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Academic Background

2000 B.E.: Environmental Engineering, Da Yeh University

2002 M.E.: Chemical Engineering, National Tsing Hua University

2006 Ph. D.: Chemical Engineering, National Tsing Hua University

Professional Career

2007 - present Research Scientist, Department of Advance Fiber & Biomass Material Research, ITRI

2006 – 2007 Visiting Scholar, Biochemistry Center II, University of Cologne, Germany

Research Field:

Environmental Engineering/Biochemical Engineering/Microbiology/Cell Biology





Modulation of Kennedy Pathway Genes for Increasing Storage Lipid in *Chlorella* sp.

Speaker : Hsin-Ju Hsieh, PhD

Date: Aug, 26, 2011

MCL/ITRI

Outline

- **Overview of classification of *Chlorella* and annotation of *Chlorella* genome.**
- **Pathway of CO₂ to triacylglycerol in algae.**
- **Carbon partitioning of lipid, starch, and protein in algae.**
- **Major unresolved questions and future prospects.**

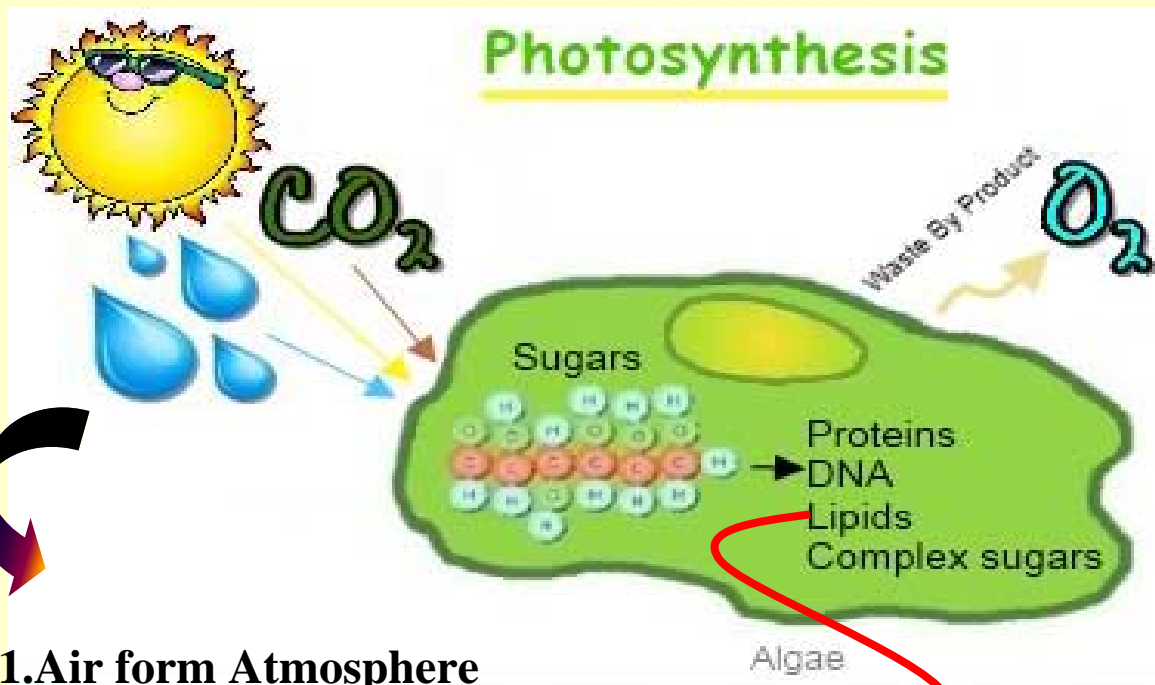
Eukaryotic Microalgae

The exploitation of naturally occurring photosynthetic microalgae, collected and isolated over the past 25 years for production of renewable liquid fuels, initially in collaboration with the Aquatic Species Program of the DOE, and subsequently at Arizona State University, to meet increasing energy needs !!

Comprehensive concepts:

- (1) an extremely diverse group of organisms (are classified into 7 phyla, described species are larger than 40,000).**
- (2) possess novel metabolic features (unique metabolic characteristics such as intracellular lipid storage).**
- (3) a growth potential with an order of magnitude greater than terrestrial crop plants (extraordinarily efficient light and nutrient utilization).**
- (4) the metabolic engineering tools application in photosynthetic microalgae has the potential to create renewable fuel (not compete with food production or arable land).**

Algal Biorefinery



1. Air form Atmosphere

-- Low CO_2 concentration
(0.033% by volume)

2. Emission from Industrial Sources

-- High CO_2 concentration
(coal-burn power plant: 13-15%)
(natural-gas-fired power plant: 8-10%)



Algae Oil
(Carbon-Neutral Oil)



Carotenoids ; Xanthophyll
Chlorophyll a & b & c
Phycocyanin
Phycoerythrin
Fucoxanthin; Peridinin

Soil Conditioners & Agrochemicals

- > Fertilizers
- > Proteins

Fine Chemicals & Bioactive substances

- > Carotenoids
- > Phycobilliproteins
- > Omega 3 & Omega 6 fatty acids
- > Polysaccharides
- > Antioxidants
- > Bactericides
- > Plant growth promoters
- > Proteins and enzymes
- > Medical treatment & Pharmaceuticals

Energy Carriers

- > Biodiesel
- > Hydrocarbons
- > Ethanol
- > Gasoline
- > Methane & Hydrogen

Part I.

- **Overview of classification and annotation of *Chlorella* genome.**
- Pathway of CO₂ to triacylglycerol in algae.
- Carbon partitioning of lipid, starch, and protein in algae.
- Major unresolved questions and future prospects.

Classification of *Chlorella*



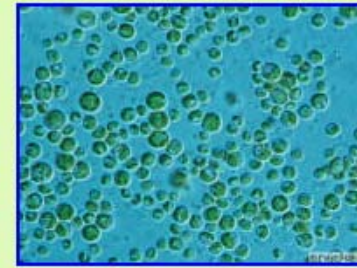
Mychonastes homosphaera
(= *Chlorella homosphaera*)



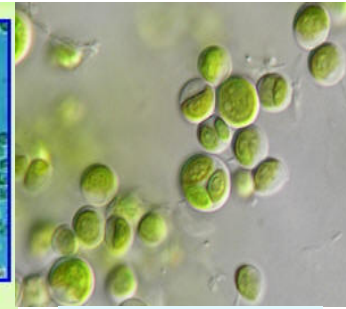
Scenedesmus rubescens
(= *Chl. fusca*, var. *rubescens*)



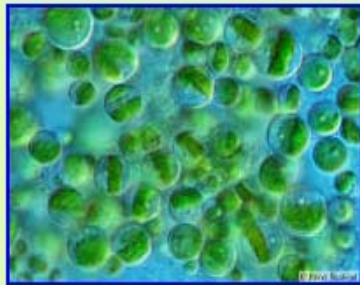
Scenedesmus vacuolatus
(= *Chl. fusca*, var. *vacuolata*)



Chlorella minutissima



Chlorella mirabilis



Chlorella luteoviridis



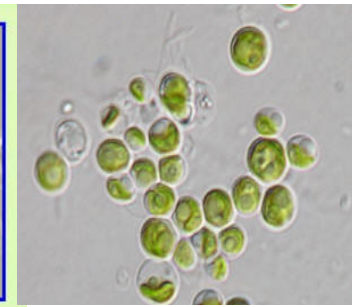
Chlorella lobophora



Chlorella saccharophila



Chlorella vulgaris



Chlorella sorokiniana

- (1) The 6 kingdoms are: *Archaeobacteria*, *Eubacteria*, *Protista*, *Plants*, *Animal*, and *Fungi*.
 (2) Some algae are in the *plantae kingdom* (green algae-plant), but some are also in the *protista kingdom* (green algae-protist).
 (3) Similarities and differences among algal, fungal, and protozoan groups have led scientists to propose major taxonomic changes, and *these changes are continuing* !

Read more: <http://wiki.answers.com/>

Ref: www/botany.natur.cuni.cz/algo/determin.html

Taxonomic Changes of *Chlorella*

H 6201 *Neodesmus pupukensis* (KALINA et PUNČOCHÁŘOVÁ) HEGEWALD et HANAGATA

Syn.: *Kermatia pupukensis* KALINA et PUNČOCHÁŘOVÁ

Ident.: UTEX 2219 (as *Chlorella minutissima*), DSIR 145/79, CCAP 211/52, CCAO 256

Note: Reclassification according to Hegewald & Hanagata (2000, *Algol. Stud.* 100:29-49.)

H 1901 *Parachlorella kessleri* (FOTT et NOVÁKOVÁ) KRIENITZ et al.

Syn.: *Chlorella kessleri* FOTT et NOVÁKOVÁ

Ident.: UTEX 262, CCAP 211/11g, IAM C-208 (all as *Chlorella kessleri*)

Note: Reclassification based on Krienitz et al. (2004)

H 1904 *Chloroidium ellipsoideum* (GERNECK) DARIENKO et al.

Syn.: *Chlorella ellipsoidea* GERNECK, *Chlorella trebouxioides* PUNČOCHÁŘOVÁ

Originally designated as: *Chlorella saccharophila* var. *ellipsoidea*

Ident.: SAG 3.95, LABIK 837, IPPAS C-8

Note: Reclassification based on Darienko et al. (2010) - *Eur. J. Phycol.* 45(1): 79-95

H 1912 *Chloroidium saccharophilum* (KRÜGER) DARIENKO et al.

Syn.: *Chlorothecium saccharophilum* KRÜGER, *Chlorella saccharophila* (KRÜGER) MIGULA

Ident.: SAG 211-9a, UTEX 2469, CCAP 211/9a, ATCC 30408, UTCC 91

Note: Reclassification based on Darienko et al. (2010) - *Eur. J. Phycol.* 45(1): 79-95

H 1983 *Choricystis parasitica* (BRANDT) PRÖSCHOLD et DARIENKO

Bas.: *Zoochlorella parasitica* BRANDT

Note: formerly (until 2010) as *Chlorella sorokiniana*, reclassification according to Pröschold et al. (2010) - *Environmental Microbiology*, *in press*

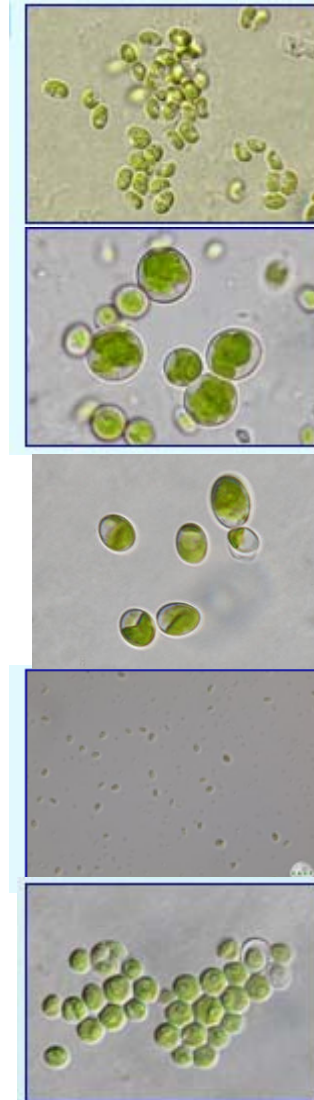
H 1990 *Pseudochlorella pringsheimii* (SHIHIRA et KRAUSS) DARIENKO et al.

Syn.: *Chlorella pringsheimii* SHIHIRA et KRAUSS

Originally designated as: *Chlorella ellipsoidea* GERNECK

Note: Reclassification based on Darienko et al. (2010) - *Eur. J. Phycol.* 45(1): 79-95

Ref: www/botany.natur.cuni.cz/algoc/caup-list.html



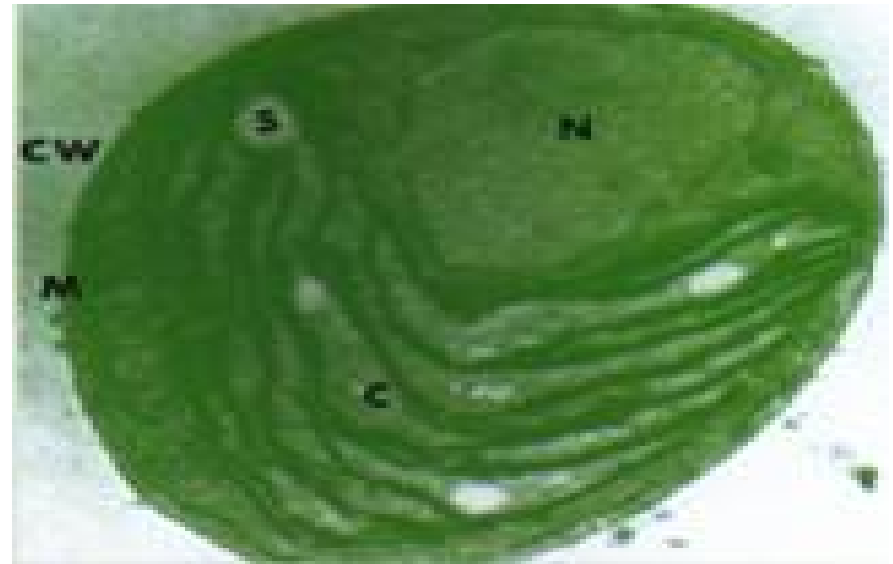
Cell Structure of *Chlorella*



Chlorella Cells

- (1) round cell in of about **6 mm** diameter.
- (2) a form of life that emerged over **2 billion years ago**, coming after photosynthetic bacteria.
- (3) is asexually reproduced.
- (4) second-generation biodiesel thanks to its **high lipid content (20~50 wt%)**.

Ref: <http://protist.i.hosei.ac.jp/>

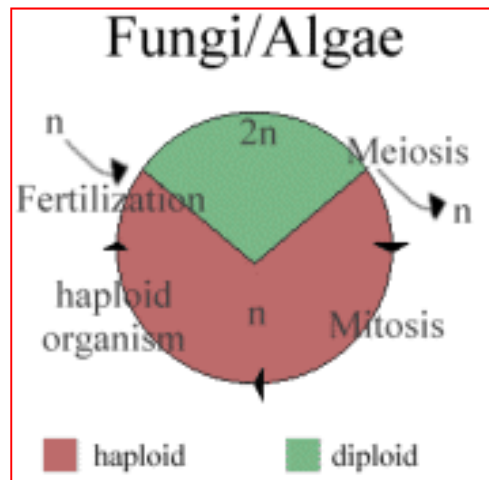


- (CW) which is mostly cellulose
- (N) Nucleus
- (S) Starch grains
- (C) Belt shape chloroplast
- (M) Mitochondria produces the energy

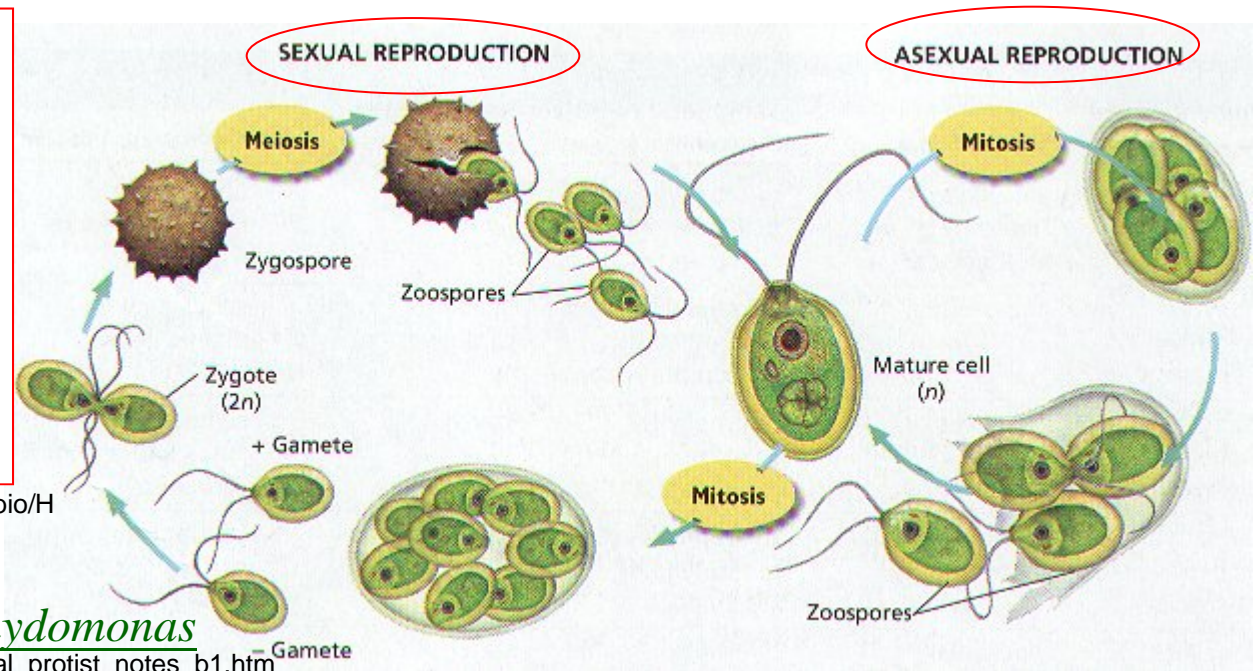
Moisture (5.77%), Crude protein (56.24%),
Crude fat (13.38%), Carbohydrate (16.95%),
Crude fiber (1.02%), Crude ash (6.64%),
Chlorophyll (1.7%), Calorie (417 cal)
-- Saturated FA (14.7%), Unsaturated FA (85.3%)

Annotation of *Chlorella* Genome

- Elucidation of the complete genome of *Chlorella*, has been completed, which is a team work collaborated with format including France (CNRS), American and Japanese laboratories (Sep, 2010). → *Chlorella variabilis* NC64A (*Unicellular Algae*)
- This analysis has brought a **light unexpected finding, which has suggested that *Chlorella* could have a sexual cycle** (which had gone unnoticed so far) and that **a virus probably gave it the capability to synthesize chitin-rich cell wall**, a unique property in algae.



http://www.stmary.ws/highschool/science/apbio/Heredity/types_of_sexual_life_cycles.htm



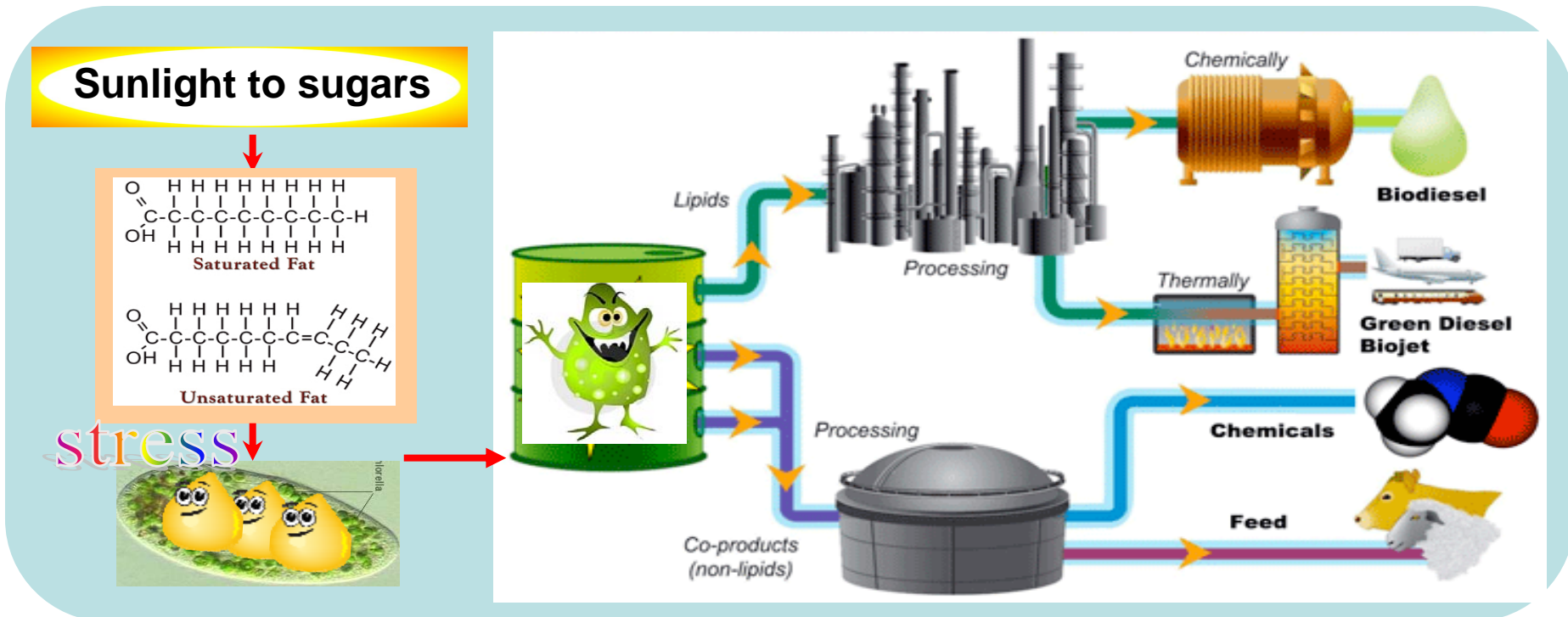
Life Cycle of *Chlamydomonas*

http://www.biologyjunction.com/algal_fungal_protist_notes_b1.htm

Part II.

- Overview of classification and annotation of *Chlorella* genome.
- **Pathway of CO₂ to triacylglycerol in algae.**
- Carbon partitioning of lipid, starch, and protein in algae.
- Major unresolved questions and future prospects.

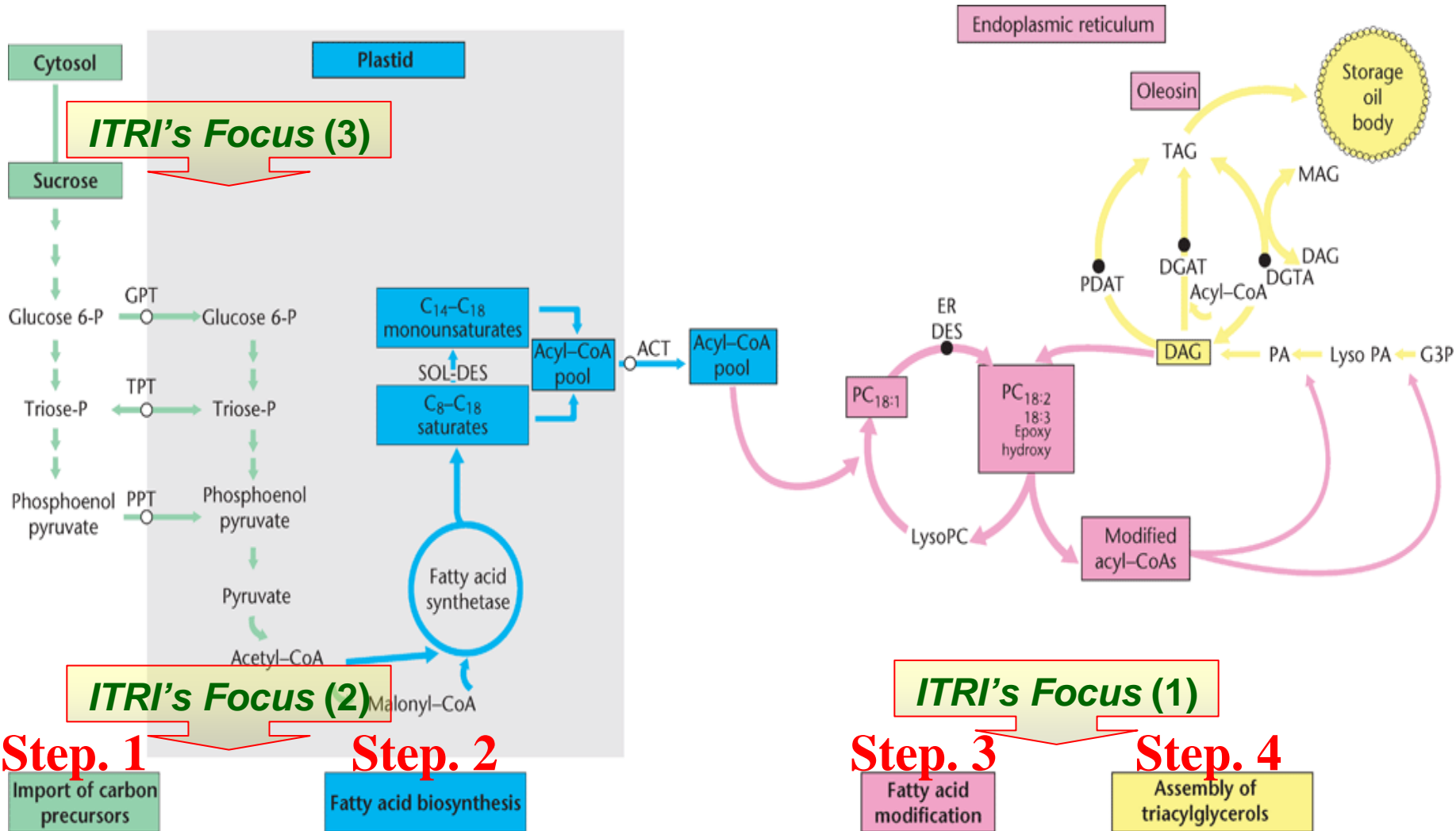
How Algae Make Oil



Algae break down larger sugar molecules two carbons at a time and use them to make fatty acids.

- (1) When algae are over eat or **under stress will limit their growth**, fatty acids are converted to a **storage form as triacylglycerols** by bonding three fatty acid molecules to a three carbon glycerol molecules.
- (2) This **turns the triglycerides into a fat stored as oil.**

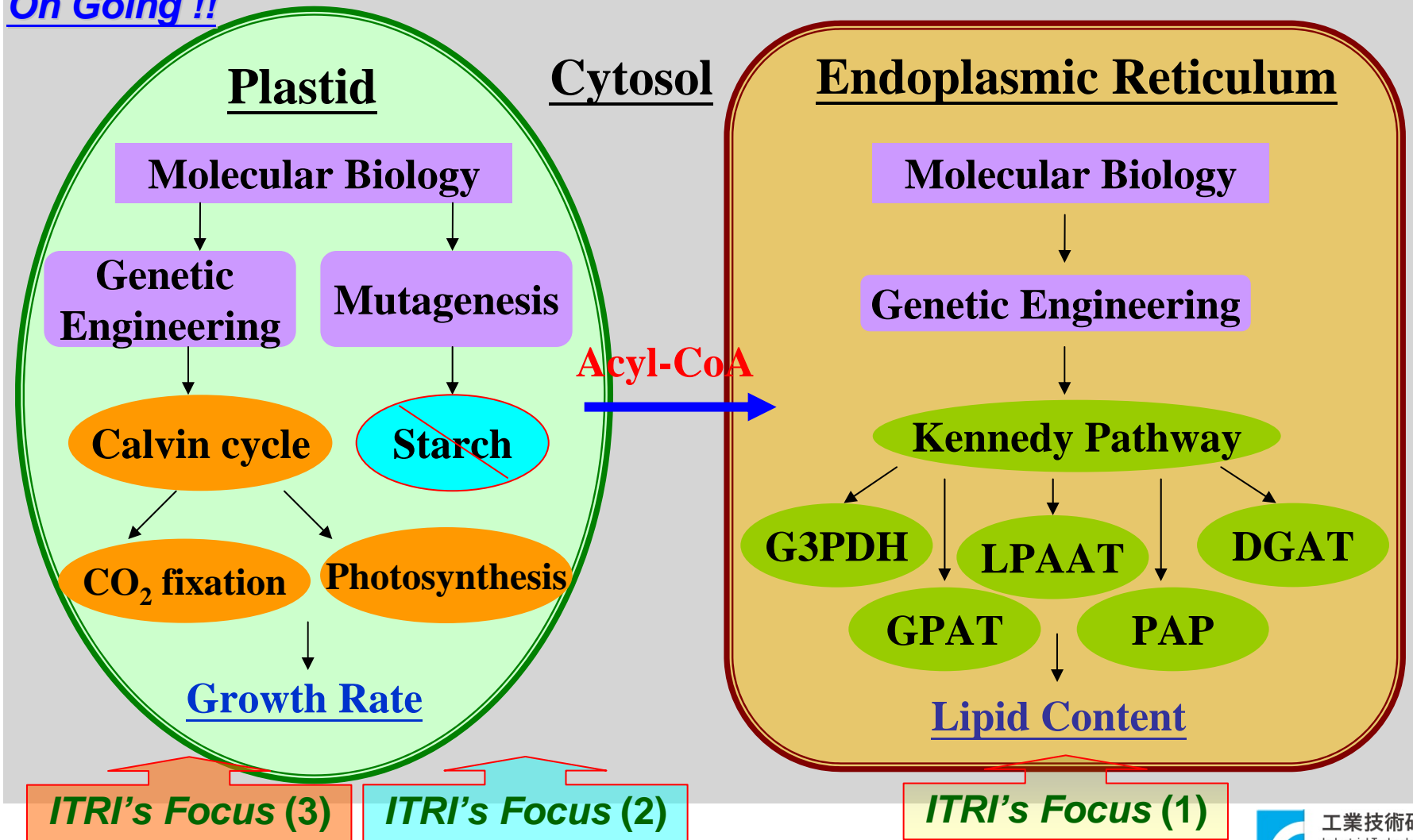
Metabolic Pathways of Microalgal Storage Lipids



Ref: Denis J Murphy, University of Glamorgan, Pontypridd, Wales, UK

Strategy of the Lipid Productivity Increment in ITRI

On Going !!



Modification of Lipid Content in Microalgae

*It is **reasonable** to believe that some of the **strategies** that result in **increased oil seed content in terrestrial plants** may be able to increase the lipid content in **microalgal cells** as well.*

*→ it would be of great benefit to develop **genetic strategies** to increase the cellular **tolerance to a variety of stress factors**.*

ITRI's Focus (1) & (2)

It will be interesting to see how overexpression of lipid synthesis pathway genes and abolish of starch synthesis pathway genes will enhance lipid content of microalgae.

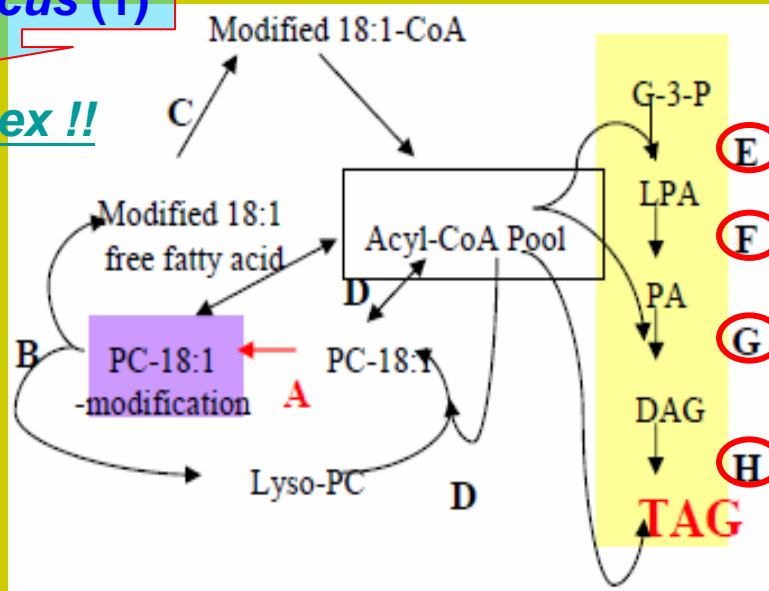
ITRI's Focus (3)

Improve growth capacity through increased stress tolerance or photosynthetic efficiency against the burden of existing **overexpression**.

Cloned Genes Encoding Enzymes in Triacylglycerol Biosynthesis

ITRI's Focus (1)

Complex !!

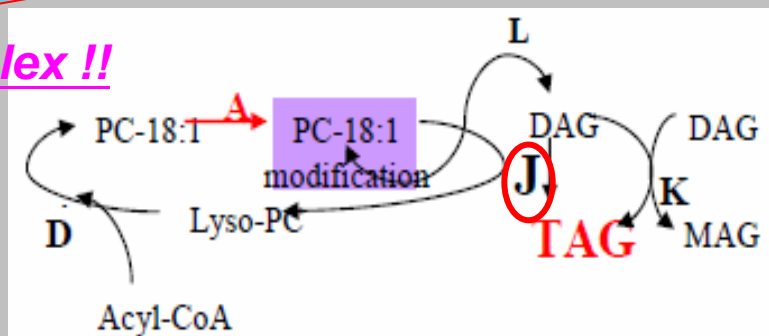


Acyl-CoA dependant pathway.

- A Oleate hydroxylase.
- B Putative phospholipase A2 (PLA2).
- C Acyl-CoA synthase (ACS).
- D Lyso-PC acyltransferase (LPCAT).
- E Glycerol-3-phosphate acyltransferase (GPAT).
- F Lysophosphatidic acid acyltransferase (LPAAT).
- G Phosphatidic acid phosphatase (PAP).
- H Diacylglycerol acyltransferase (DGAT1 and DGAT2)

ITRI's Focus (1)

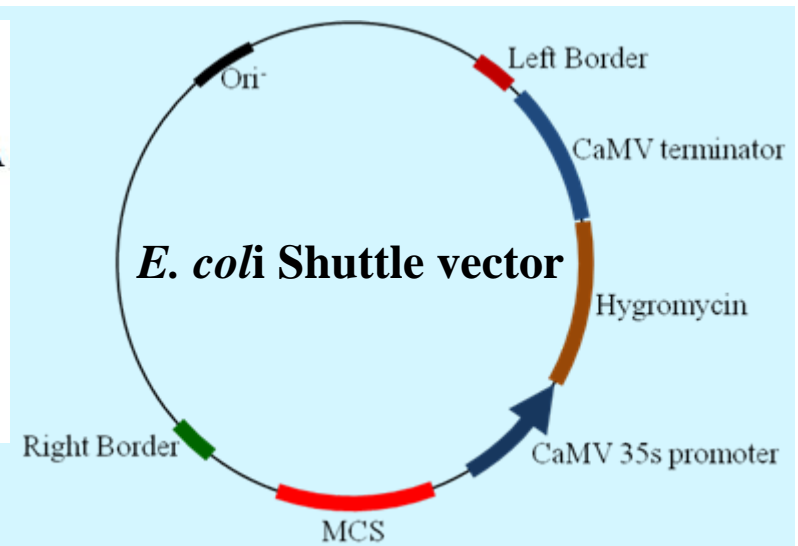
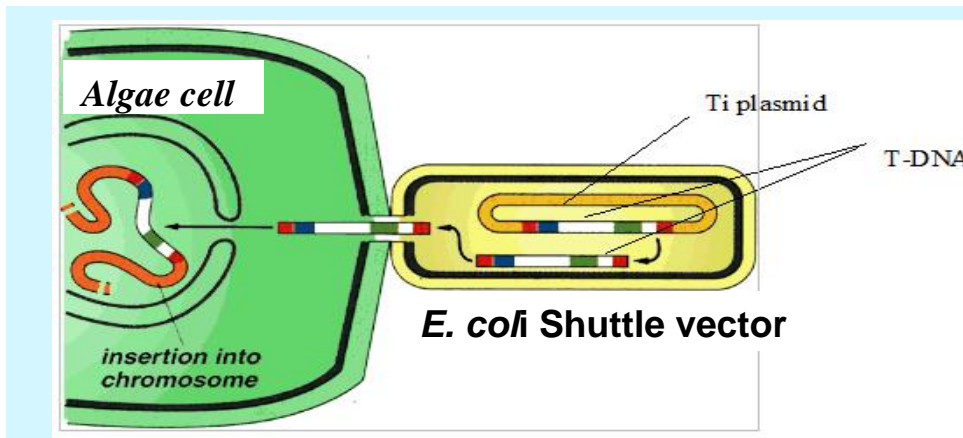
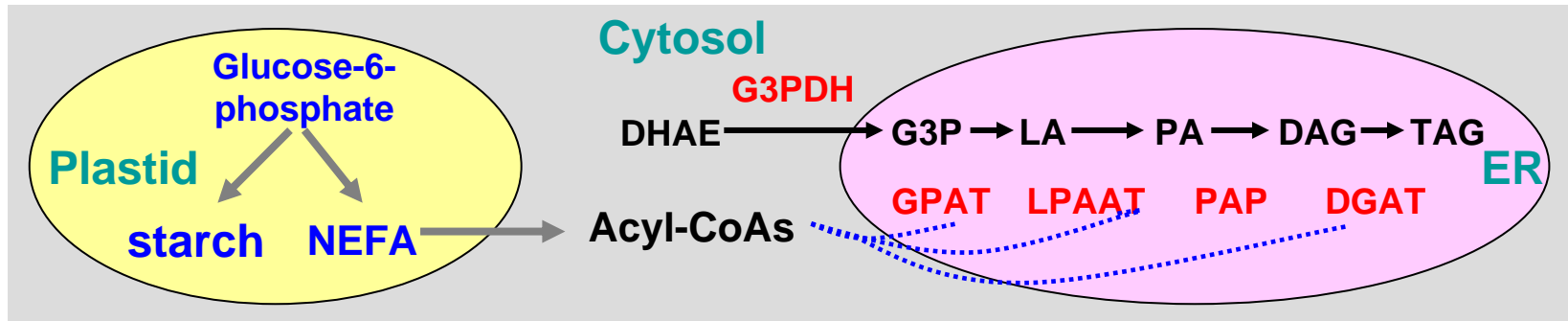
Complex !!



Acyl-CoA independant pathway.

- A Oleate hydroxylase.
- D Lyso-PC acyltransferase (LPCAT).
- J Phospholipid:Diacylglycerol acyltransferase (PDAT).
- K Transacylase.
- L CDP-choline diacylglycerol choline phosphotransferase (CPT)

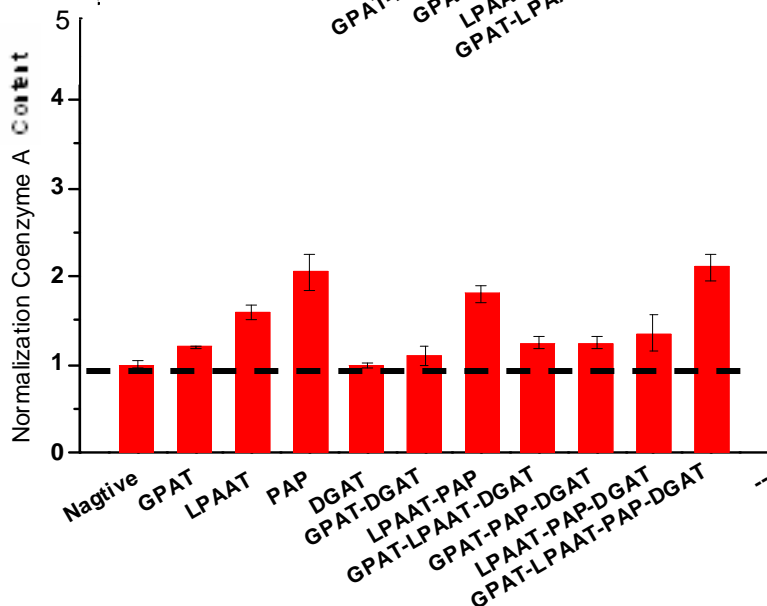
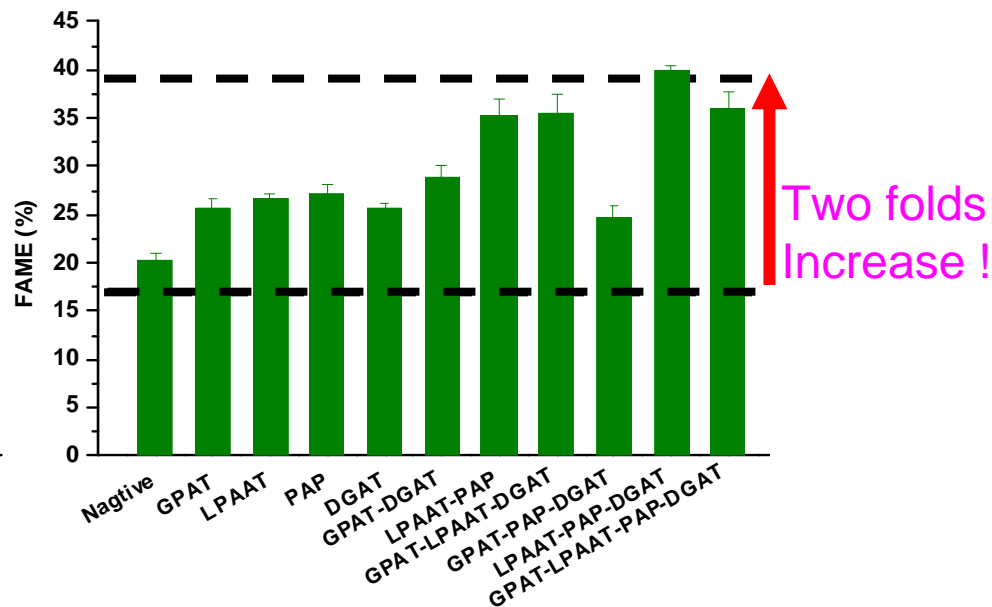
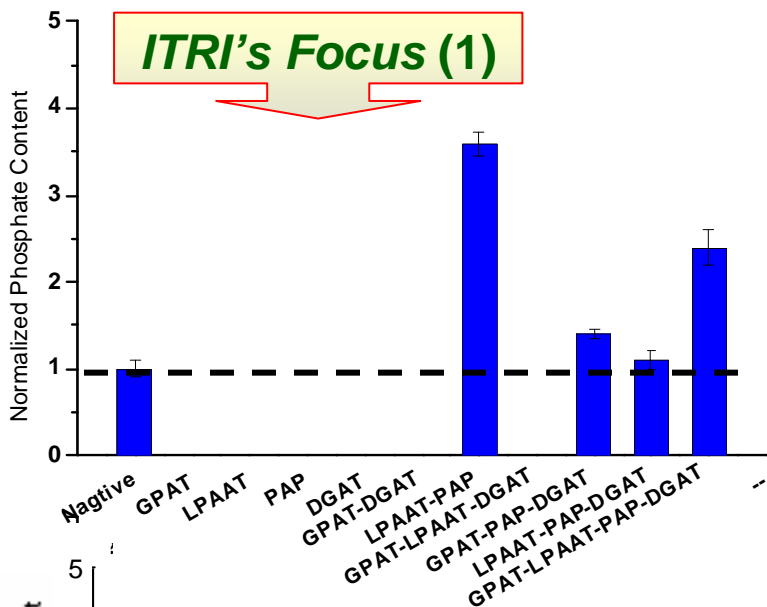
Developing the Tool for Algal Engineering in ITRI



- (1) Genetic transformation of algae is used by homologous crossover.
- (2) Nuclear transformation proceeds by random integration, which has chance targeting proteins to plastids, cytoplasm, or export.

➔ mRNA expression level
Protein expression level
Enzyme activity
Oil content

Status of Lipid Accumulation of Domestic *Chlorella* in ITRI



- (1) Promoter replacement
- (2) Copy number incensement
- (3) Signal peptide impartment

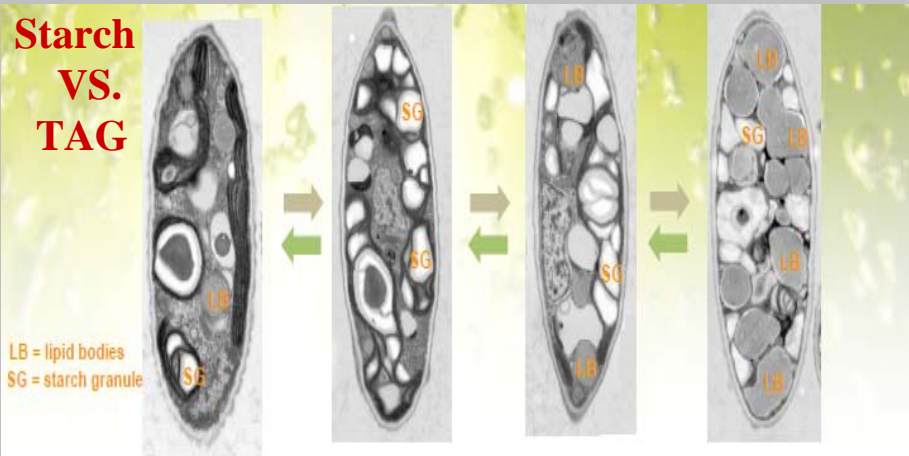
On Going !!

Part III.

- Overview of classification and annotation of *Chlorella* genome.
- Pathway of CO₂ to triacylglycerol in algae.
- **Carbon partitioning of lipid, starch, and protein in algae.**
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Carbon Partitioning of Microalgae

Strong evidence: the abolishment of the starch synthesis pathway rerouted photosynthetic carbon flux toward TAG synthesis, resulting in hyper-accumulation of TAG. (Prof. Qiang Hu, ASU)

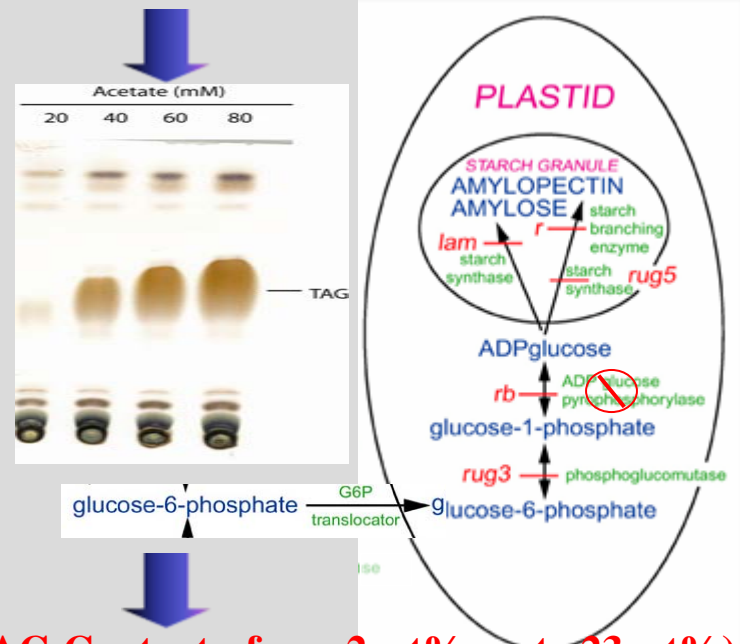


Reversible transformation of a *Scenedesmus* cell under various culture conditions

Lipid (% dwt)	10	15	20	45
Starch (% dwt)	8	35	25	15
Protein (% dwt)	55	30	20	10
Cell wall (% dwt)	10	12	14	16

Ref: SARC

Chlamydomonas starchless mutant defective in ADP-glucose pyrophosphorylase hyper-accumulates triacylglycerol

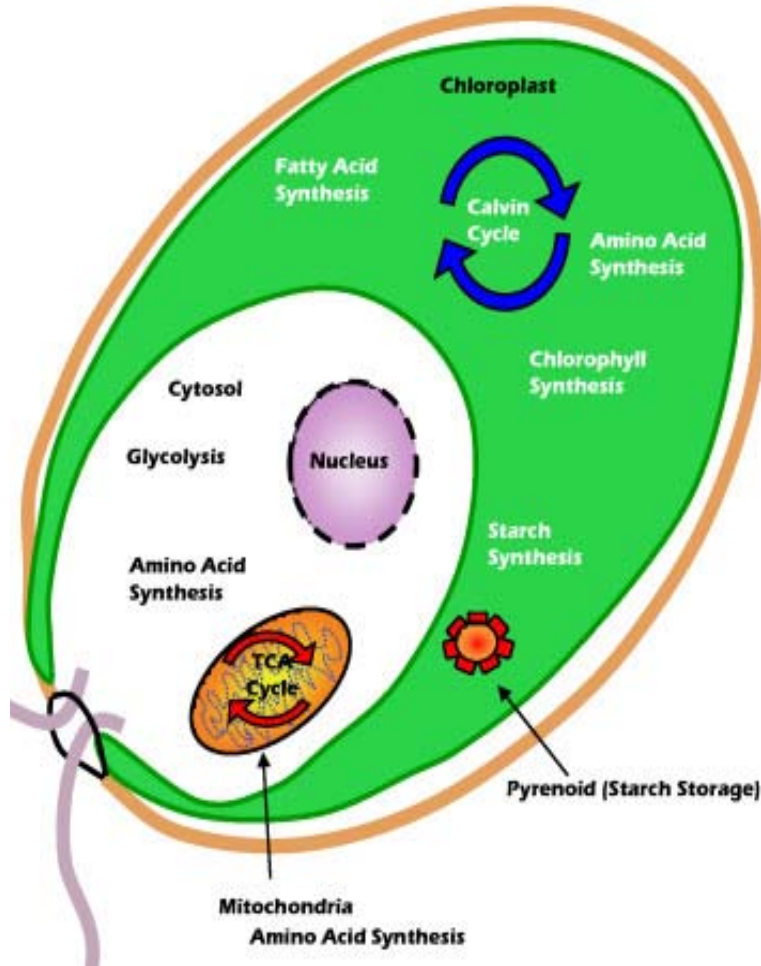


TAG Content : from 2 wt% up to 23 wt%

Metabolic Engineering 12(4), 2010, p387-391

Mutagenesis Modification of Carbohydrate Metabolism

ITRI's Focus (2)



Decreasing starch accumulation by mutagenesis

Cell number $\approx 2.0 \times 10^4$

20,000 mw-sec/cm²

Irradiated by UV light for different seconds

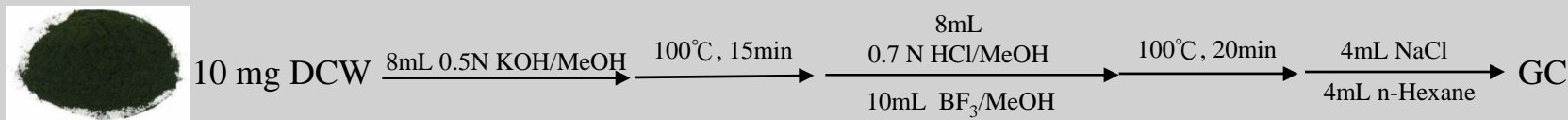
Iodine vapor assay

The figure shows a high-throughput assay for starch content. It starts with three petri dishes of algae cells, which are irradiated by UV light at a dose of 20,000 mw-sec/cm² for varying durations. The resulting cultures are then analyzed using an iodine vapor assay, shown in a 96-well plate. The color change from dark blue to yellow indicates a decrease in starch accumulation. Red circles highlight specific wells in the plate.

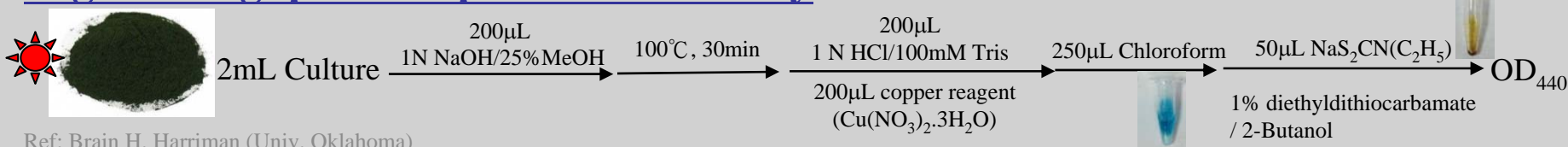
Highthroughput of Starch Content

Strain Improvement of Lipid Content in ITRI

Traditional Analysis of Lipid Content Assay

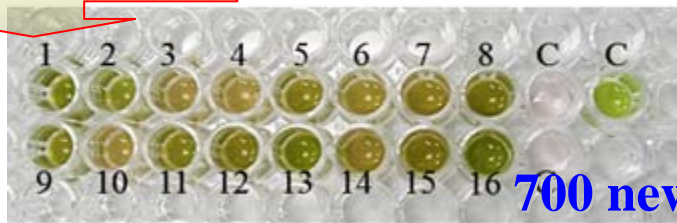


High throughput of Lipid Content Assay



Ref: Brain H. Harriman (Univ. Oklahoma)

ITRI's Focus (2)



700 new strains screened **On Going!**

Strain	Starch Content (%)
C	22.3 ± 0.8
2	21.9 ± 0.5
4	18.3 ± 0.1
13	19.4 ± 0.1
16	18.5 ± 0.2

(68/288)

Strain	Lipid Content (%)
C	21.3 ± 0.6
2	19.5 ± 0.7
4	31.5 ± 0.3
13	39.3 ± 0.4
16	20.6 ± 0.1

(31/288)

Part IV.

- Overview of classification and annotation of *Chlorella* genome.
- Pathway of CO₂ to triacylglycerol in algae.
- Carbon partitioning of lipid, starch, and protein in algae.
- **Major unresolved questions and future prospects.**

Future Works of the Engineered *Chlorella*

Goal 3

High Lipid Productivity of Engineered Microalgae



Carry out a Large Scale Model after Test and Verify

On Going !!

Goal 2

Molecular Biology

Lipid Productivity

Process Engineering

Genetic Engineering + Mutagenesis



Cultural Optimization + Simulation

On Going !!

Goal 1

Molecular Biology

Lipid Content

Process Engineering

Genetic Engineering + Mutagenesis



Cultural Optimization + Simulation

Unresolved Questions

1. **How is the carbon partitioning between starch and TAG controlled, and how it be manipulated ?**
2. **Do overexpress growth related-genes have chance to conquer the existing overexpression (lipid biosynthesis) for pushing more photosynthate into lipid than the wild-type ?**
3. **How are the regulating in gene expression and enzyme activities, and how it be fine-tuned the composition of nutrient during oil accumulation?**

Questions and Answer

Thank You For Your Attention !

Financial support provided by the Bureau of Energy, MOEA, R.O.C. is acknowledged

Cooperative research teams:

- Microalgae Technology Platform for the Establishment of Gene Expression System
- Mutagenesis method to explore the starch abolishment on the algal oil production (MCUT, Assistant Professor Liang-Jung Chien)