milestone A.3.ML.2: Down selected best strain(s) and process(es) for maximum lipid and ethanol yields for scale up testing. **Completed September 2012**

Milestone A.3.ML.3: Evaluation of alternative uses for algal protein fraction for enhanced production of biofuels **Completed February 28, 2013**

Milestone A.3.ML.4: Report on exploration for novel, algae specific enzymes **Completed February 28, 2013 (Submitted with Final Report)**

Milestone B.ML.1: Report on chemical analysis and ASTM standards testing for algal biofuels. **Completed May 2013 (Submitted with Final Report)**

Milestone C.ML.1: Submit final report on project progress including measure of fuel yields using a biochemical or a combined chemical-biochemical approach, data for inputs into technoeconomic models, and identification of critical elements for future yield improvements. **Completed April 2013 (for TEA modeling input); Final Report in May 2013.**

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Technical Accomplishments and Results

Subtask 1.1 Feedstock Development and Production

Subtask 1.2: Characterization

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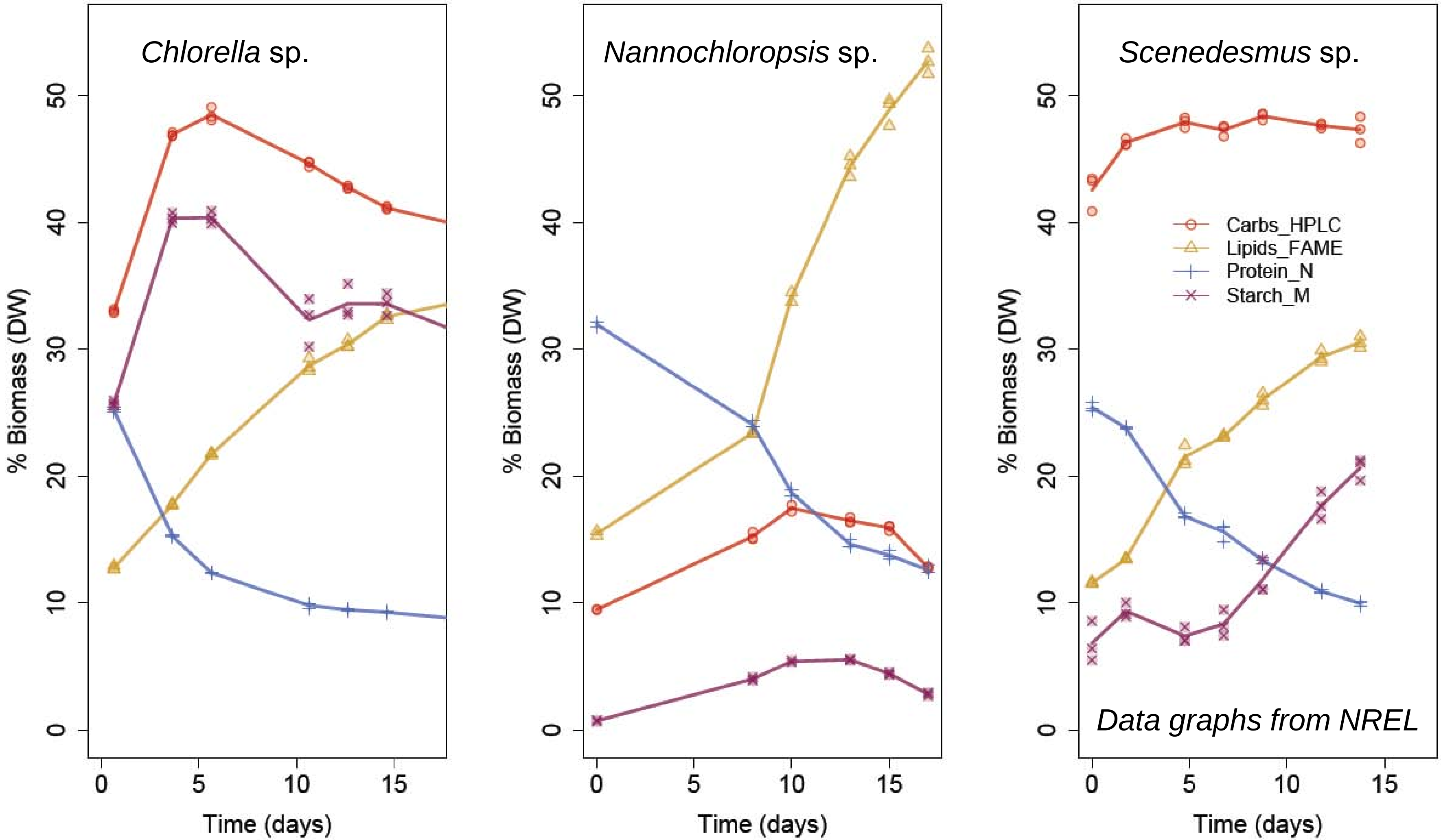
Feedstock Development and Production

Biochemical Composition of Algae is both Species and Condition-Dependent



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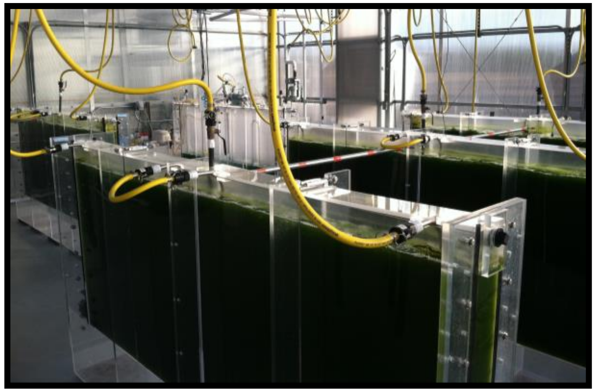
Subtask 1.1 Biomass Production and Materials Supply

Outcome: Supported biomass growth of 10’s g to kg quantities (per selected species/growth condition) and production of fractionated biomass for biochemical conversion and fuel production



Yr1: 10-100g’s scale
Yr2: 10’s kg scale.

- From Feb ‘11 through Aug ’11: **8 kg** of biomass harvested (2 strains)
- From Sept ‘11 to Feb ‘12: **80 kg** of biomass harvested (3 strains)
- From Mar ‘12 to June ’12: **60 kg** of biomass harvested (3 strains)
- From June ‘12 to September ’12, **60 kg** of down selected strain produced for larger scale integrated testing
- Through December 2012, total biomass harvested for project >**300 kg**.

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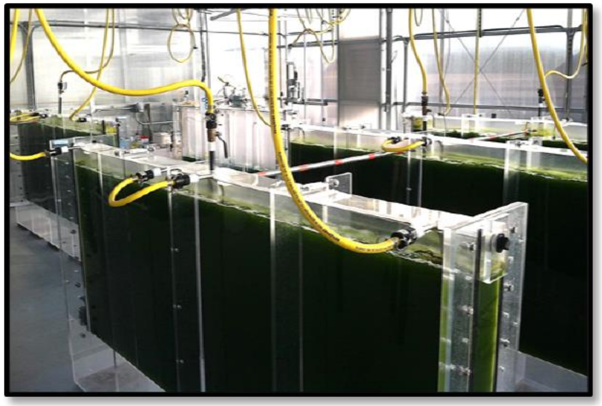
Biomass Production Process Flow

1. **Culture Vessel:** Columns (800 mL)

Location: Indoor lab
Typical batch size: 10-12 columns
Batch time: 6-10 days

2. Culture Vessel: 2'x2' flat panel PBR (15 L/PBR)
Location: Indoor lab
Typical batch size: 10-12 tanks
Batch time: 6-10 days



3. Culture Vessel: 4'x8' flat panel PBR (110 L or 240 L)
Location: Greenhouse
Typical batch size: 5 tanks (550 or 1200 L total)
Batch time: 5-7 days

4. Culture Vessel: PBR pilot array
- 4'x48' production rows
- 1500 L / row: 4" optical path length (OPL)
- 660 L / row: 1.5" OPL

Location: AzCATI testbed site
Typical batch size:
- 1-4 rows for biomass growth (1500-6000 L)
- 3-8 rows for lipid production (1980-5280 L)

Batch time: 5-7 days for 4" OPL, 4-18 days in 1.5" OPL (depends on target composition desired)

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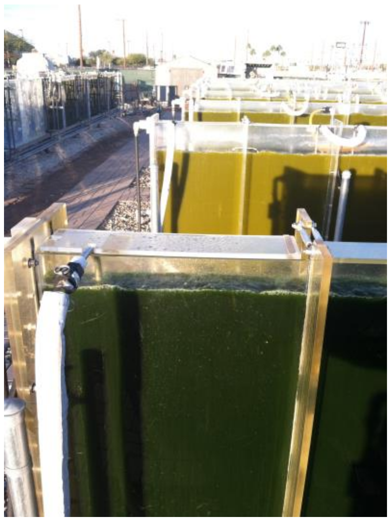
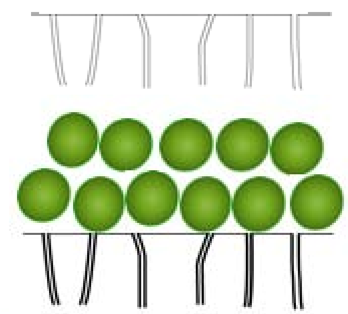
Biomass Production Process Flow



Pilot-Scale Membrane Algal Harvesting unit (Litree) at (ASU)

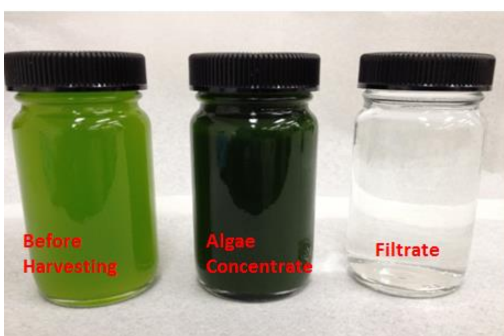
Permeate flow rate: 1~3 gpm
Biomass recovery: ~85-95%
Solids content: 4~6%

Pore size: ~10 nm



Lavin Continuous Centrifuge

Permeate flow rate: ~0.1 gpm
Biomass recovery: ~80%-90%
Solids content: ~20-30%



Biomass and Total Lipid Productivity
Chlorella sp. and *Scenedesmus* sp.

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Strain	Production Batch Date (Start – End)	Biomass Productivity (g/L*day^{-1})	Total Lipid Productivity (g/L*day^{-1})
Chlorella sp. (LRB-AZ-1201)	11/17/2011 - 12/05/2011	0.19	0.064
	05/23/2012 – 05/31/2012	0.32	0.097
Scenedesmus sp. (LRB-AP-0401)	01/13/2012 – 01/30/2012	0.17	0.076
	08/01/2012 – 08/07/2012	0.37	0.15

Nutrient deplete, batch mode, OPL 1.5”

Biomass Composition Changes *Scenedesmus* sp.

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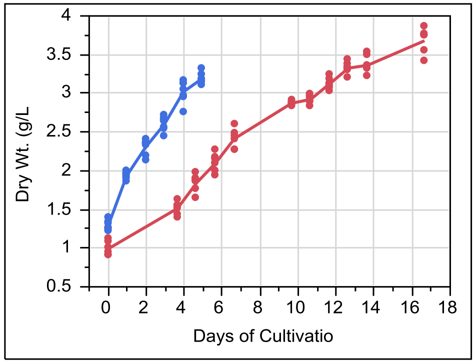
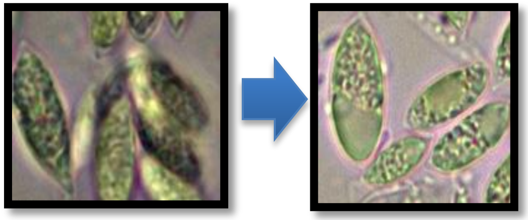
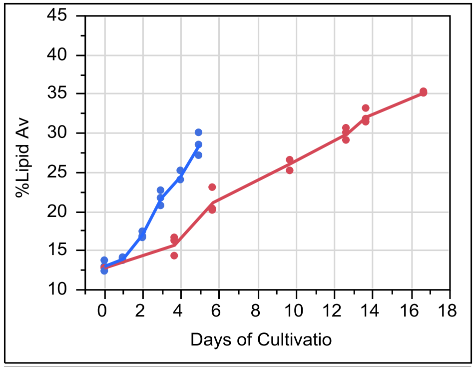


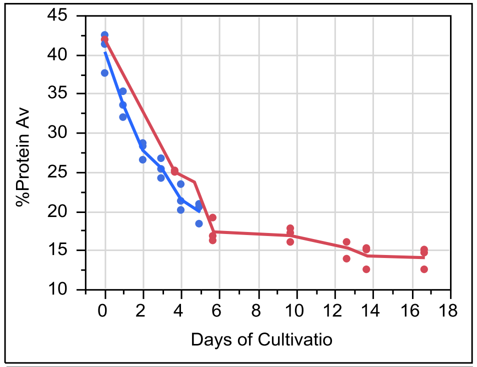


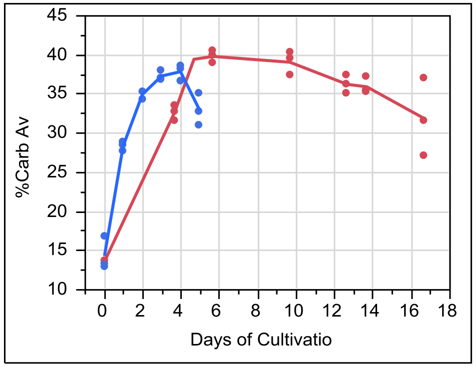
Micrographs courtesy of NREL

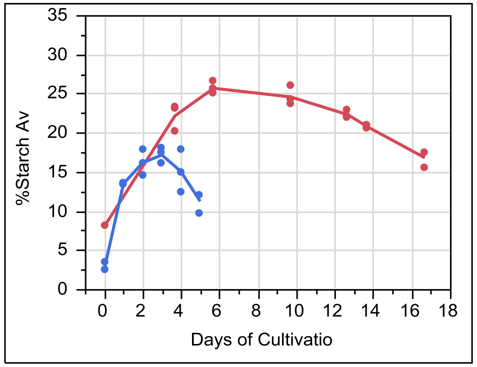
Batch ID

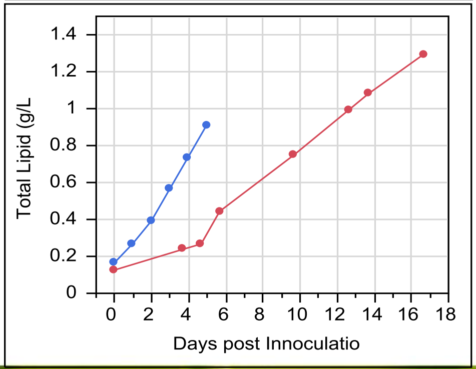
- 0401_01132012_PBR (red)
- 0401_08012012_PBR (blue)

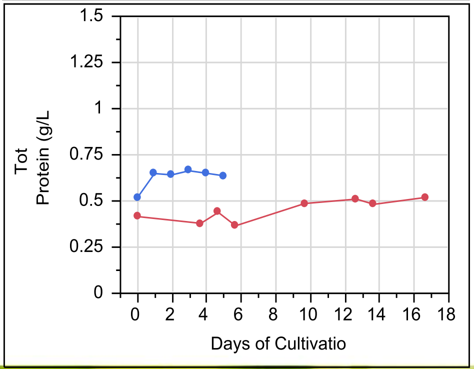


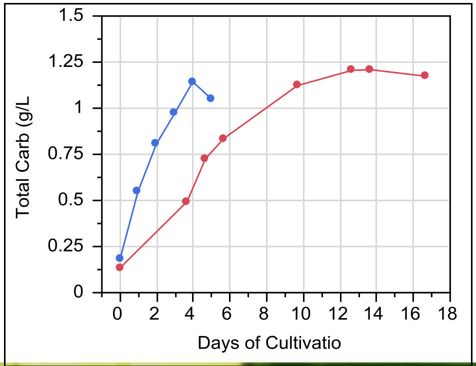


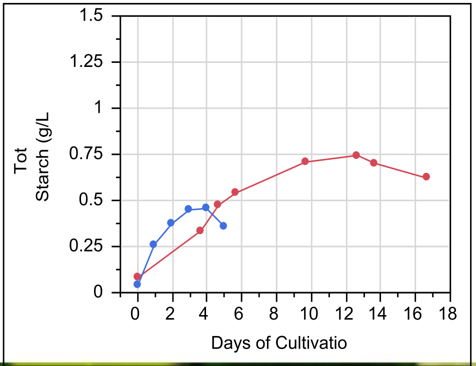












Biomass Production for Down Select Milestone July '12 – September '12

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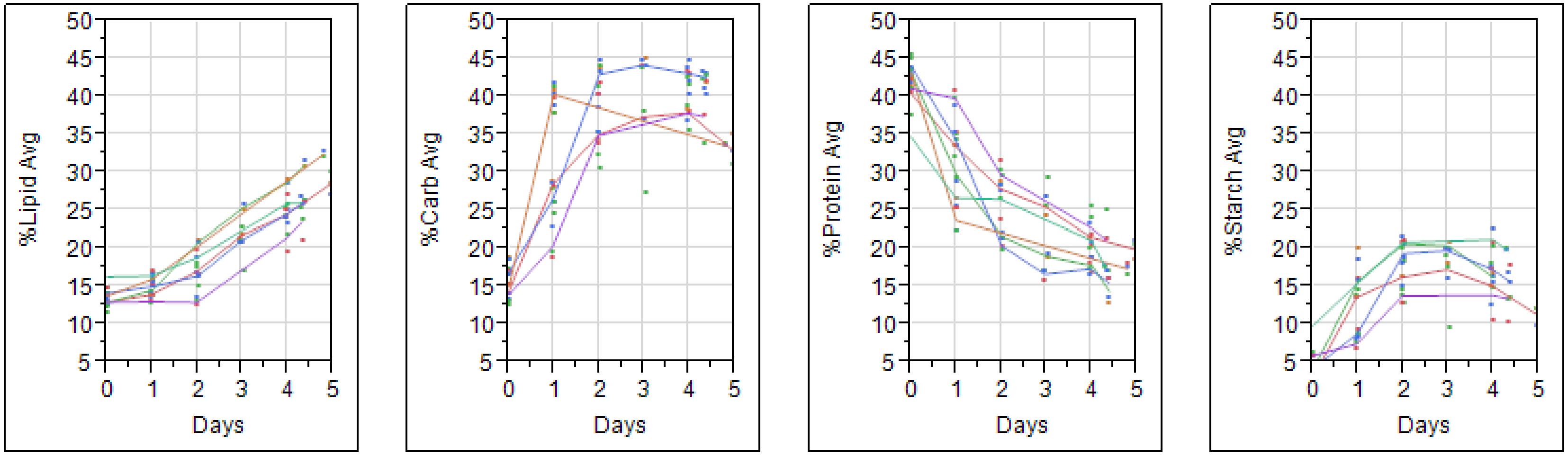
Batch ID	Harvest Date	Harvest No.	Volume Harvested (L)	g/L @ Harvest	Actual Yield (kg)	Expected Yield (kg)	Harvest % Yield	Paste Solids Content
0401_07122012_PBR	7/17/2012	62	5760	1.33	6.54	7.66	85%	31.8%
0401_08012012_PBR	8/8/2012	64	4550	3.13	11.26	14.24	79%	32.1%
0401_08082012_PBR	8/14/2012	65	3325	2.23	6.26	7.41	84%	33.0%
0401_08162012_PBR_A	8/21/2012	66	3125	2.37	5.77	7.41	78%	30.7%
0401_08222012_PBR	8/28/2012	67	3125	2.32	6.26	7.25	86%	34.1%
0401_08292012_PBR	9/4/2012	68	3125	1.94	5.79	6.06	95%	32.8%
0401_09052012_PBR	9/11/2012	69	4550	2.39	10.39	10.87	96%	33.4%
0401_09122012_PBR	9/18/2012	70	4550	2.49	8.54	11.32	75%	26.0%
				Total	60.8			



LRB-AP-0401 Scenedesmus sp.

Biomass Production for Down Select Milestone July '12 – September '12

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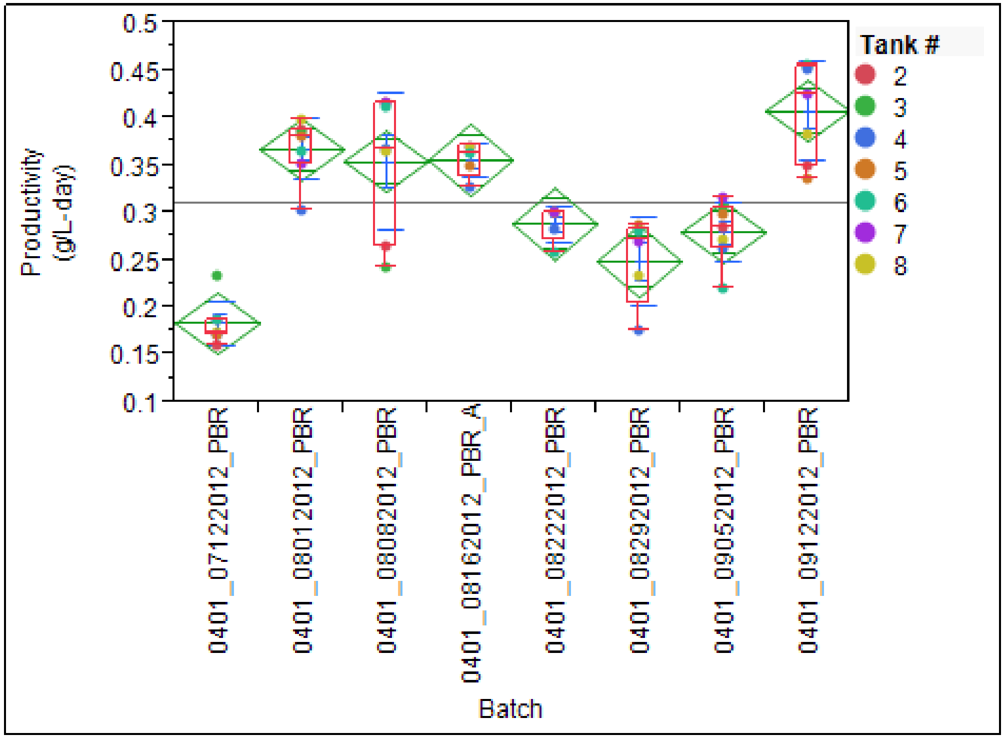
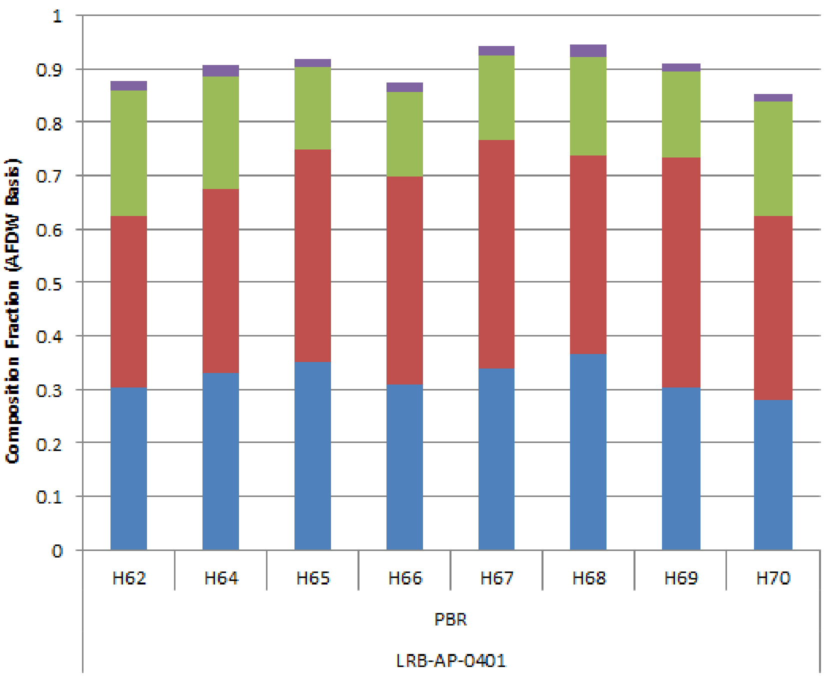


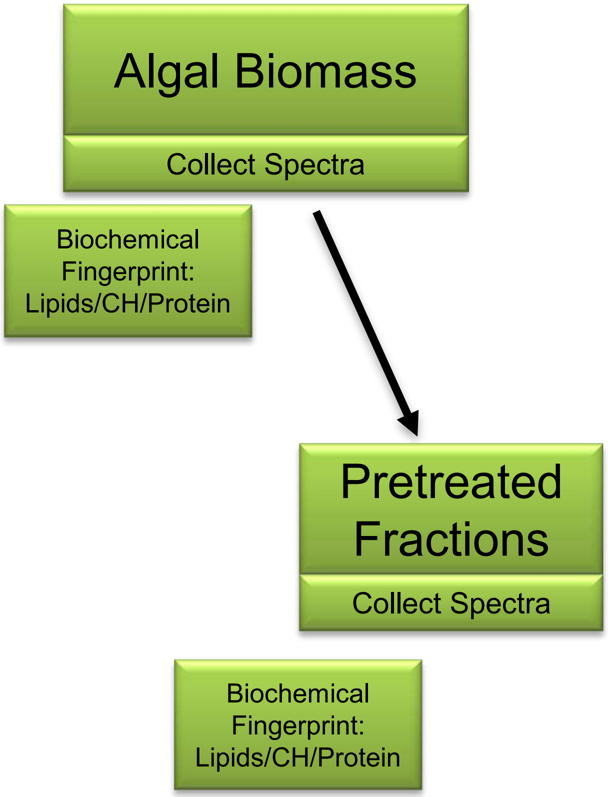


LRB-AP-0401 Scenedesmus sp.

Subtask 1.2 Characterization

Outcomes:

- Completed compositional analysis of algal biomass with the generation of a compositional library as a function of species and growth conditions
- Biochemical fingerprinting and routine measurements after individual "treatments" with standardized and validated methods to ensure reproducibility between shipments, lots, labs, and researchers
- Provided solutions for routine and challenging analytical measurements improving the understanding of process chemistry and mass balance closure
- Developed real-time high throughput methods shortening compositional analysis time from days to minutes
- Provided data for systems analysis, techno-economic models, and LCAs



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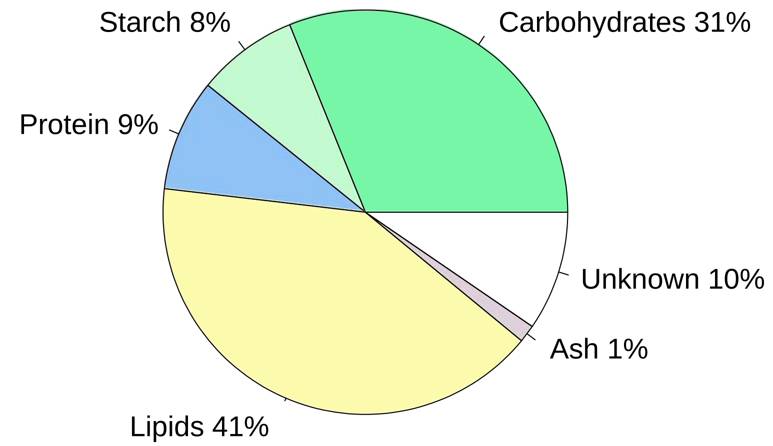
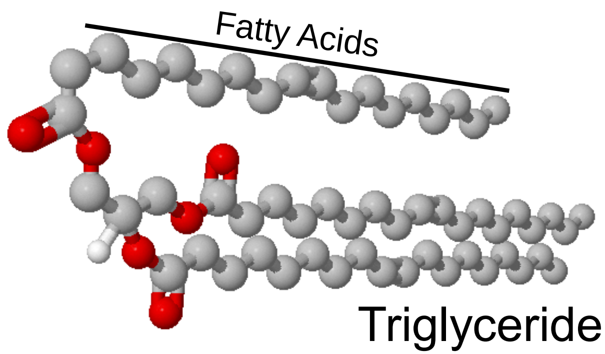
Subtask 1.2 Characterization

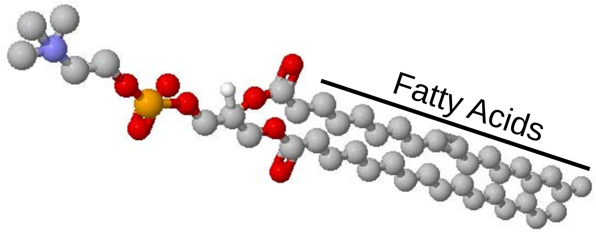


Characterization Subtasks

- **Algae Characterization**
  - Consequences of lack of standardization
    - Round-Robin experimental framework
    - Biological and physiological measurement variability
    - Yield calculations and process economics dependent on individual measurements
    - Process and biomass mass balance determine target metrics
    - Common measurement language lacking
    - Establishment of standardized measurements for unambiguous mass closure
- **Characterization of Biomass Conversion**
  - Solutions for challenging measurements of pretreatment samples
  - Structural changes of process chemistry through advanced spectroscopic characterization



Phospholipid

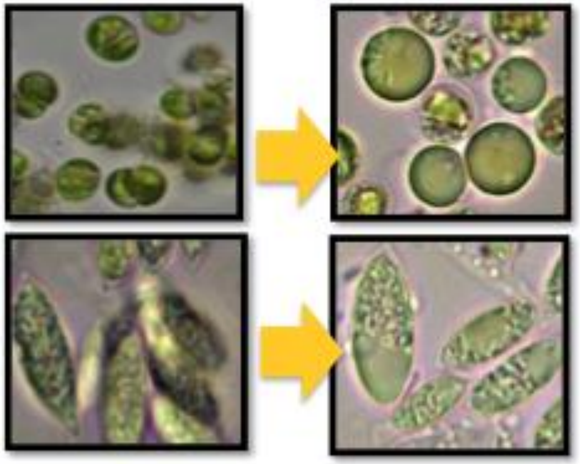


Compositional Analysis in Algae

Outcome:
Summative mass analysis, avoiding double counting of constituents

Complexity of Algal Biomass:

- Measurement and biological variability
- Lack of standardization of analytical procedures
- compositional mass balance closure 10-30% short of 100%



Measurement Uncertainties – 2011 Analytical Round Robin Experiment

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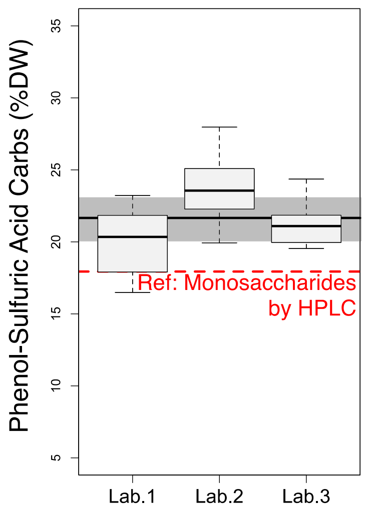
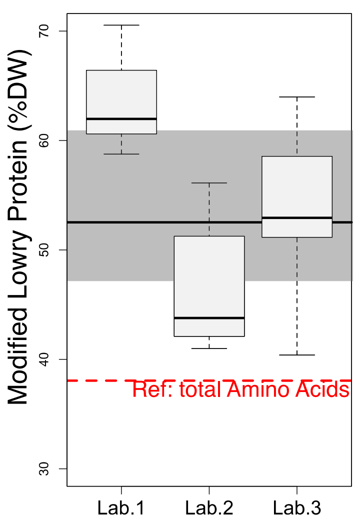
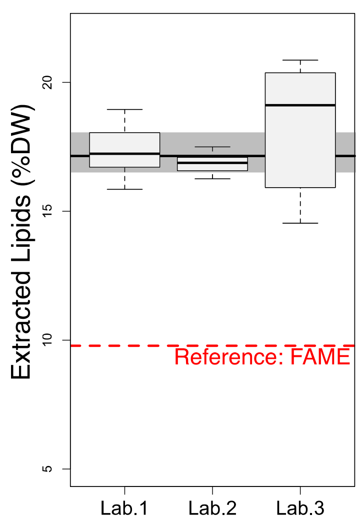


Goal

Quantify uncertainty and difference between methods and laboratories

- Survey of routinely used methods for proteins, lipids and carbohydrate measurements
- Comparison of yields, accuracy and precision of each method
- Design of Round Robin experiment



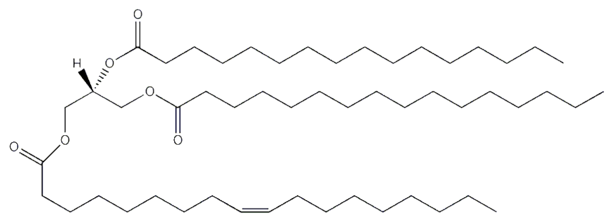


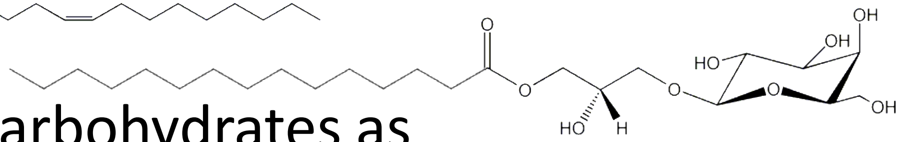
8 analysts, 12 days, 9 methods, 5 replicates
1 biomass sample (*Chlorella* sp.)

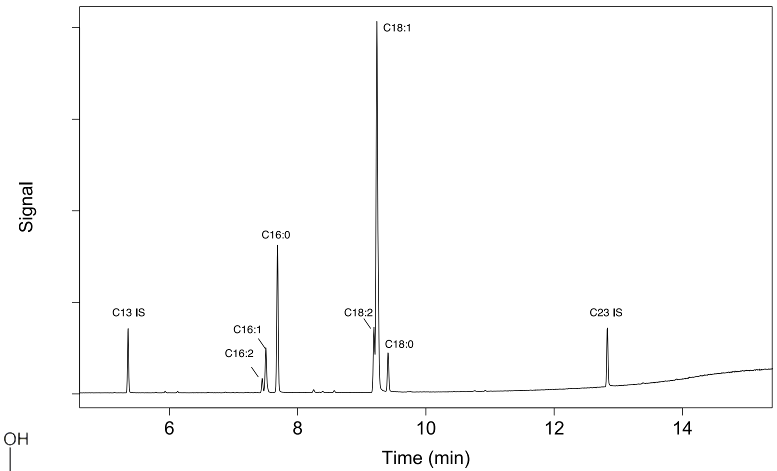
Anal. Chem. (2012) 84(4):1879-87

Common Measurement Language?

- Unambiguous measurements
  - Lipids as fatty acids

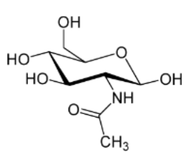




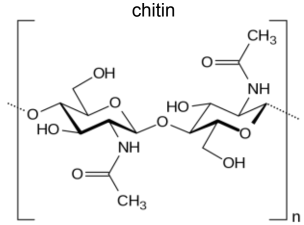


  - Carbohydrates as monosaccharides

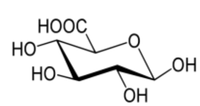
N-acetyl glucosamine



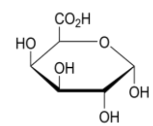
chitin

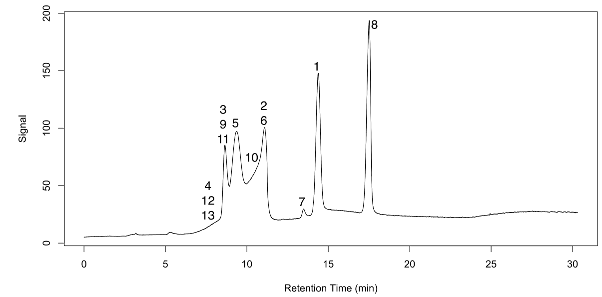


β-D-glucoronic acid

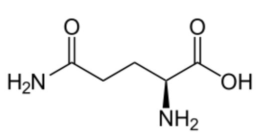


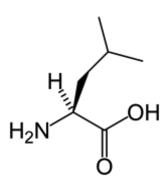
galacturonic acid

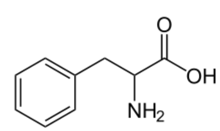


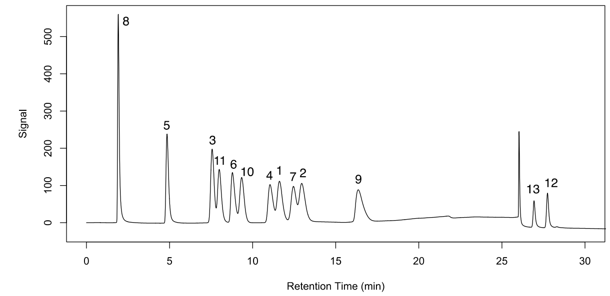


  - Proteins as amino acids

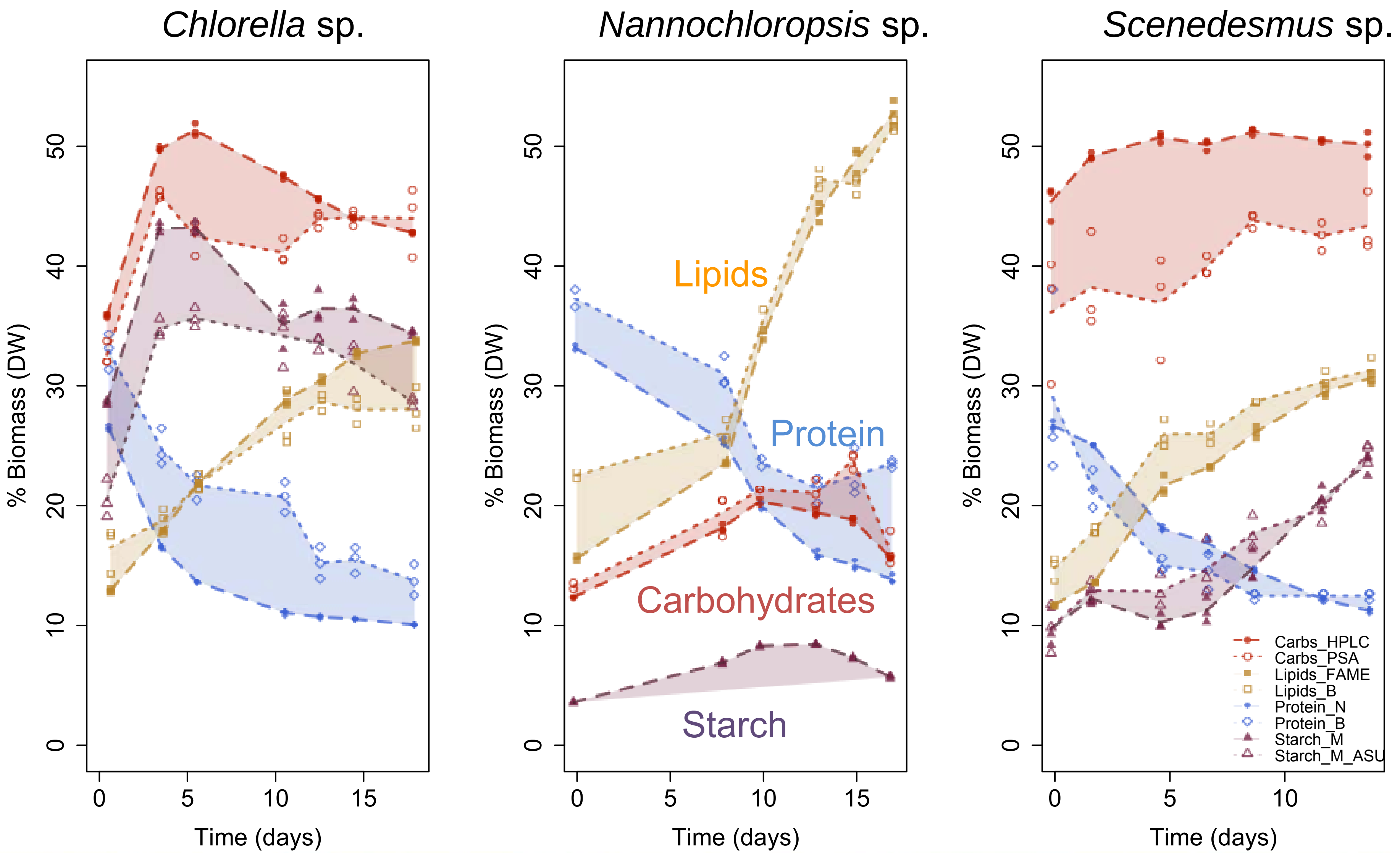








Measurement Variability between methods and sample matrix



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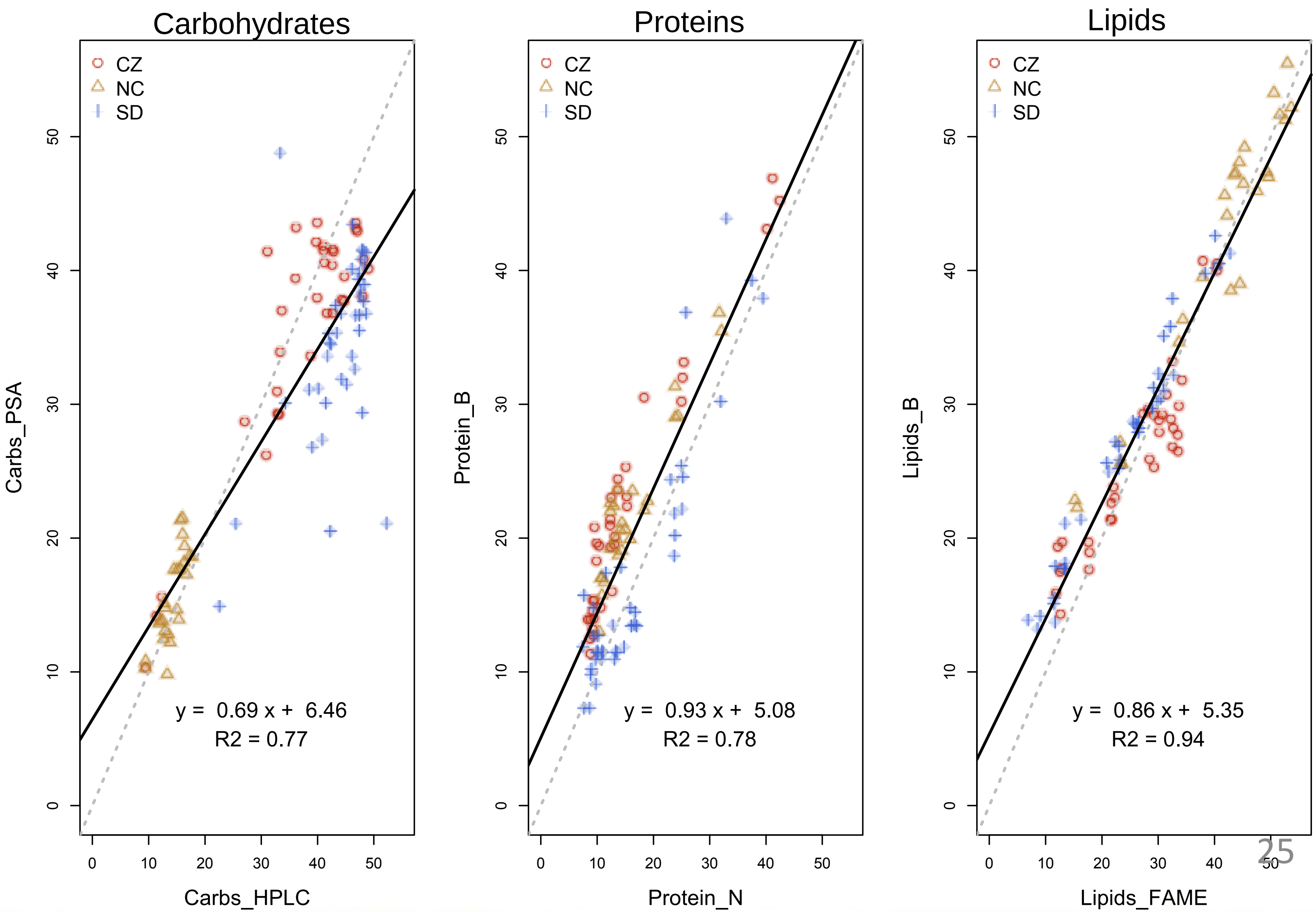
Measurement Variability between methods and sample matrix



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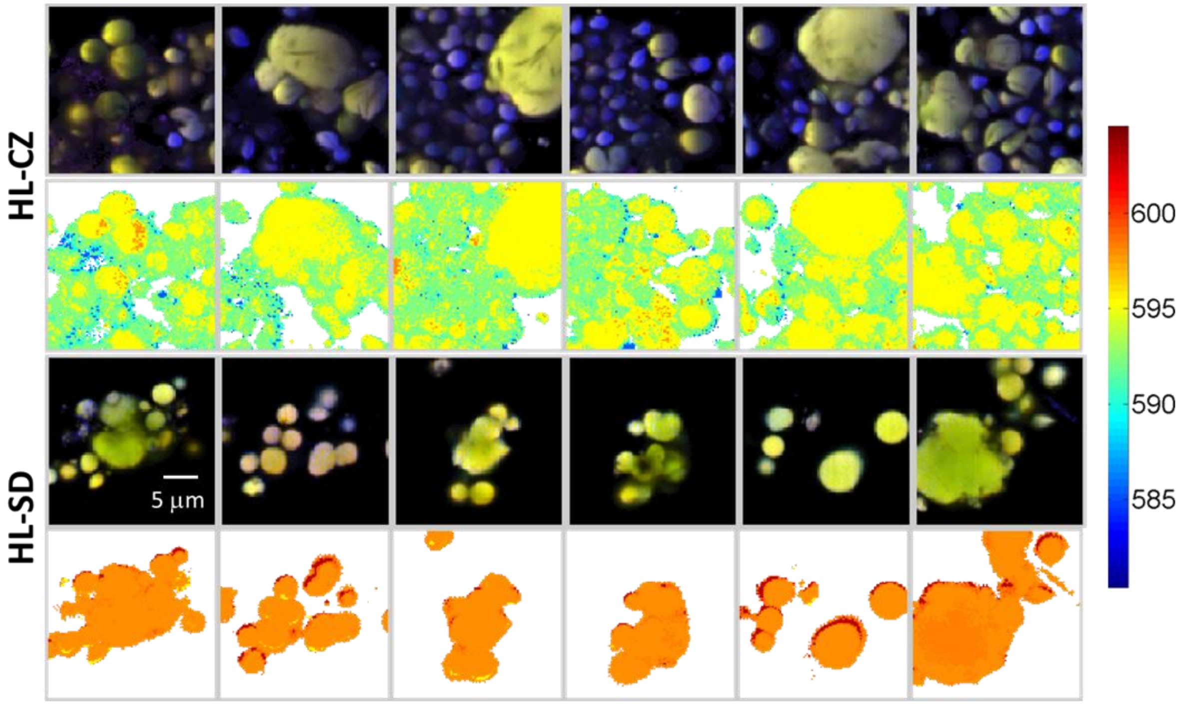
Advanced Characterization

- Whole biomass characterization
- Lipids *vs* Fatty acids
  - Fraction distribution
  - Extractable *vs* bound fatty acids
  - Recovery during conversion process
- Carbohydrates
  - Monosaccharide release
  - Recovery *vs* degradation
  - Fermentable carbohydrate fraction
  - Fermentation toxicity of breakdown products
- Spatial, temporal and biochemical microscopy
  - Hyperspectral Raman-enhanced imaging

Spatial Resolution of Lipid Chemistry



- Shifts consistent with TAG/FFA concentration in SD/CZ oil droplets
- Aggregates of lower polarity material surrounding the lipid droplets in the HL-CZ which is not present in the HL-SD

RGB images of HL-CZ and HL-SD

- NR Polar Lipid = Red
- NR Neutral Lipid = Green
- Chloroplast Membranes = Blue
- Lipid droplets appear yellow (red+green = yellow)

Technical Accomplishments and Results
Subtask 1.3 Biochemical Conversion

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Subtask 1.3 Investigation of Biochemical Conversion Routes for Whole Algae and Algae Residues

- **Pretreatment and Commercial Enzyme Screening:** Determine optimal pretreatment and conditions for algal deconstruction and demonstrate impact of algal cultivation on bioconversion
- **Exploration for Novel Algae Specific Enzymes:** Discover and develop algal-specific hydrolytic enzymes for lipids, carbohydrates and protein complexes by mining thermophilic fungal genomes and metagenomes of microbial communities for novel lipid & carbohydrate-active genes for cloning, expression and evaluation
- **Lipase/Protease-Based Biochemical Conversion:** Discover and develop algal-specific hydrolytic enzymes for lipids, carbohydrates and protein complexes and develop new pathways for industrial algal deconstruction
- **Integrated Algal Biomass Processing:** Explore integrated approaches to biochemical conversion of whole cell and algal residuals and develop new pathways for industrial algal deconstruction

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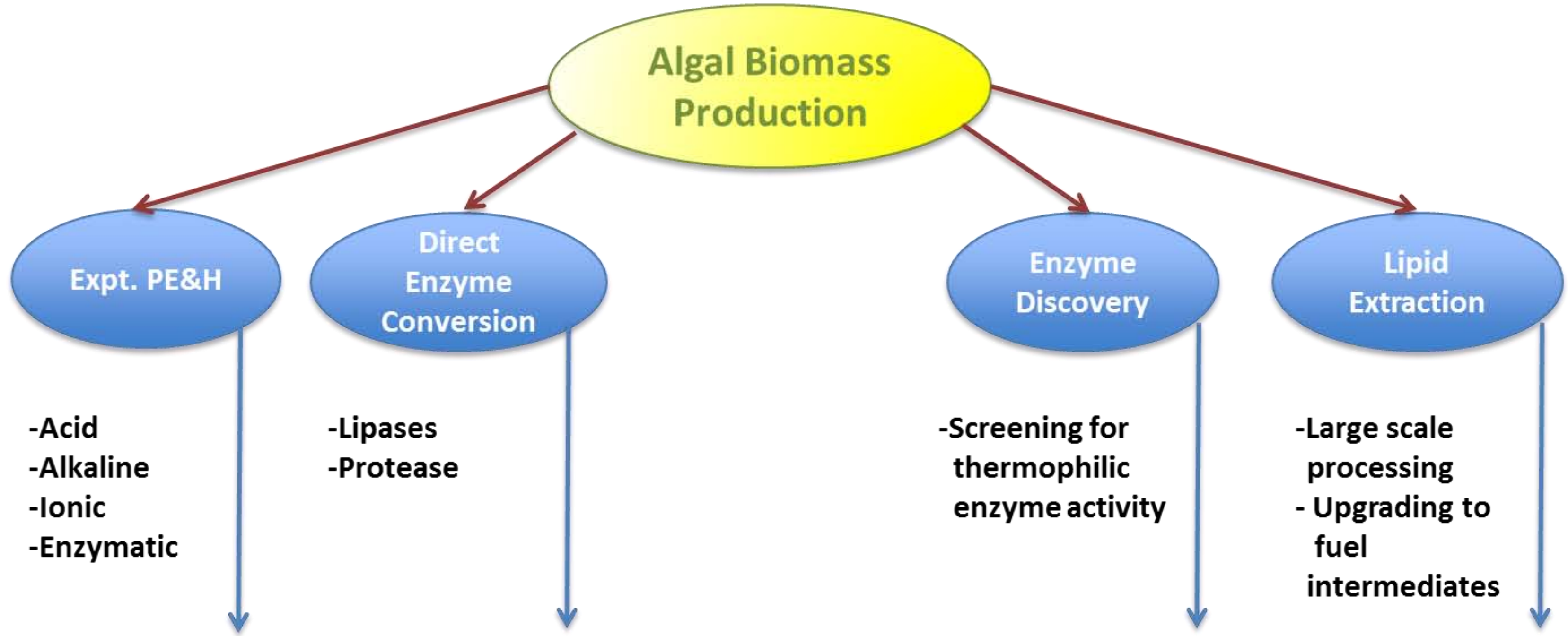
Subtask 1.3 Biochemical Conversion Initial Concept



	Expt. PE&H	Direct Enzyme Conversion	Enzyme Discovery	Lipid Extraction
Year 1 Activity	Screening	Screening	Screening	Screening
Down Select				
Year 2 Activity	Integrated processing	FAME production	Isolation of new enzymes	Scale-up

Reduce process costs, increase biofuel yields, and enhance understanding of process.

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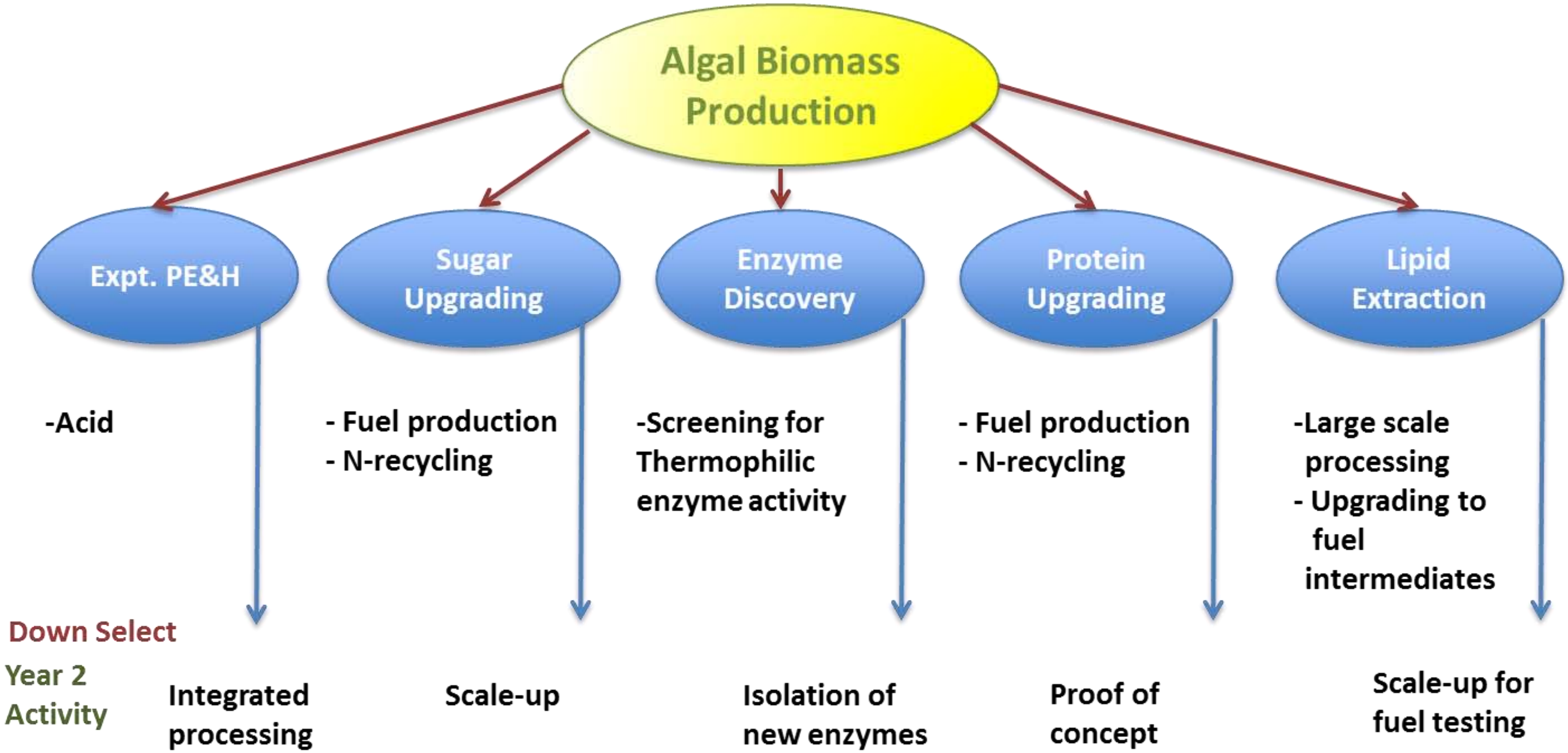
Subtask 1.3 Biochemical Conversion Final Concept



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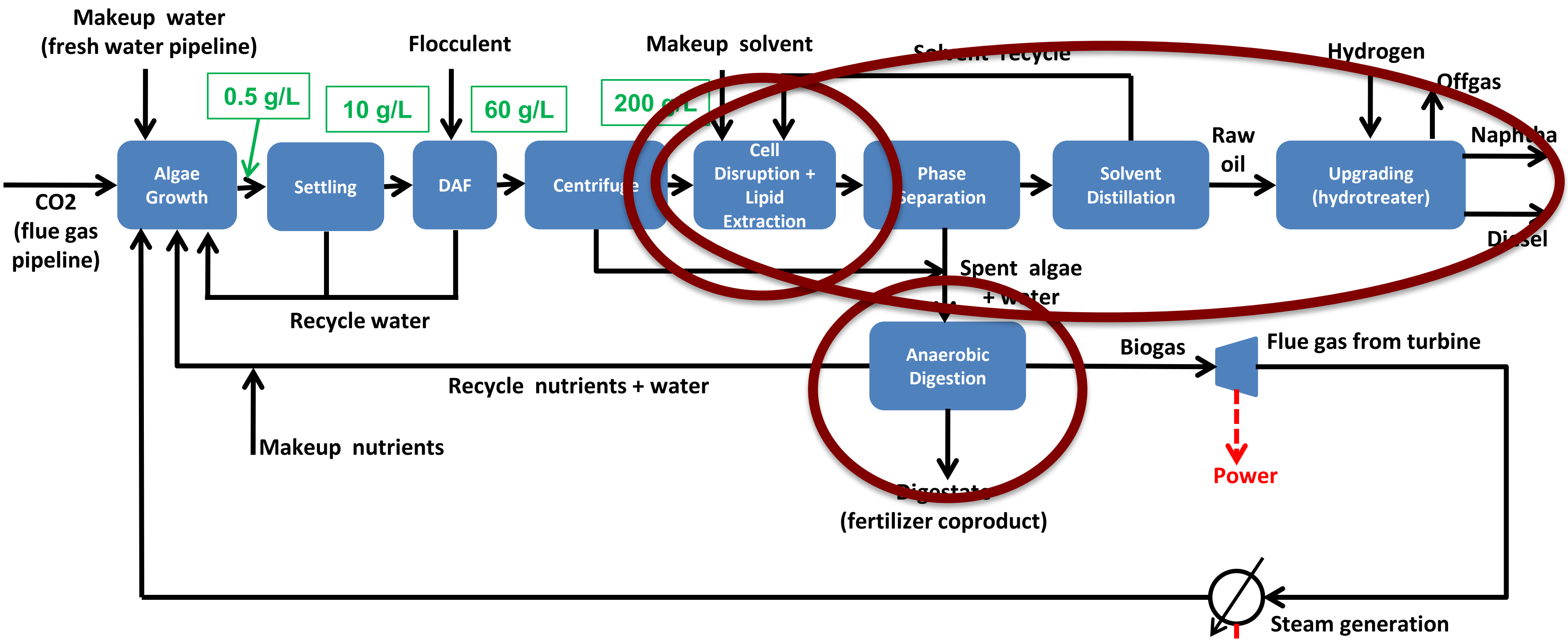
Harmonized Process Design Configuration

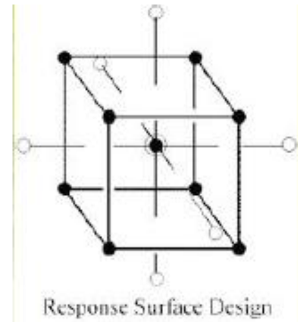


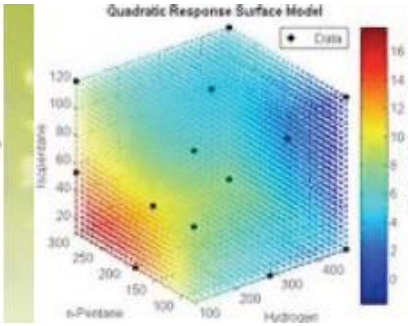
Green = algae cell density

Pretreatment and Commercial Enzyme Screening

Outcomes:

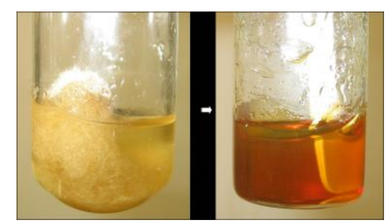
- Several commercial enzymes identified which degrade cell walls but no combination enhance lipid extraction efficiency
- Bioprospecting for novel enzyme activities in secretomes of thermophilic fungi showing promise
- Ionic liquid pretreatment showed promise but work discontinued due to scale up issues
- Dilute acid and base pretreatment provided basis for further work. Both processes demonstrated effectiveness but base pretreatment discontinued due to complications with downstream carbohydrate analysis.






4-L Steam Digester


ASE 350


Ionic Liquid Pretreatment

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Protease-based algal cell wall degradation

Enzyme	*Chlamydomonas* CC-1690	*Chlorella*	*Scenedesmus*
Papain	2%	ND	ND
Protease from aspergillus	8%	ND	ND
Protease from Streptomyces	7%	ND	ND
Trypsin	8%	ND	ND
Rennet	ND	ND	ND
Alcalase	20%	ND	ND
Subtilisin	17%	ND	ND
Protamex	17%	ND	ND
Glucanex	6%	ND	ND
Esperase	5%	ND	ND
Autolysin	50%	ND*+	ND*+

Release of Chlorophyll by Commercially Available Proteases

- A method was developed to monitor efficacy of cell wall degrading enzymes by monitoring release of chlorophyll into the media.
- Alcalase and Subtillisin were selected for further testing

- These enzymes are likely good candidates for addition as part of a cocktail containing other carbohydrate hydrolyases but cannot function as stand-alone enzymes for cell wall disruption.

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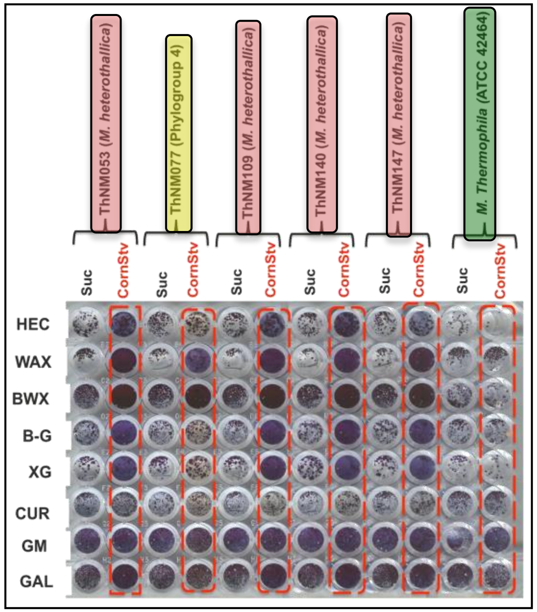
Enzyme Discovery and Development

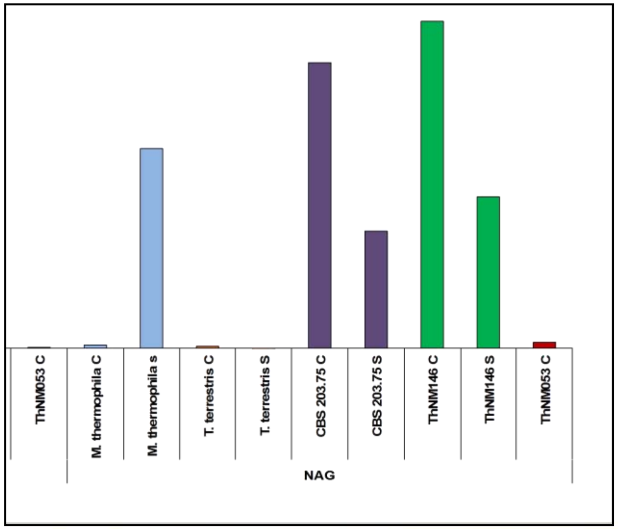
Analysis of cell wall biopolymers indicate potential for supplemental feedstocks and increased biofuel yields on algal biomass

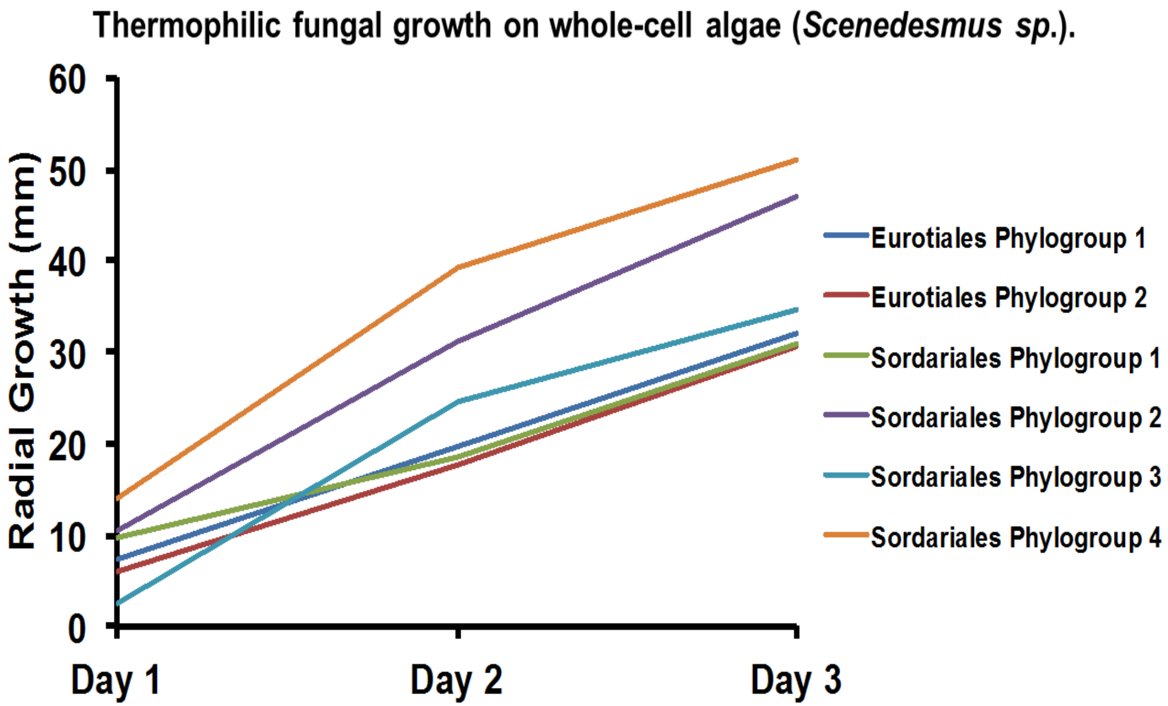
- Fermentable sugars from the cell walls of each strain have been characterized. ~16% of the *N. gaditana* cell wall appears to be cellulose
- Enzymes to facilitate biomass deconstruction have been identified
- Commodities of potential value, including carotenoids and algaenan

Species	Primary carbohydrates	Other	Enzymatic susceptibility
Chlorella sp.	rhamnose, glucose, galactose, mannose	inorganics	chitinase, lysozyme, glusulase, protease, pectinase
Scenedesmus sp.	mannose, glucose	algaenan, inorganics carotenoids	protease
Nannochloropsis gaditana	glucose	Protein, algaenan, ester-linked FAs, inorganics zeaxanthin	not determined

Thermophilic Fungi As Source for Hydrolytic Enzymes

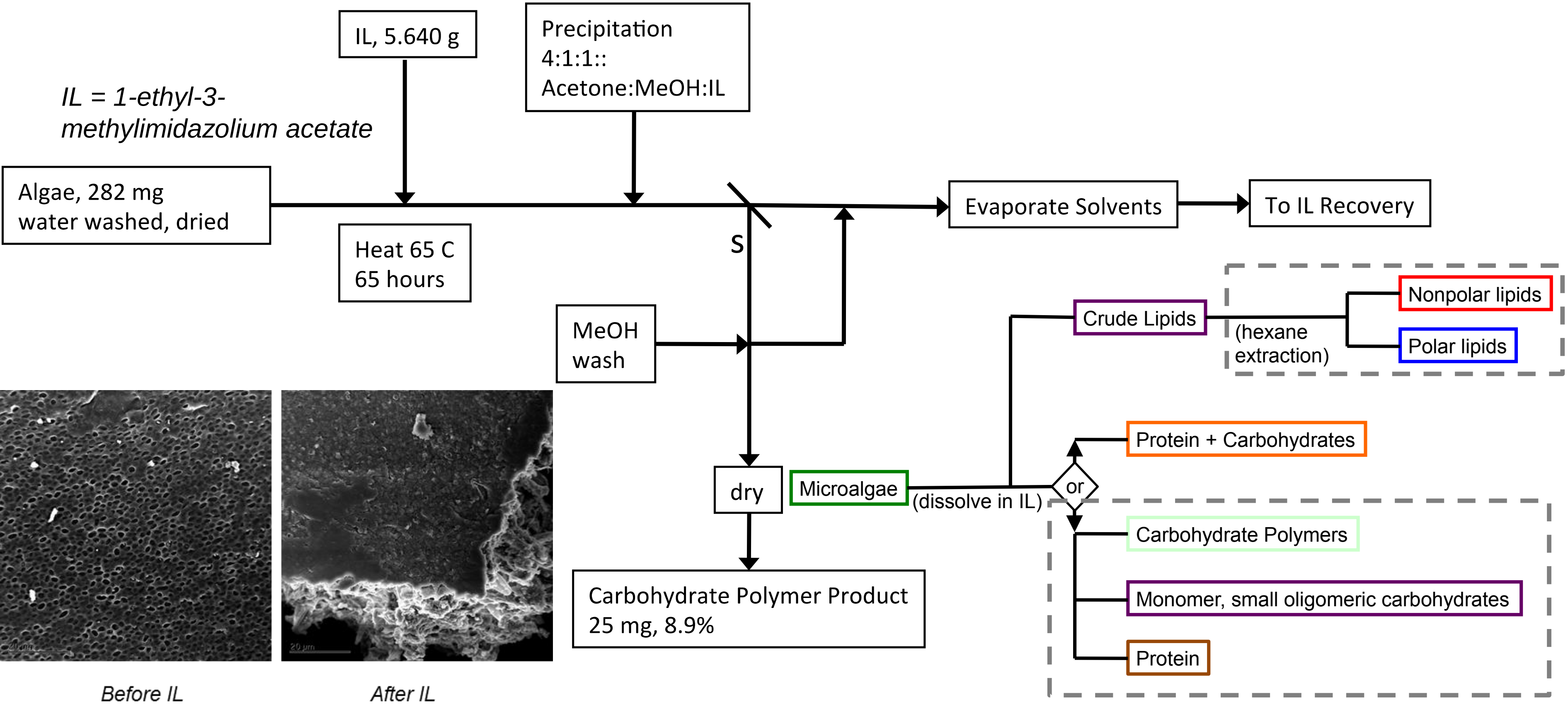






Ionic Liquid Pretreatment

IL = 1-ethyl-3-methylimidazolium acetate



Before IL *After IL*

SEM, 1000X by Bernhard Knierim

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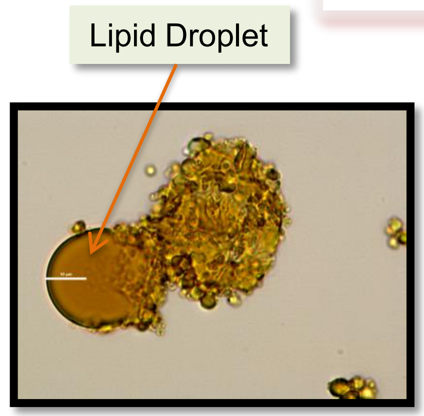
Dilute Acid/Base Pretreatment

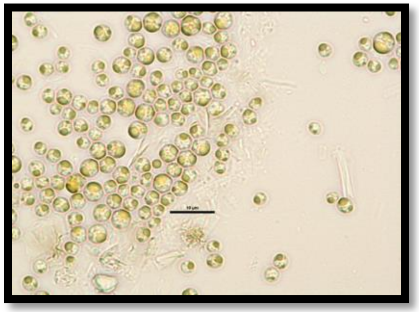
- CEM Explorer Microwave Reactor
- Automated system w/36-positions
- 300 watt output
- Stir bar mixing-rapid heating

Experimental Parameters – CCD Experimental Approach Whole Algae- Non Extracted	Low	High
Temperature	115° C	180° C
Time (min)	1	20
Catalyst conc. (% acid)	0.0	3.0
Catalyst conc. (% NaOH)	0.0	0.1

- Use pretreatment to breech cell wall
- Tracking both carbohydrate and lipid (FAME) release
- Enable enzymatic hydrolysis

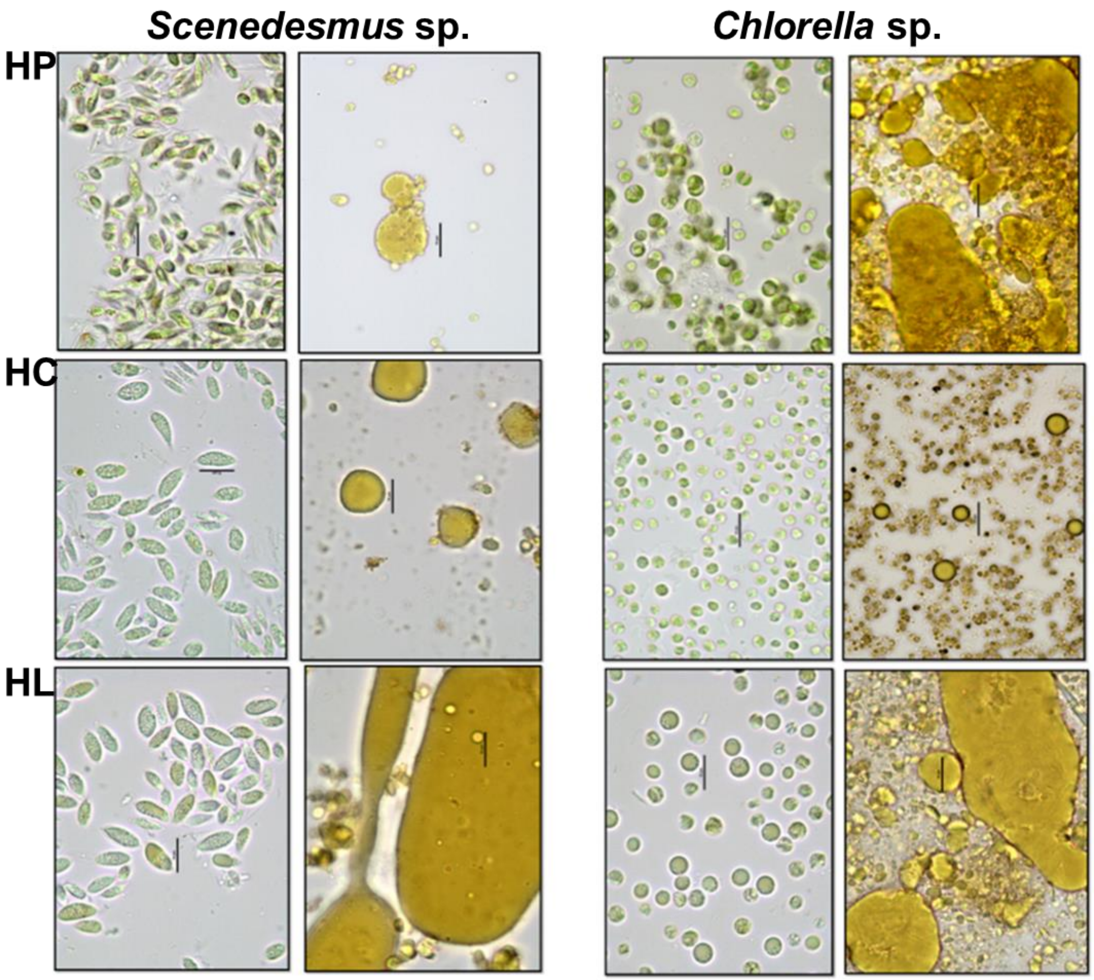


Acid Catalyzed



Alkaline Catalyzed

Dilute Acid Pretreatment Biomass Deconstruction



Lipid droplets remain entrained in cell debris. Additional steps needed for lipid recovery

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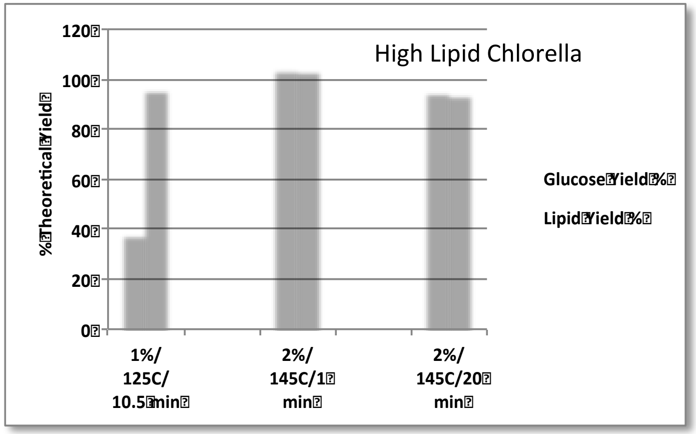
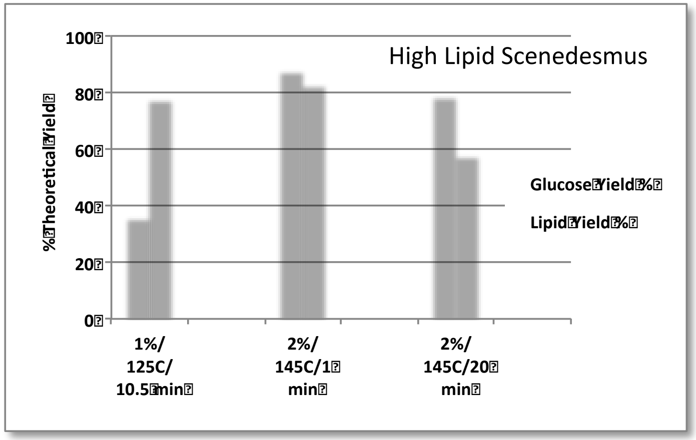
Glucose and Lipid Yield from Dilute Acid-Microwave Pretreatment

Key Findings

- Developed and demonstrated single-step dilute acid pretreatment that hydrolyzes polysaccharides to glucose without enzymes or production of inhibitors, and liberates lipids for high extraction yields.
- Hexane extraction of pretreated solids provided good lipid yields
- Alkaline pretreatment conditions confounded analysis and this approach was abandoned.
- Demonstrated carbohydrate release in both *Chlorella* and *Scenedesmus*, cultivated under high lipid and high carbohydrate regimes
- Limited efficacy with post-enzymatic digestion using commercially-available enzymes
- Scale-up of pretreatment to produce materials for fermentation





Impact of Process Sequence

Extraction >Pretreatment *Scenedesmus sp.*

	Fatty Acids in Extract (g/kg biomass)	Fatty Acid Recovery (%)	Glucose in Liquor (g/kg biomass)	Glucose Recovery (%)
High Protein SD	9	13	98	72
High Carb SD	19	7	236	68
High Lipid SD	20	6	174	67

Pretreatment > Extraction *Scenedesmus sp.*

	Fatty Acids in Extract (g/kg biomass)	Fatty Acid Recovery (%)	Glucose in Liquor (g/kg biomass)	Glucose Recovery (%)
High Protein SD	53	78	92	67
High Carb SD	236	97	185	53
High Lipid SD	268	76	180	69

Same result seen with *Chlorella* biomass.

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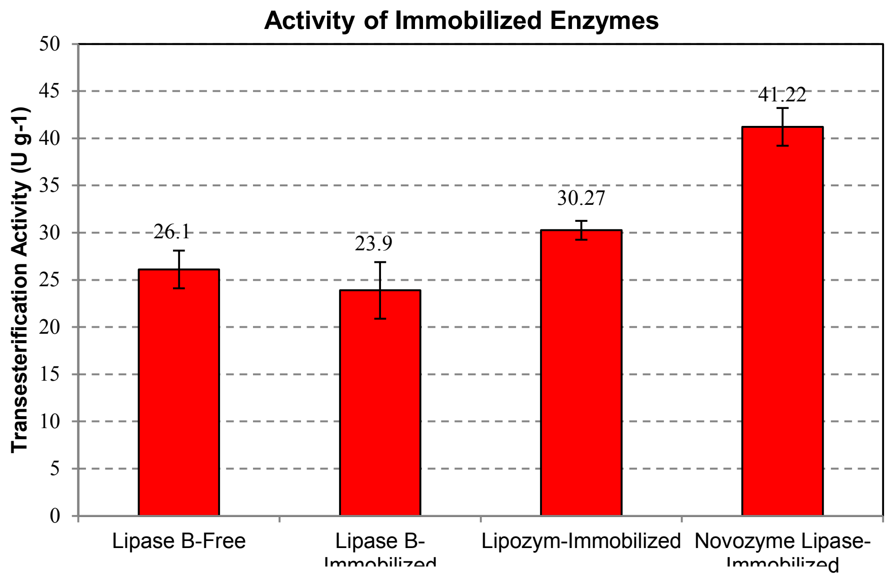
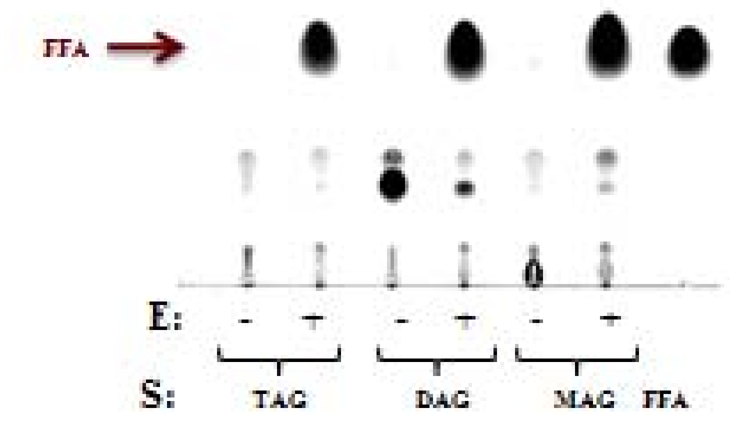
Lipase Based Conversion of Lipids to FAME

- Commercial, algal, and viral lipases were screened.
  - The Novozyme lipase performed the best.
- Immobilized lipase process
  - Amberlite XAD®-2 resin supported high transesterification activity.
  - Immobilized enzyme was reused >30 times with no loss in activity.
- Effective lipase conversion protocols were established for algal neutral lipids, polar lipids, crude algal oils.
- FAME sample analyzed for biodiesel qualities





Pretreatment and Fermentation Results-Shake Flasks

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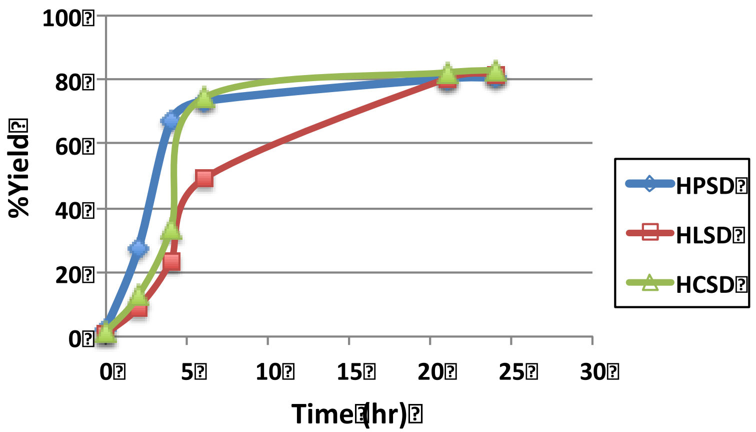
Carry out dilute acid pretreatment at 100 g scale in Zipperclave reactor.

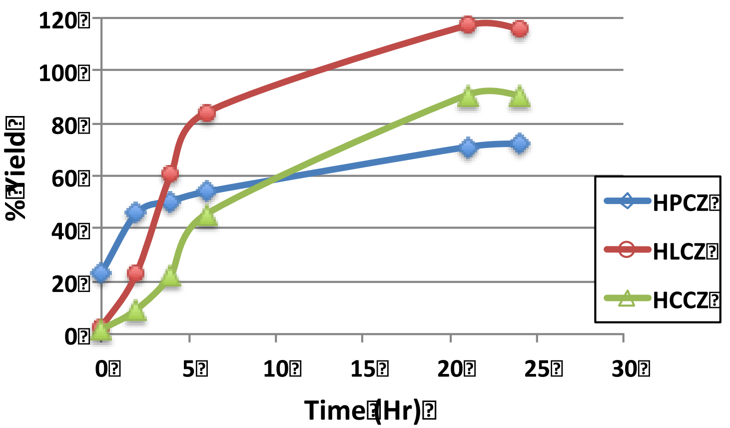
Biomass	Glucose Recovery (g/kg biomass)	Ethanol Yield (%)
HPSD	105	81
HCSD	228	83
HLSD	131	82
HPCZ	3	72
HCCZ	217	91
HLCZ	176	>100

- Fermentation completed at 6-21 hr.
- HMF concentration ranged from 0.5-1.5 g/L
- No furfural measured
- Shake flask setup using neutralized prt hydrolyzates
- YP addition at 0.5X (3-4g/L)
- Yeast (D5A) fermentation organism
- No pH control
- Triplicate fermentations

Scenedesmus



Chlorella



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Recovered Fuel Yields Per Ton of Biomass

	HP SD	HC SD	HL SD	HP CZ	HC CZ	HL CZ
Combined fermentable sugars (kg)	193	324	220	5	234	182
Ethanol yield (%)	82	100	81	73	91	100
Ethanol-fermentation (kg)	81	165	91	2	109	93
Ethanol (Gallons)	28	55	30	1	36	31
Gasoline Gallon Equivalent (GGE)	19	36	20	0	24	20
Btu equivalent (x10e3)	2206	4224	2329	43	2778	2378
Fatty Acids (FAME in ext) (kg)	36	154	210	35	35	151
Hydrocarbon (kg)	28	120	164	28	28	118
Diesel Equivalent (gallon)	10	41	56	9	9	40
Btu equivalent (x10e3)	1187	5046	6886	1158	1158	4957
Total Fuel Energy (x10e3 Btu)	**3393**	**9270**	**9215**	**1201**	**3936**	**7335**
Total GGE	**29**	**80**	**79**	**10**	**34**	**63**

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Integrated Algal Biomass Processing

Outcome:

- Selected high carb *Scenedesmus* sp. as optimal biomass based on total biofuel potential and shortened growth cycle
- Selected dilute acid pretreatment, solids liquid separation, and hexane extraction to fractionate carbs, lipids, and protein fractions
- Established biofuel potential of fractions
  - Lipid yields based on FAME analysis
  - Ethanol fermentation of carb fraction
  - Biobutanol fermentation of protein fraction
- Established value of ethanol fermentation broth for nutrient recycle

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Integrated Scale-Up Demonstration

Biomass Production (ASU)

Pretreatment (NREL)

Solid/Liquid Separation (NREL)

Solids

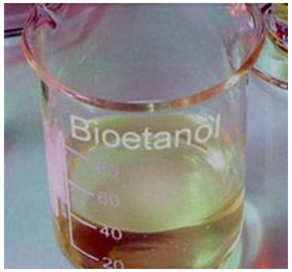
Lipid Extraction (NREL)

Lipids

Protein

Sugar Fermentation (NREL)

Nutrient Recycle (CSM)



Protein Fermentation (SNL)

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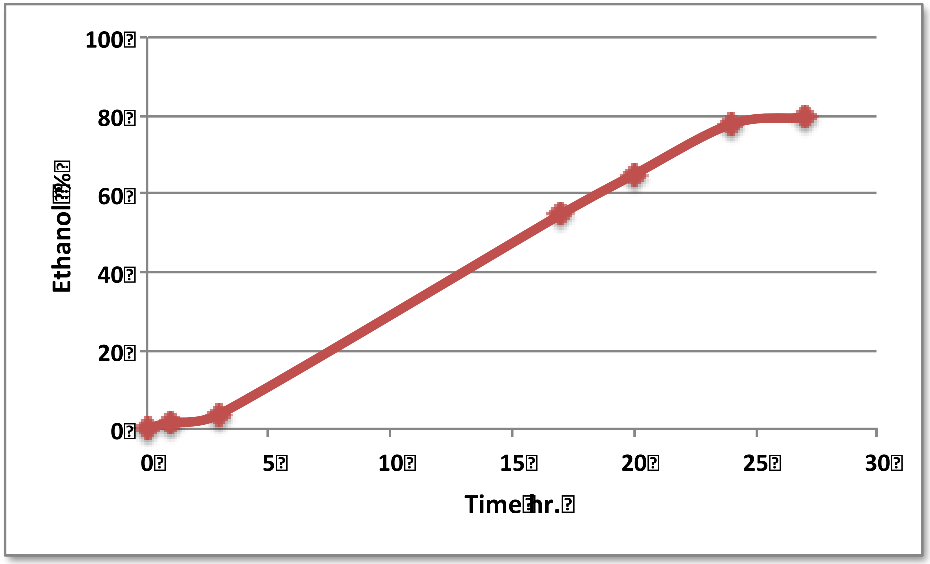
Pretreatment and Fermentation Results- Demonstration Scale

Key Findings

- Demonstrated scalable process from mg to g to kg scale
- Minimal fermentation inhibitor produced from dilute acid pretreatment
- Carbohydrate release and FAME recovery demonstrated
- Ethanol fermentation is a good proxy for other fuels/chemicals
- Pretreatment of algae followed by extraction offers a new process scheme.



Final Yield (%)

Glucose[1]	Ethanol[2]	FAME[3]
68	80	81

1 Based on initial composition
2 Based on 0.51g EtOH/g glucose
3 Recovery based on initial composition

Bioconversion of Algal Protein Fraction to Biobutanol

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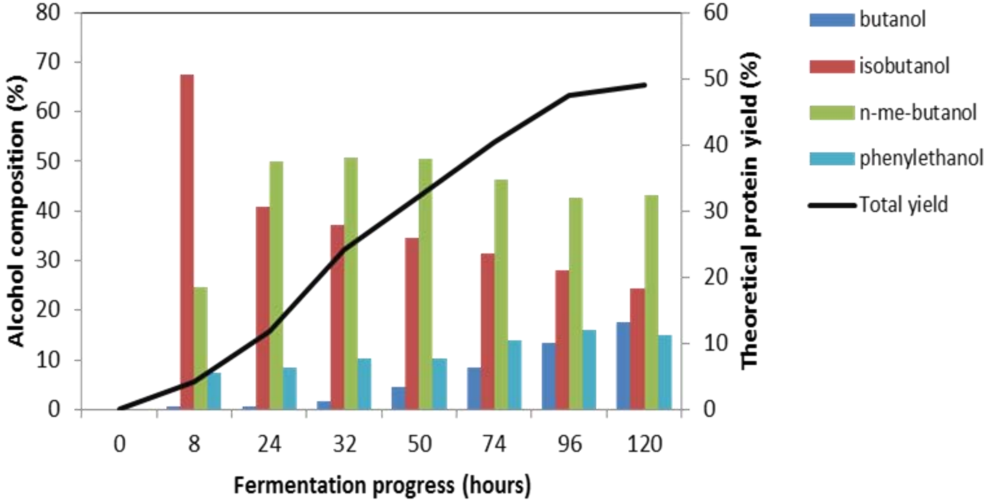
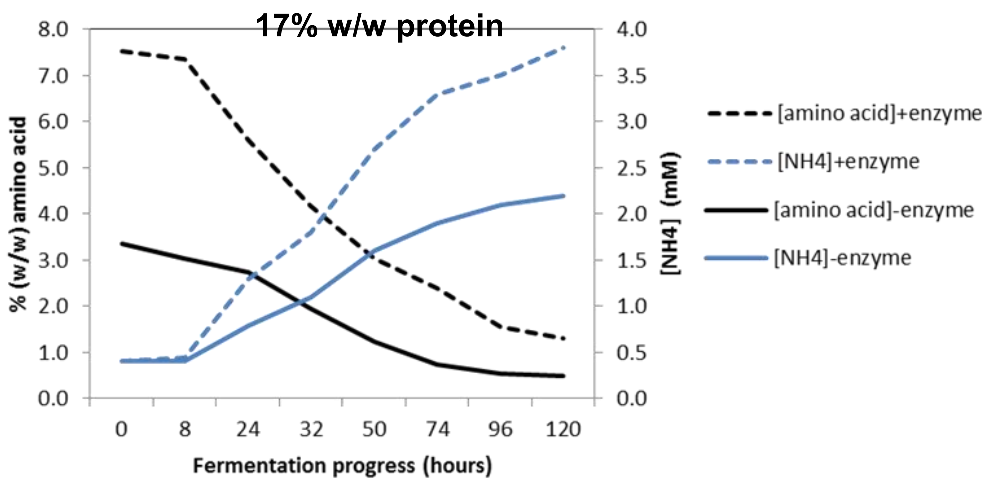
Subject protein fraction to microaerobic fermentation using an alcohol tolerant metabolically engineered *E. coli* strain, developed by Prof. James Liao at UCLA.



Results

1) Higher yields with enzymatic digestion
2) Achieved 50% theoretical yield
3) Alcohol components do not significantly vary with biomass type
4) Accumulation of alcohols proceeds in distinct temporal phases
5) Ammonium is accumulated in fermentation liquor as amino acid breakdown product



Huo et al. “Conversion of proteins into biofuels by engineering nitrogen flux, Nature Biotechnology 29, 346351 (2011)

Support of Algal Growth By Recycle of Ethanol Fermentation Broth

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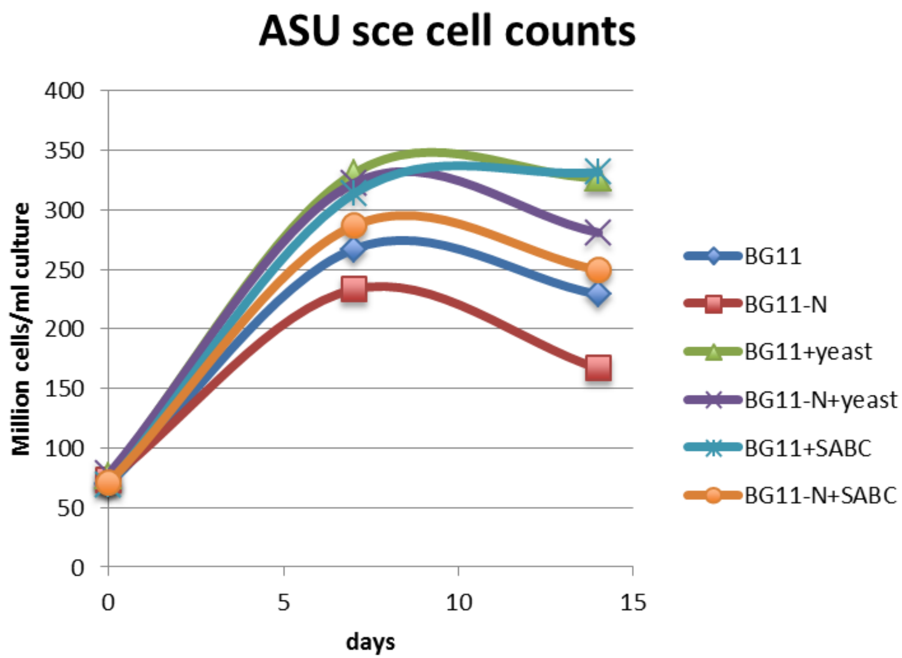
Processing of biomass residues to enable algal growth

- Scenedesmus residues after lipid extraction and fermentation are heavily pigmented and contained inhibitors of new culture growth.
- Commercial polymer resins effectively removed growth inhibitors, resulting is a clarified extract with minimal loss of nitrogen content.
- Medium supplemented with treated algal residues supports the growth of SABC *Scenedesmus sp.*, as effectively as commercial yeast extracts.



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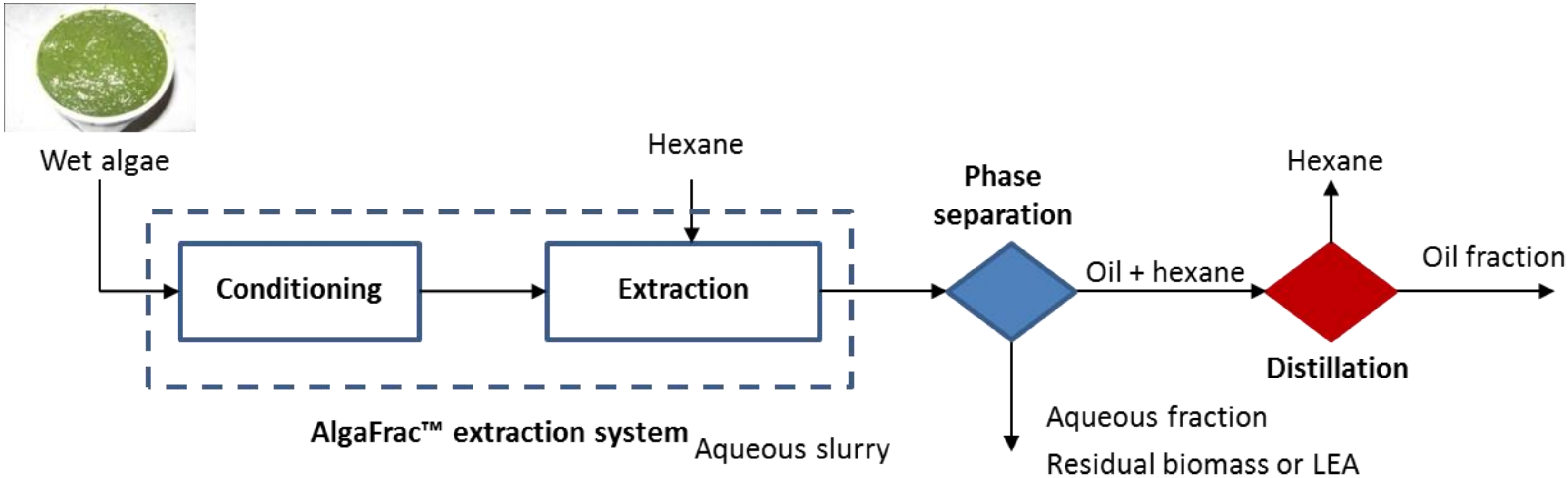
Task 2 Product Performance of Fuels and Blend Components

Outcomes:

- Large scale biomass processed by proprietary Valicor technology to provide lipid streams for upgrading
- Lipids hydrotreated and hydroisomerized by EFT for fuel testing. Impurities inhibited latter step but problem mitigated by distillation.
- NREL evaluated algal hydrocarbons, biodiesel and biobutanol fit-for purpose properties.

Valicor Renewables Pilot Scale Processing

- Algae processed (6-7 kg AFDW basis)
  - *Chlorella* sp.
  - *Nannochloropsis* sp.
  - *Scenedesmus* sp.
- Provided oil to EFT for processing



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Characterization of Algal Oils

SABC-*Chlorella*	Wt% of total fatty acids		
	I	**II**	**I/II**
C16:0 Palmitic	21.4	25.3	0.85
C16:2 Hexadecadienoic	7.7	7.2	1.07
C18:1-cis Oleic	26.7	28.9	0.92
C18:2 Linoleic	25.4	20.8	1.22
C18:3 Linolenic	13.6	9.8	1.39
	94.8	**92.0**	**1.03**

SABC-*Scenedesmus*	Wt% of total fatty acids		
	I	**II**	**I/II**
C16:0 Palmitic	14.0	12.7	1.10
C16:1n7 Palmitoleic	4.3	4.0	1.08
C18:1-cis Oleic	54.7	54.7	1.00
C18:2 Linoleic	8.1	6.7	1.21
C18:3 Linolenic	10.1	9.2	1.10
	91.2	**87.3**	**1.04**

%	[redacted]	
C	77.97	77.85
H	11.74	12.10
N	0.04	0.03
O	11.03	10.57
S	0.01	0.01
Ash	< 0.1	< 0.1

ppm	[redacted]	
Al	0.3	0.8
As	<0.1	<0.1
Ba	<0.1	<0.1
Ca	0.9	3.0
Cr	<0.1	<0.1
Cu	1.8	4.0
Fe	<2	10
Mg	0.2	1.2
Mn	<0.1	0.1
Mo	<0.01	<0.01
P	4	4
K	<1	1
Na	3	10
Ti	0.4	0.5
V	<0.1	<0.1
Zn	0.3	2.6

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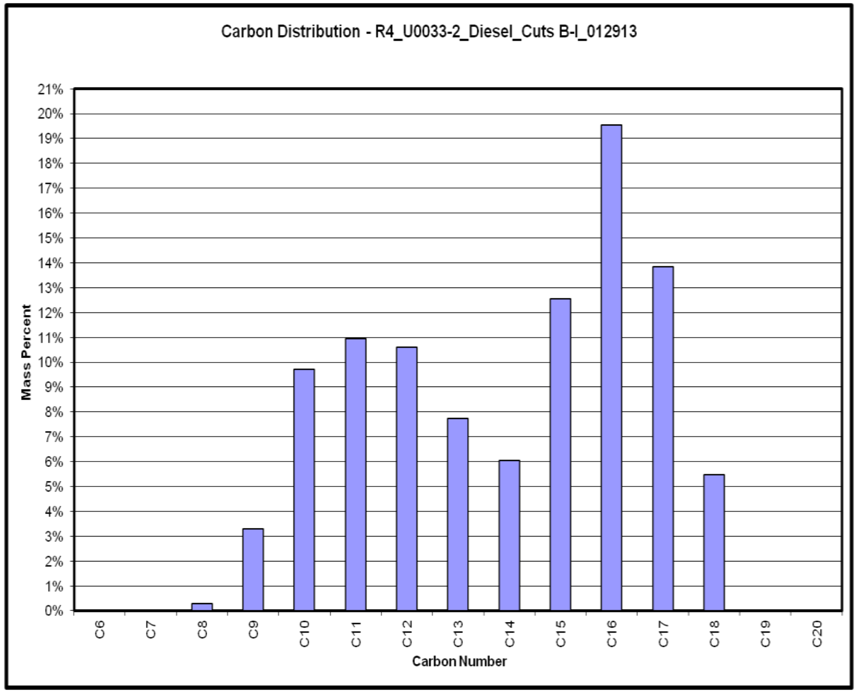
Catalytic Upgrading

1. Catalytically convert the raw algae oil to a hydro-deoxygenated ("HDO") product. Process involves cleaving the fatty acid components from the triglyceride backbone and removing oxygen from the fatty acids as water to generate a generally paraffinic product.
2. Process the "HDO" product generated in step 1 using a catalyst system that will both crack and isomerize a portion of the paraffins in the HDO product into a diesel range base stock. This process improves the HDO characteristics to meet ASTM D-975 specifications.
3. Distill the product generated in step 2 to a final product meeting the ASTM D-975 carbon distribution requirement as defined by the required distillation specifications.

Catalytic Upgrading



Carbon #	Mass%	Isomer Mass%	Normal Mass%	Iso/Normal Ratio by Carbon #
C6	0.0%	0.0%	0.0%	
C7	0.0%	0.0%	0.0%	
C8	0.3%	0.2%	0.1%	2.65
C9	3.3%	2.7%	0.6%	4.76
C10	9.7%	8.4%	1.3%	6.58
C11	10.9%	9.3%	1.7%	5.59
C12	10.6%	8.5%	2.1%	4.09
C13	7.7%	6.3%	1.4%	4.48
C14	6.0%	4.5%	1.5%	2.96
C15	12.6%	8.6%	3.9%	2.19
C16	19.5%	11.1%	8.5%	1.31
C17	13.8%	11.3%	2.5%	4.53
C18	5.5%	4.2%	1.3%	3.20
C19	0.0%	0.0%	0.0%	
C20	0.0%	0.0%	0.0%	
Totals	100.0%	75.2%	24.8%	

- Lipid fractions from *Scenedesmus and Chlorella* all were effectively upgraded to hydrocarbons.
- Hydroisomerization was not successful. Nitrogen-containing contaminant considered to be problem

- Hydroisomerization catalyst poison was removed by distillation in first trial
- Distillation was not successful in removing catalyst poison in second trial

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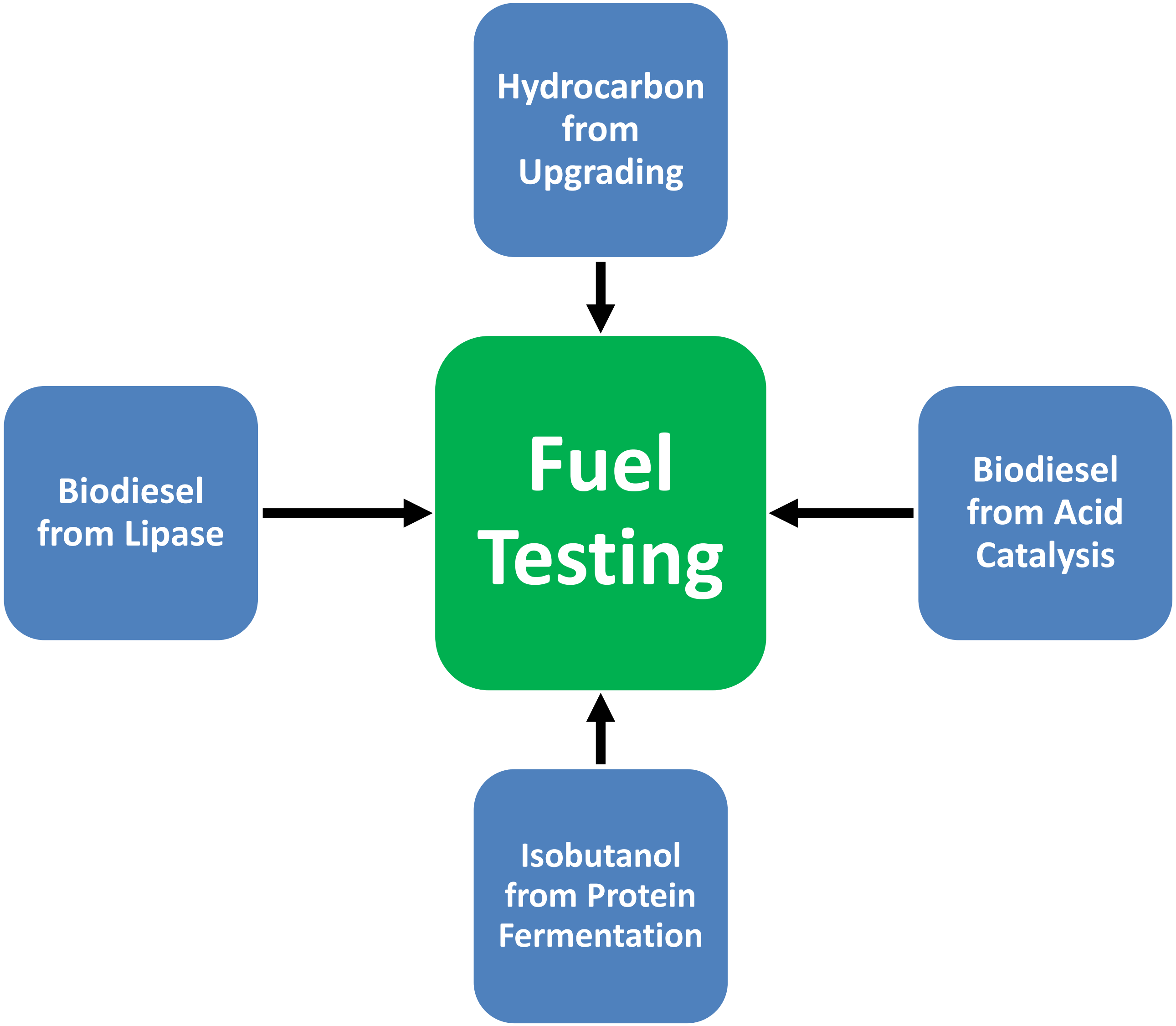
Fuel Property Testing



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Product Performance of Algal Biofuels

- FAME Biodiesel by acid esterification
  - *Scenedesumus* sp. lipid fraction
  - Product had cetane number of 58 and cloud point of 0.6 C.
  - Very low glycerin and glycerides
  - High total acid number
  - Could meet spec for ASTM D6751 with lower acid content
- FAME Biodiesel by lipase transesterification
  - Lipase catalyzed transesterification with methanol
  - 32% saturated FAME, 51% C18:1
  - Very low poly-unsaturates (expect good intrinsic stability)
  - Glycerin and glycerides very low, consistent with vacuum distillation
  - Cloud point 16°C (similar to palm oil FAME)
  - Predicted cetane number of 65 (based on iodine value of 47)

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Product Performance of Algal Biofuels

- Deoxygenated diesel blendstock
  - *Scenedesumus* sp. lipid fraction hydrogenated to remove oxygen
  - Product had cetane number of 110, but cloud point of 17°C.
  - High cloud point limits the market for this material
  - May be marketable as a blend component for increasing cetane of low cetane refinery streams
- Hydrocarbon renewable diesel
  - Deoxygenated and isomerized products produced from *Scenedesumus* sp. and *Chlorella* sp. lipid fractions were combined
  - This hydrocarbon material met the requirements of ASTM D975 with a cetane number of 69 and cloud point of -22°C

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Product Performance of Algal Biofuels

Biobutanol gasoline blendstock

- Alcohol mixture tested as 17% (2.7% oxygen) blend in conventional gasoline
- Relatively low octane number because of low octane pentanol and hexanol components
- Phenylethanol is not currently a legal gasoline component
- Future work should focus on production of isobutanol and 2-butanol or isolation of other alcohols as value added products

Alcohol	% vol
Isobutanol	14
2-butanol	33
1-pentanol	22
2-methyl-1-pentanol	14
2-phenylethanol	16

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Relevance

- **Relevance**:
  - Provides basis for integrated algal biorefinery with broad biofuel portfolio
- **Approach**:
  - Multiple biomass samples provided opportunity to explore biofuel opportunities from all major algal components
  - Improvement of current compositional analysis methods as well as development of game-changing real-time methods for evaluation of productivity in growing cultures
  - Pretreatment steps eliminate need for drying biomass and offer lower cost and lower energy route to lipid extraction and conversion
  - Fractionation and development of biofuel routes based on lipids, sugars, and proteins can expand total yields and reduce production costs
  - Enzyme discovery supplemented commercial enzyme evaluation to facilitate conversion with heretofore unexplored biomass components
  - Fuel conversion and testing provided assurance that work is relevant

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Critical Success Factors

- Establishment of robust biomass production system with multiple productive strains facilitated work on downstream processing
- Management structure provided mechanism to track progress and to identify and overcome hurdles.
- Well integrated team allowed for seamless handoff of materials and information with academic, national lab, and industrial partners
- Familiarity with techno-economic modeling provided guidance for experimental priorities
- Flexible project plan allowed for both discovery and process research but focus on overall goals maintained emphasis on high payoff activities
- Project leveraged substantial experience set of all partners
- Establishment of high standards for analysis enhanced confidence in value of data.

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Critical Success Factors (cont.)

- Expand strain/biomass sampling to understand breadth of production opportunity
- Improve analytical methods to better track proteins and carbs
- Fill in gaps in pretreatment/fractionation/conversion table to confirm utility of this method in integrated experiment
- Improve protein upgrading process for enhanced yields and product distribution
- Gain detailed knowledge of relationships between biomass characteristics and lipid extract quality and between lipid extract quality and catalytic upgrading efficiency.

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Future Work

Project has officially ended. SABC fractionation/conversion process concept is centerpiece for proposal to ABY FOA.

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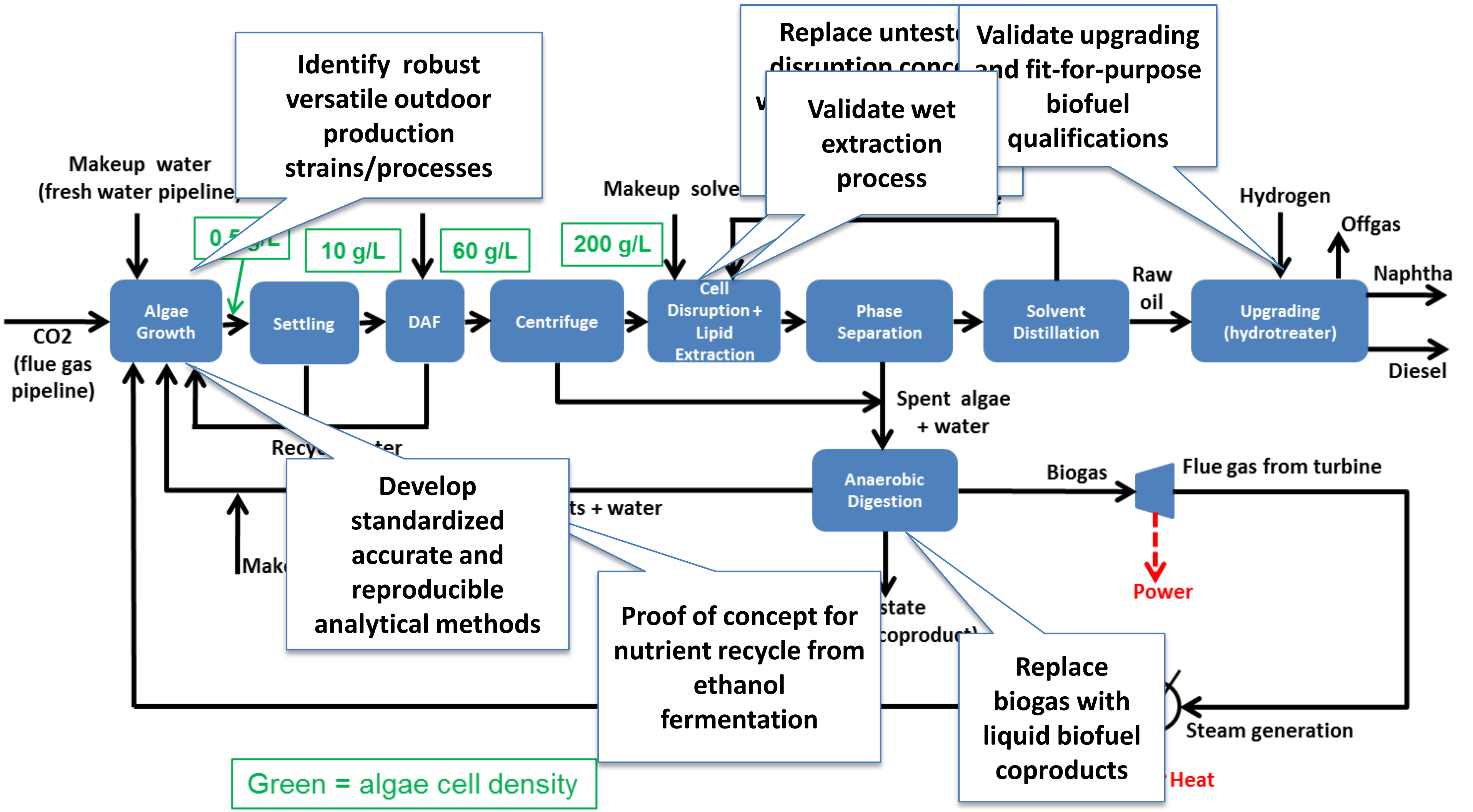
Summary: Impact of Work On Critical Path Elements



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Summary: Algal Biofuel Potential Based on Yield Data

Theoretical Yields	HP SD	HC SD	HL SD	HP CZ	HC CZ	HL CZ
Total Carbohydrates	220	435	356	112	378	246
Glucose/Mannose	190	420	343	52	333	214
Ethanol	97	214	175	27	170	109
Ethanol (gallon)	32	72	59	9	57	36
Gasoline equivalent (gallon)	21	47	39	6	37	24
Btu equivalent (x10e3)	2478	5481	4476	678	4344	2787
Total FAMEs	60	240	371	118	200	367
Hydrocarbon	47	187	289	92	156	286
Diesel equivalent (gallon)	16	64	99	31	53	98
Btu equivalent (x10e3)	1959	7865	12139	3858	6559	12021
Amino Acids	266	69	60	304	104	82
Butanol	106	28	24	122	42	33
Butanol (gallon)	34	9	8	39	13	11
Gasoline equivalent						
Btu equivalent (x10e3)	3425	890	775	3914	1341	1057
Total fuel energy (x10e3 Btu)	**7861**	**14236**	**17390**	**8451**	**12244**	**15865**
Total GGE/short ton	**68**	**123**	**150**	**73**	**105**	**137**

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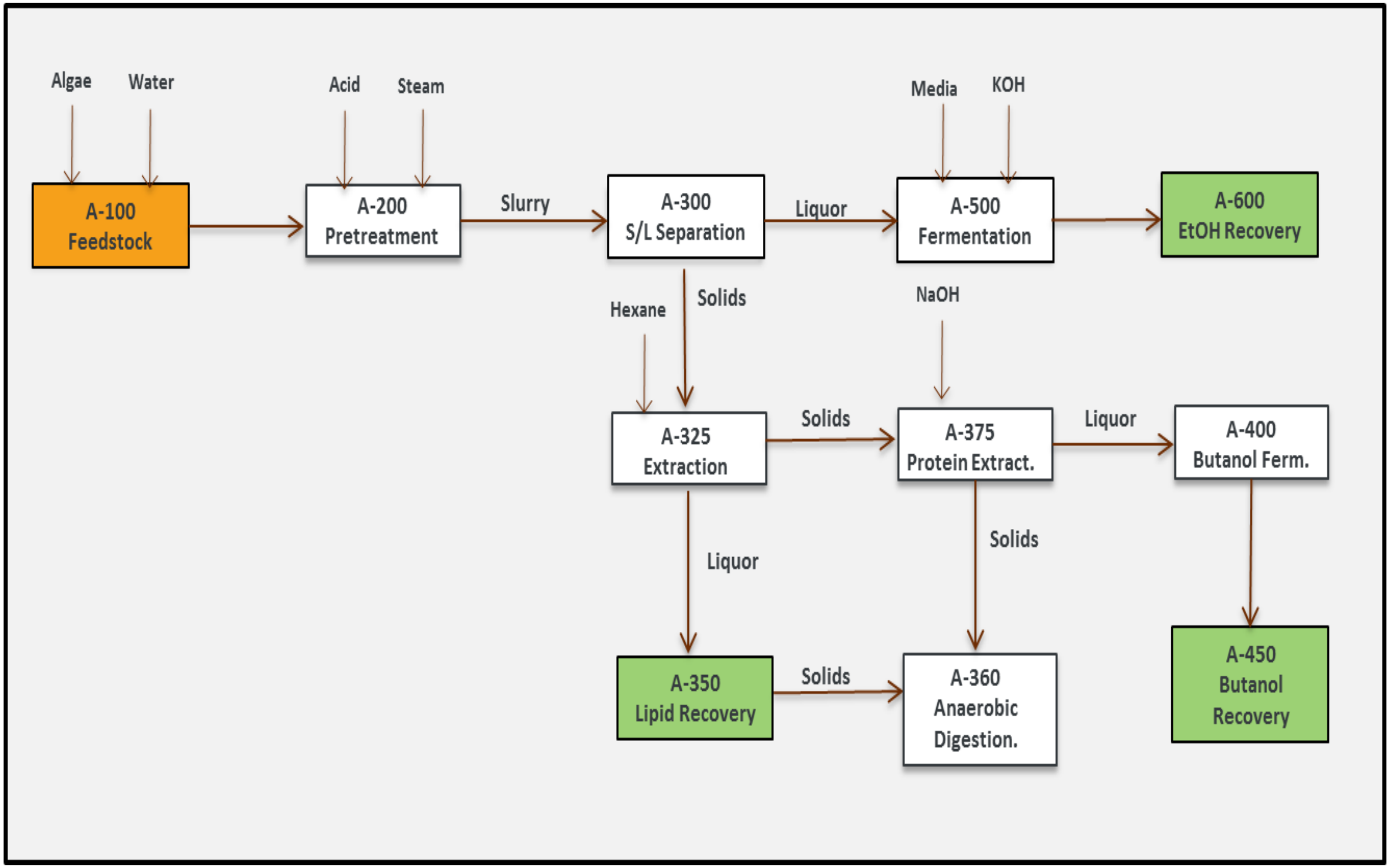
Summary: PFD for SABC Integrated Bioprocess



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Acknowledgements

U.S. DEPARTMENT OF ENERGY

ASU

NREL
NATIONAL RENEWABLE ENERGY LABORATORY

Sandia National Laboratories

Valicor Renewables

School of Mines Colorado 1874

GT

Emerging Fuels Technology

Additional Materials

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Responses to Previous Reviewers' Comments

Many of the reviewers' comments reflected the early stage of the project in 2011, but were complimentary of the project focus and planning. Here we will respond to three relevant critical comments:

Comment 1: Specific quantitative goals for the conversion processes should be defined and expressed.

Response 1: Once we understood the potential value of all three components for biofuel production pathways, we established yields on carbon input as our primary quantitative goal and gallons gasoline equivalent (GGE) as our combined goal. While we were unable to do much more than establish a baseline for all conversion processes, we could use these values to establish potential for this concept.

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Responses to Previous Reviewers' Comments

Comment 2: Growing biomass appears to be a bottleneck. Partnership with facility that can supply kg quantities might be valuable.

Response 2: Supply of well-characterized algal biomass is a constant issue for large scale experimental plans. That could have been especially true in this project where biomass production was not specifically called out in the funding process. However, ASU's knowledge of the production strains and their expertise and capacity at outdoor cultivation meant that reproducible multi-kg batches of high protein, high carbohydrate, and high lipid biomass were delivered to partners in a timely fashion.

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Responses to Previous Reviewers' Comments

Comment 3: Work on converting FAME, FFA, and sugars to fuels is not relevant to overcoming hurdles to algal biofuel commercialization. Algal biofuels will not fail because of fuel conversion process viability

Response 3: Although conversion of lipids to finished fuels has been demonstrated at scale, the details are not available to the public and technology development will be enhanced by publically available data. Furthermore, overall process viability will only be achieved with significant cost reduction. Though the additional biofuels that can be obtained from the sugars and protein fractions do not bring the high value of foods or nutraceuticals, they are scalable with the lipid based biofuels and they match the demand for low cost/low quality inputs needed for process economics. Initial TEA evaluation indicates that this approach can provide significant cost reductions over basecase.

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Responses to Previous Reviewers' Comments

Comment 4: Project aims to develop a site of rapid, accurate, and reproducible analytical methods for determination of algal biomass composition that can be applied to whole cell biomass as well as fractions generated by processing of the biomass (and) to apply optimization of biomass pretreatment, including enzymatic hydrolysis and conversion operations of the biomass with integration into a single, efficient process. These items are a tall order to be achieved in this short period of time.

Response 4: We believe that we have succeeded in achieving most if not all of our goals, though we have by no means completed the process of analytical method or process optimization, but rather, have established a new set of standards for future work. Not all of our approaches paid off, but our downselect process was robust , allowing us to focus the bulk of our efforts on high value outcomes.

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Publications and Presentations

- Lieve M.L. Laurens[*], Thomas A. Dempster, Howland D. T. Jones, Edward J. Wolfrum, Stefanie Van Wychen, Jordan S. P. McAllister, Michelle Rencenberger, Kylea J. Parchert and Lindsey M. Gloe (2012). Algal Biomass Constituent Analysis: Method Uncertainties and Investigation of the Underlying Measuring Chemistries. *Analytical Chemistry* 84: 1879-1887.
- Yoon, K. Han, D. and Li, Y. Sommerfeld, M. & Hu, Q. (2012) Phospholipid:diacylglycerol acyltransferase is a multifunctional enzyme involved in membrane lipid turnover and degradation while synthesizing triacylglycerol in the unicellular green microalga *Chlamydomonas reinhardtii. The Plant Cell* 24: 3708–3724.
- Peter E. Zemke, Milton R. Sommerfeld, & Qiang Hu (2013) Assessment of key biological and engineering design parameters for enhanced production of *Chlorella* sp. (Chlorophycease) in an outdoor photobioreactor. *Applied Microbiology and Biotechnology* (in press)
- Li, Y., Han, D., Yoon, K., Zhu, S. M. Sommerfeld & Hu, Q. (2013) Molecular and cellular mechanisms for lipid synthesis in microalgae and biotechnological implications, pp. 545-565. In Richmond and Hu (eds.) *Handbook of Microalgal Culture*, 2nd edition, Wiley-Blackwell, West Sussex, UK.
- Li, Y. Han, D. Sommerfeld, M. & Hu, Q. (2013) DGAT2, a gene encoding type-2 diacylglycerol acyltransferase, is involved in hyper-accumulation of triacylglycerol in a *Chlamydomonas reinhardtii* starchless mutant. (submitted)
  - Provisional Patent Filed (2011) “Novel multi-functional enzyme with very high conversion efficiency and broad substrate specificity” Qiang Hu, Kangsup Yoon, and Milton Sommerfeld Inventors. AzTE Case # M11-087L

Publications and Presentations

- Han, D., Yoon, K, Li, Y., Sommerfeld M. & Hu, Q.† (2013) Cloning and characterization of an Acyl-CoA:Glycerol-3-phosphate acyltransferase in *Chlamydomonas reinhardtii.* (submitted)
- Wei Chen, Sarah Arrowsmith, Milton Sommerfeld & Qiang Hu (2013) An improved phenol sulfuric acid-based method for quantitative measurement of carbohydrate in microalgae. (in preparation)
- Danxiang Han, Jing Jia, Jian Xu, Milton Sommerfeld & Qiang Hu (2013) Global Analysis of Glycerolipids of the Oleaginous Microalga *Nannochloropsis oceanica* by Electrospray Mass Spectrometry. (in preparation).
- Yongsheng Chen, Wei Zhang, Wen Zhang, Xuezhi Zhang, Qiang Hu, Milton Sommerfeld, Pasquale Amendola. Identification and Characterization of Ultrafiltration Membrane Fouling for Algal Biomass Harvesting" (in preparation and to be submitted to *Algal Research).*
- "Physiological variability of composition of algal biomass as feedstocks for carbohydrate and lipid-based fuels" [NREL and ASU authors]
- "Characterization of structural and chemical changes of biomass during pretreatment" [NREL and Sandia authors]
- "Algal biomass pretreatment for simultaneous lipid and carbohydrate-based biofuel production" [NREL authors]
- John McGowen, Jordan P. McAllister, Stefanie Van Wychen, Lieve M. L. Laurens, Thomas A. Dempster, Sarah Arrowsmith, David Cardello, and Theresa Belisle. Biomass Yield and Biochemical Composition of Algal Biomass Over the Course of Nutrient Depletion in a Pilot Production Photobioreactor Array (in preparation).

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Publications and Presentations

Extractability

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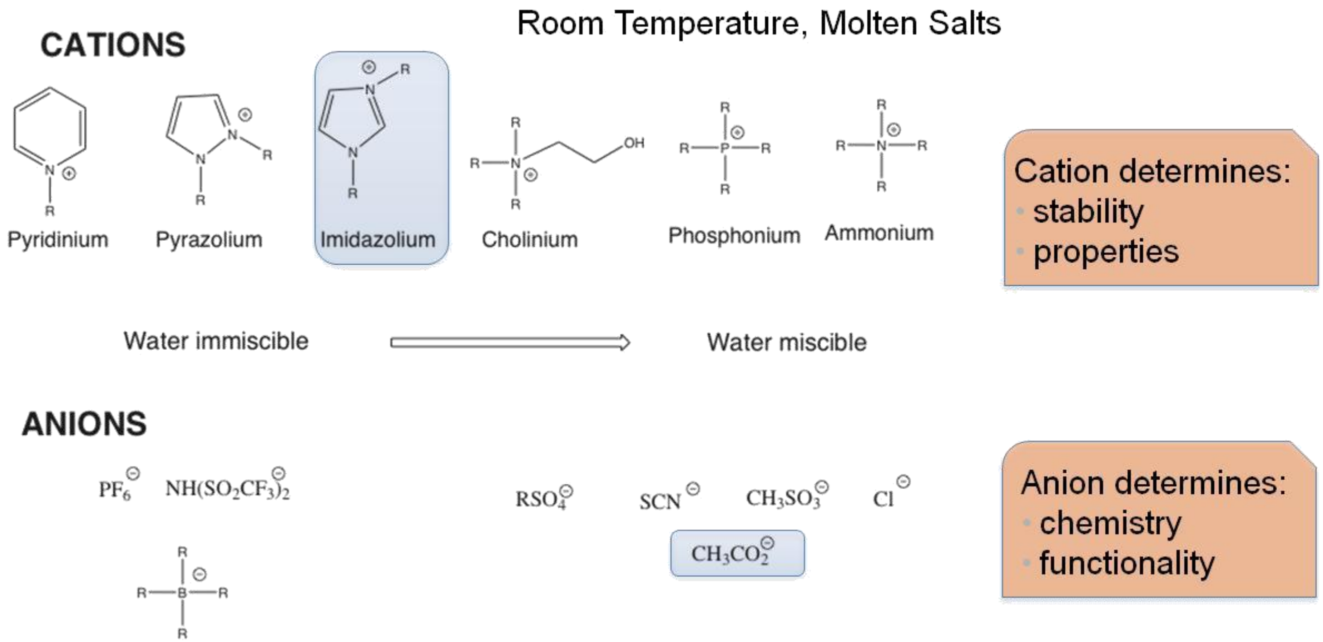
- Main biochemical difference between CZ and SD is the amount of starch present
- Stable, non-separable starch-oil composites can be formed at conversion temperatures[1]
- Composites of starch and oil have oils embedded in microdroplets of between 1-10 µm diameter
- ‘Embedding’ of the oil droplets in starch-composites could explain why the CZ oils are less extractable than SD oils
- Spatial and chemical characterization of aggregates
  - Oil composition
  - Possible microdroplet location

[1] Eskins et al., (1994) US Patent 08233174 | Eskins et al., (1996) Carbohydrate Polymers, 29:233-239

Ionic Liquids



e.g., Emim (1-ethyl-3-methyl imidazolium) acetate dissolves > 20 wt% cellulose

- Salts that are liquid rather than crystalline at near room temperature
- Cause swelling of cell walls and breakdown of lignocellulosic matrix
- Can hydrolyze carbohydrates to monomeric sugars with minimal toxic product formation
- Can lead to separation of biomass polymers with appropriate anti-solvent

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Ionic Liquid Pretreatment

Emim Ac Biomass

Mix

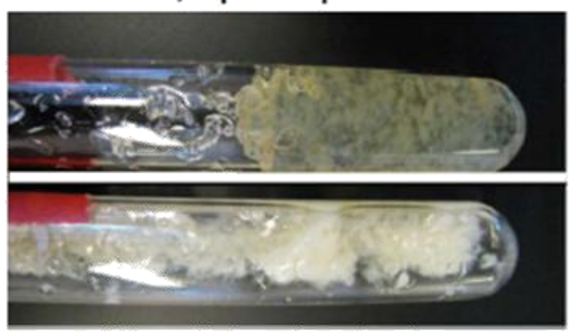
Heat
Stir

Heat
Time

Solubilized biomass

Anti
Solvent

Top: traditional anti-solvent
- Forms gel
- Solid/liquid separation difficult



Bottom: novel anti-solvent (JBEI IP)
- No gel
- Filters easily

Filter
Dry

Recovered product:
- High surface area
- Amorphous cellulose
- Decreased lignin

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Impact of Process Sequence

Extraction >Pretreatment *Chlorella* sp.

g/kg biomass	FAME	Glucose	Total
High Protein CZ	19	22	41
High Carb CZ	3	264	267
High Lipid CZ	13	155	169

Pretreatment > Extraction *Chlorella* sp.

g/kg biomass	FAME	Glucose	Total
High Protein CZ	42	30	72
High Carb CZ	42	280	321
High Lipid CZ	180	167	346

Pretreatment before extraction:

-> Increase in lipid extraction efficiency up to 10-fold

-> No loss in glucose release

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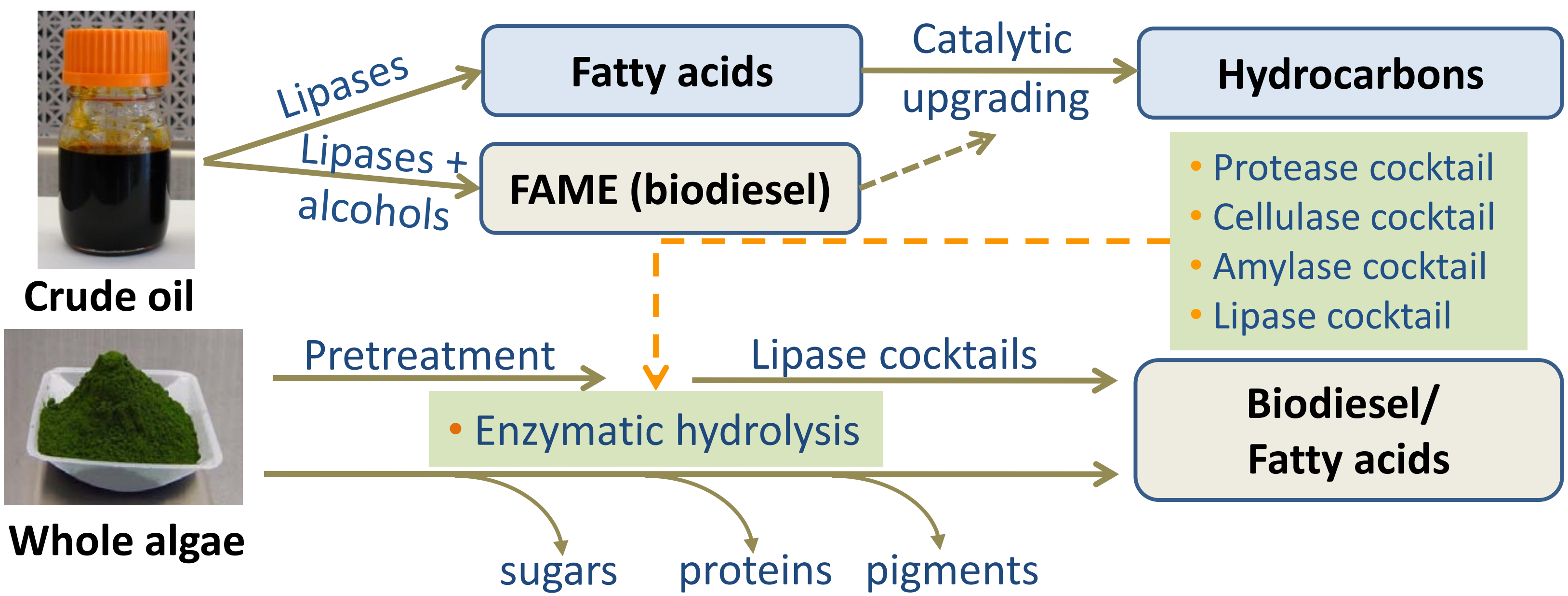
Lipase-/Protease-Based Biochemical Conversion

Expected Outcome: Discover and develop algal-specific hydrolytic enzymes for lipids, carbohydrates and protein complexes and develop new pathways for industrial algal deconstruction.



Approaches

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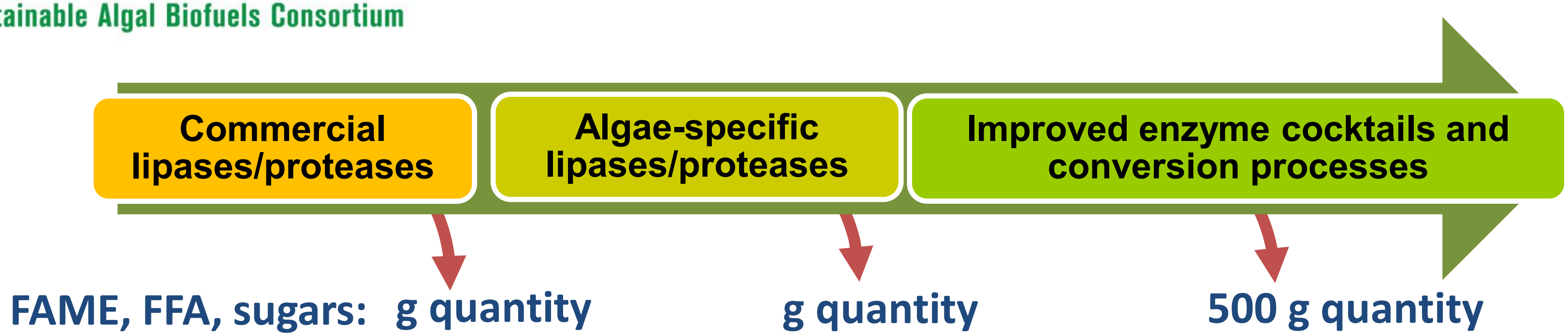

- Evaluation of commercial enzymes Screen and selection of commercial lipases and proteases
  - Evaluation of commercial lipase and protease cocktails
- Bioprospecting for novel enzyme systems Screen and selection of algae-derived lipases and proteases
  - Production and evaluation of promising enzymes
  - Cloning and characterization of selected genes/enzymes
  - Production of the enzymes in a host system
  - Evaluation of recombinant enzymes with crude algal oils and algae cells
- Process development and production of fuels and fuel intermediates
  - Optimization of enzyme combinations and reaction conditions
  - Preparation of FAME/FFA/sugars for fuel conversion and testing

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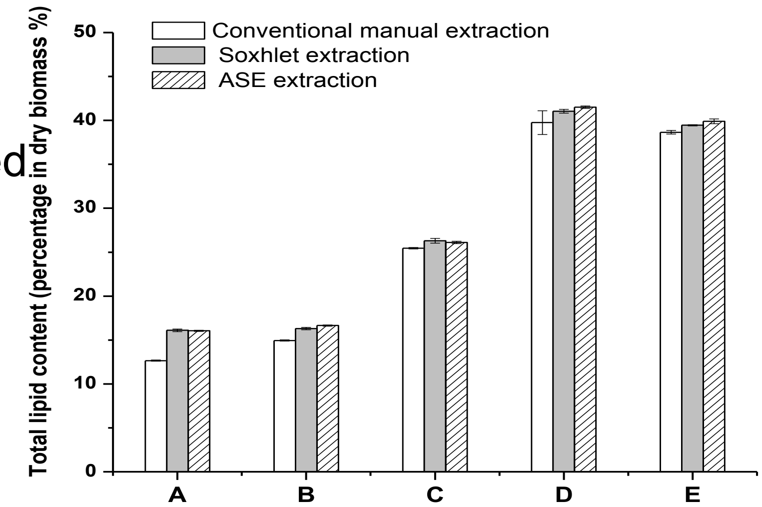
Lipase Based Conversion of Lipids to FAME

Method development

- ASE method for total lipid analysis was developed.
- Internal standard quantification method for the determination of FAME content was developed .
- Methods for determination of FAME and FFA recovery rates were developed.



A) *Chlorella sp*. (reference material)
B) SABC-P12 *Pseudochlorococcum Sp*.
C) LRB-3100 *Isochrysis galbana*
D) LRB-1201 *Chlorella zofingiensis*
E) SABC-P6 *Nannochloropsis Sp*.

Preparation of *g* quantity FAME

- 50g of FAME were prepared for testing
- Fractionation protocols were developed

Evaluation and development of pretreatment protocols for lipase conversion of whole biomass into biodiesel

- Pretreatment of dry algal biomass and lipase conversion
- Pretreatment of wet algal biomass and lipase conversion