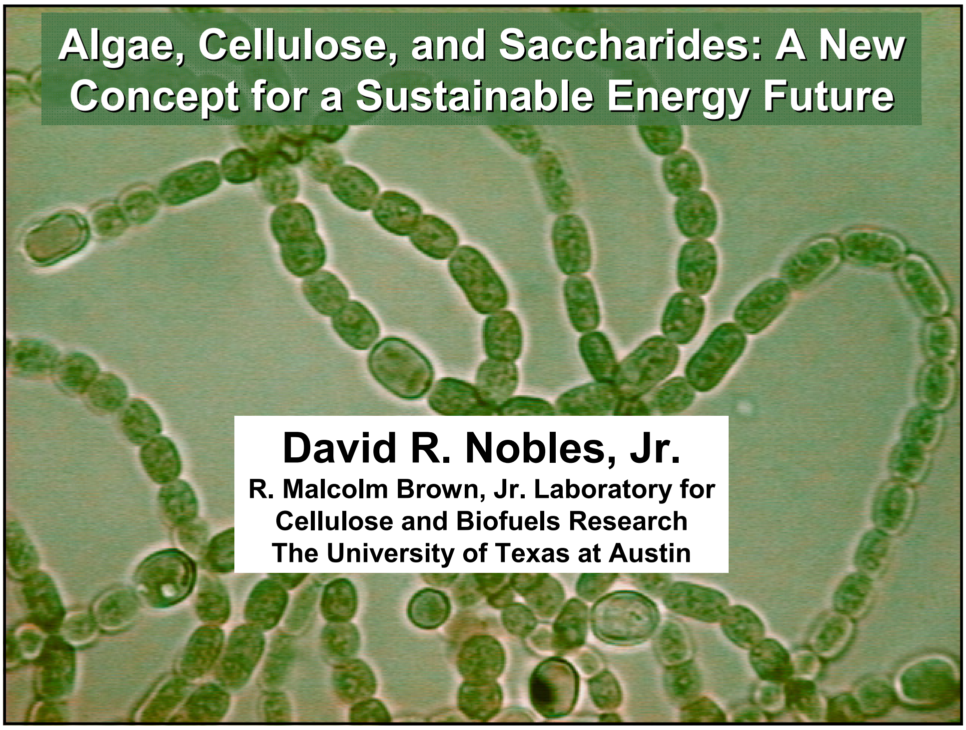


Algae, Cellulose, and Saccharides: A New Concept for a Sustainable Energy Future

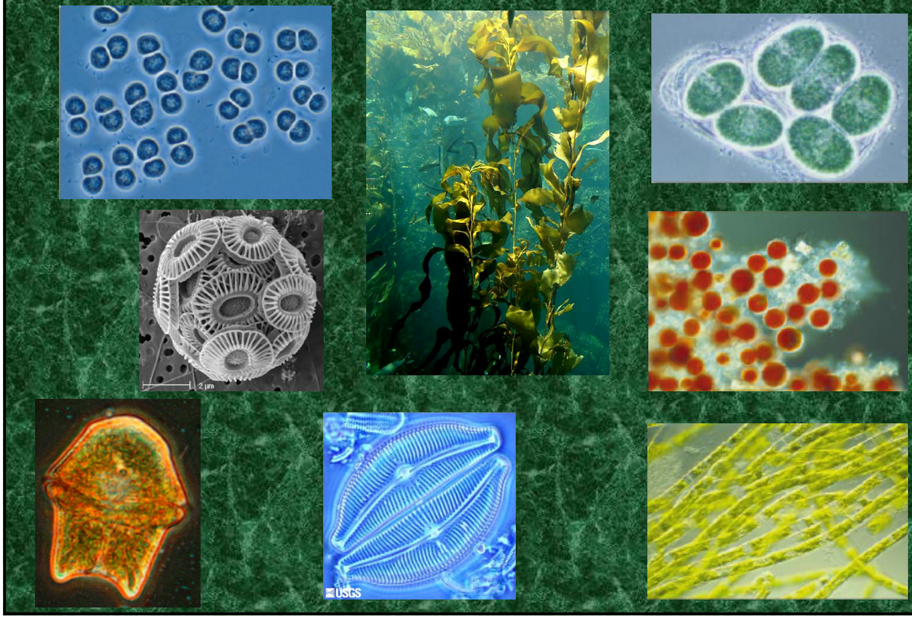
David R. Nobles, Jr.

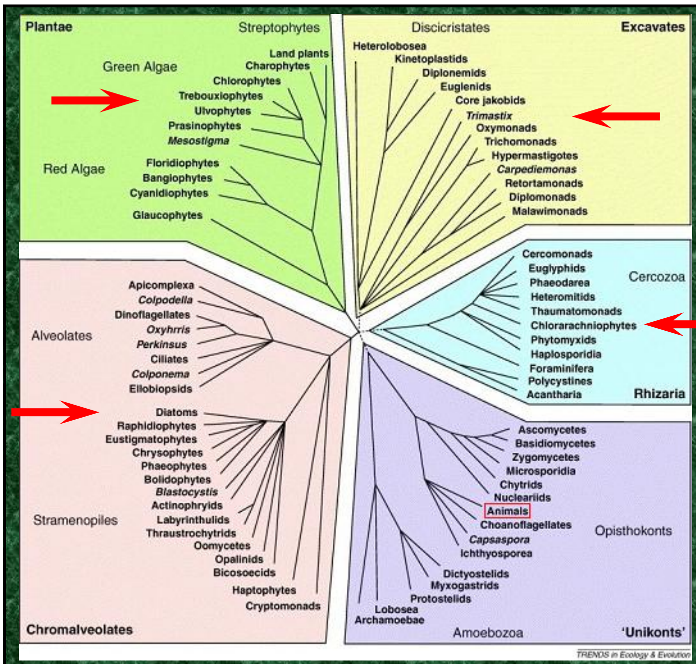
R. Malcolm Brown, Jr. Laboratory for
Cellulose and Biofuels Research
The University of Texas at Austin

Malcolm and Ann Brown

What are algae?

Algae are incredibly diverse

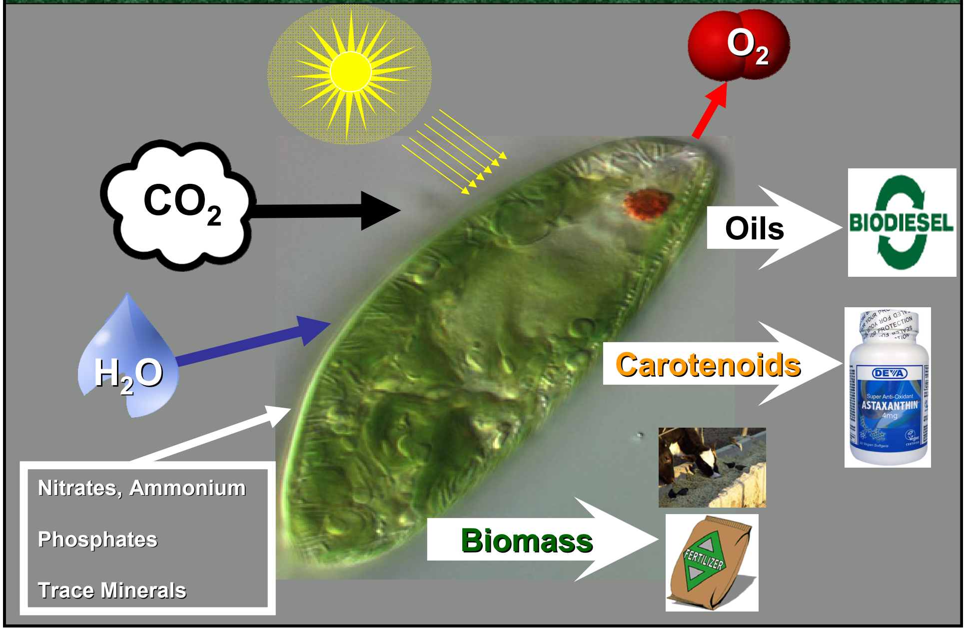




Keeling PJ, et al. (2005) The tree of Eukaryotes. Trends in Ecology & Evolution 20(12): 670-676

Algae occupy many branches on the Tree of Life

Algae are solar micro-factories



O_2

CO_2

Oils

BIODIESEL

H_2O

Carotenoids

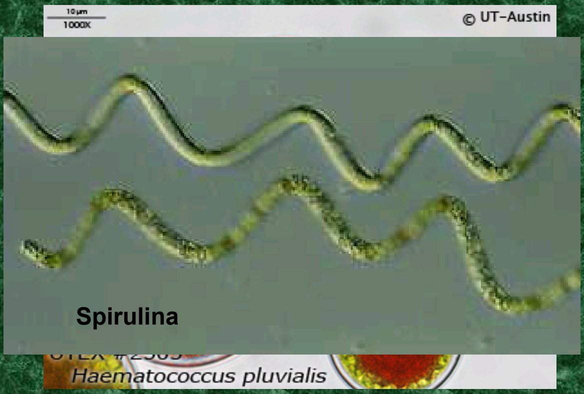
Biomass

Nitrates, Ammonium

Phosphates

Trace Minerals

A few species of algae are already commercially cultivated on a large scale



Spirulina production at Earthrise Farms

Astaxanthin production by *Haematococcus pluvialis* at Parry Nutraceuticals

What are the some potential advantages of using photosynthetic microbes for the production of biofuels and biofuel feedstocks?

- High growth rates (e.g. 1–3 doublings per day for eukaryotic algae, can be as high as 8 -10 for some cyanobacteria)
- Ability to thrive in saline/brackish water/coastal seawater for which there are few competing demands
- Potential for growth on marginal lands (e.g. desert, arid- and semi-arid lands) that are not suitable for conventional agriculture)
- Ability to utilize growth nutrients such as nitrogen and phosphorus from a variety of wastewater sources providing the additional benefit of wastewater bio-remediation
- Capable of capturing carbon dioxide from flue gases emitted from fossil fuel-fired power plants and other sources, thereby reducing emissions of a major greenhouse gas
- Produce value-added co-products or by-products (e.g. biopolymers, proteins, polysaccharides, pigments, animal feed, fertilizer and H_2
- Very high annual biomass productivity, on an area basis, far exceeding terrestrial plants

Qiang Hu et al. (2008) The Plant Journal (2008) 54, 621–639

GREENFUEL
TECHNOLOGIES CORPORATION

SOLIX
biofuels

AURORA
BIOFUELS

valcent™
products inc.

GENERAL ATOMICS

OriginOil

SYNTHETIC GENOMICS®

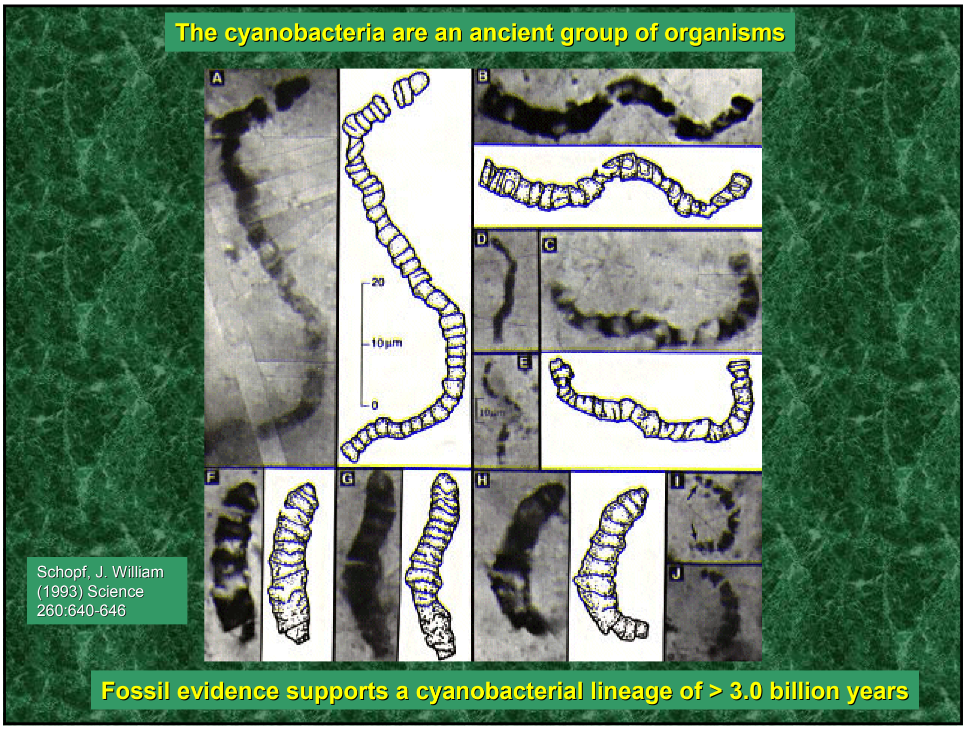
LiveFuels Inc.
From Pond To Pump (TM)

ALGENOL
BIOFUELS
HARNESSING THE SUN TO FUEL THE WORLD™

solazyme

Sapphire
Energy

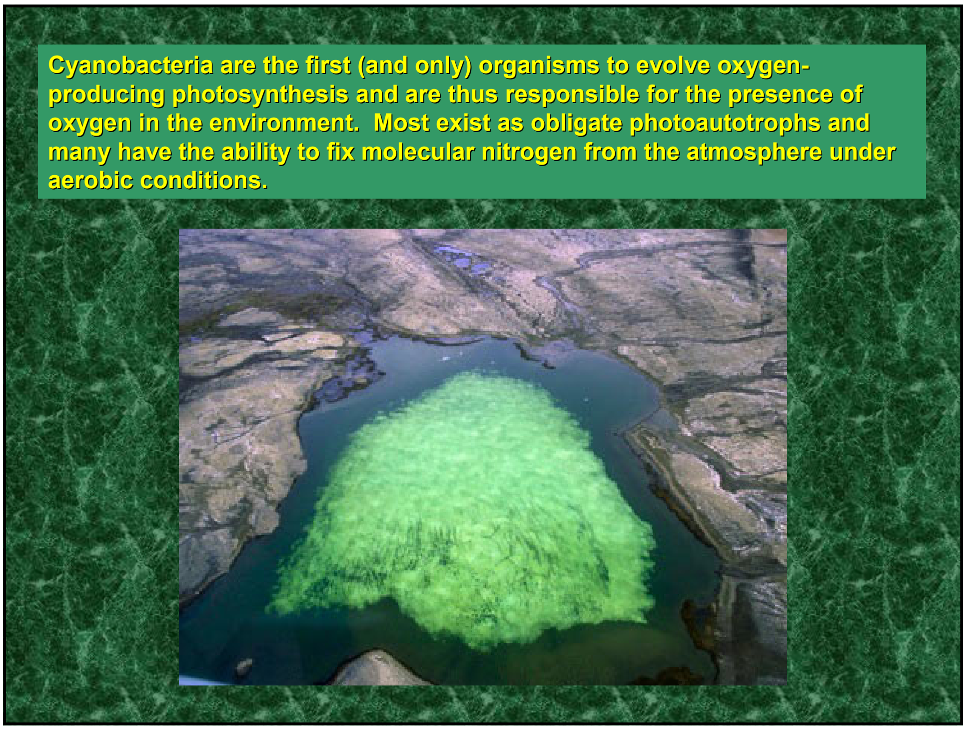
What are cyanobacteria and how do they fit into this?



The cyanobacteria are an ancient group of organisms

Schopf, J. William (1993) Science 260:640-646

Fossil evidence supports a cyanobacterial lineage of > 3.0 billion years



Cyanobacteria are the first (and only) organisms to evolve oxygen-producing photosynthesis and are thus responsible for the presence of oxygen in the environment. Most exist as obligate photoautotrophs and many have the ability to fix molecular nitrogen from the atmosphere under aerobic conditions.

But aren’t plants and other algae photosynthetic AND don’t they produce Oxygen?

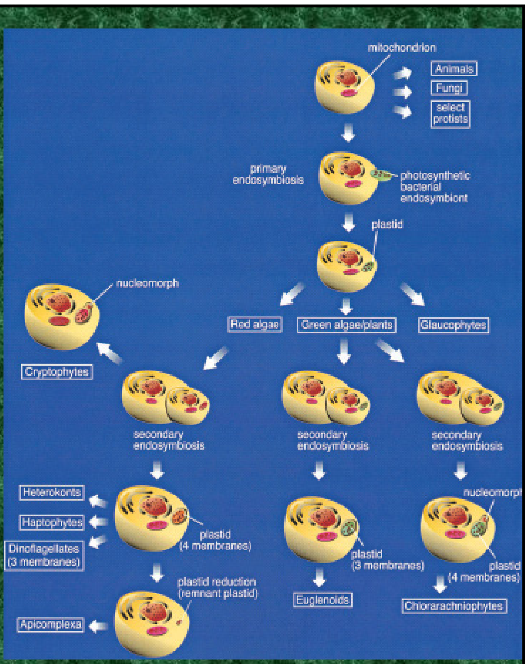
ANSWER:

Yes. But they didn’t invent it – they stole it!

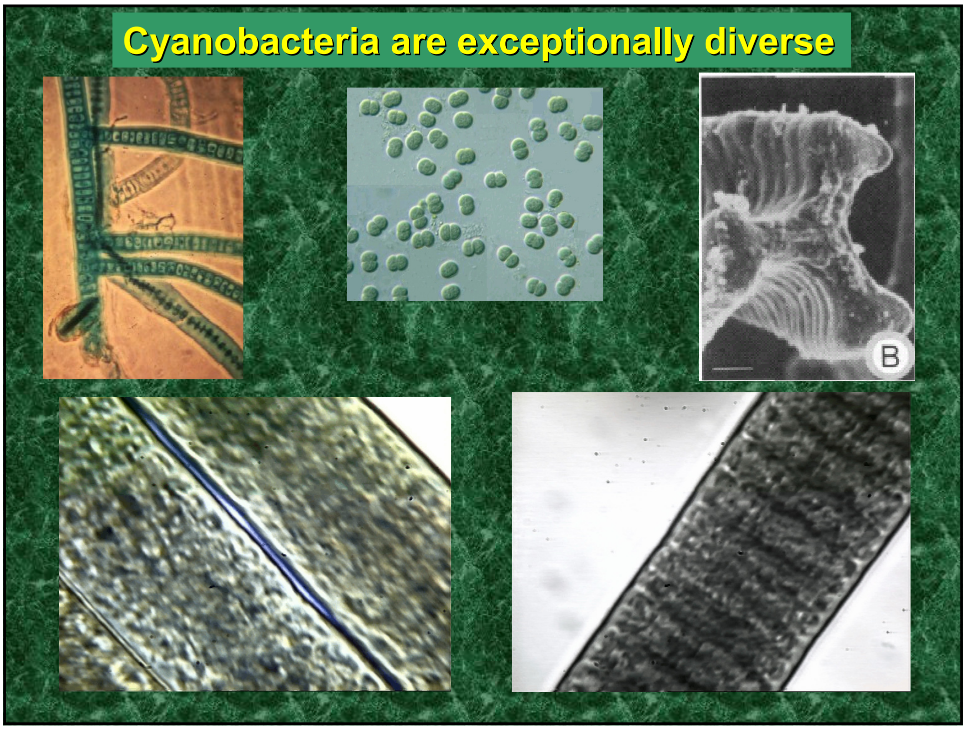
Photosynthesis in plants and algae arose from the endosymbiotic capture of a cyanobacterium

http://www.youtube.com/watch?v=DE4CPmTH3xg

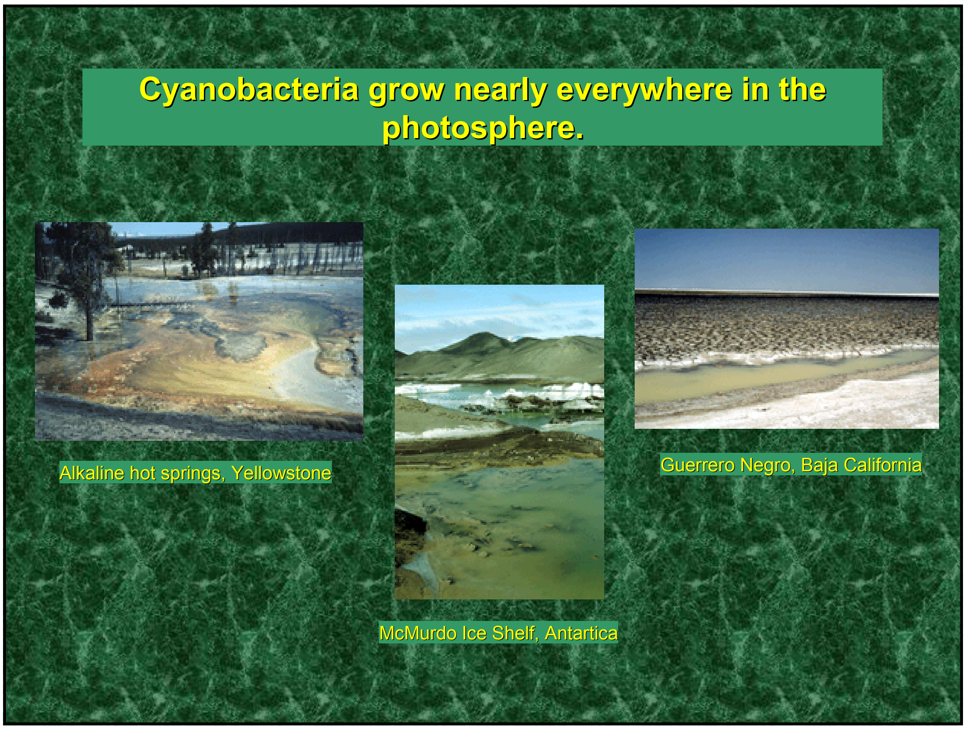
The diversity photosynthetic algae and plants is due to multiple endosymbiotic captures.



McFadden GI (2001) J. Phycology 37:951



Cyanobacteria are exceptionally diverse



Cyanobacteria grow nearly everywhere in the photosphere.

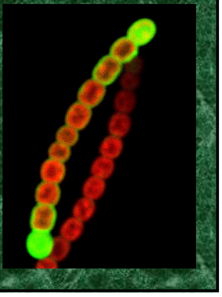
Alkaline hot springs, Yellowstone

McMurdo Ice Shelf, Antartica

Guerrero Negro, Baja California

Cyanobacteria offer all of the potential advantages of eukaryotic algae plus……

Nitrogen Fixation



Relative ease of genetic manipulation

Biological Nitrogen Fixation

$$N_2 + 8\,H^+ + 8\,e^- + 16\,ATP \xrightarrow[\text{Nitrogenase}]{} 2\,NH_3 + H_2 + 16\,ADP + 16\,P_i$$

Nitrogen fixation (diazotrophy) is a strictly prokaryotic phenomenon occurring in many groups of bacteria and archaea e.g. Rhizobia, Frankia, etc…

Nitrogen fixation is no mean feat for photosynthetic organisms

Nitrogenase, the enzyme responsible for catalyzing the reaction, is permanently deactivated by exposure to oxygen

How do cyanobacteria manage this?

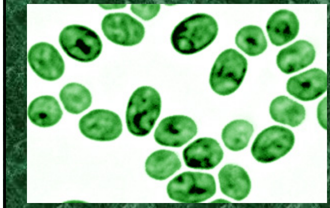
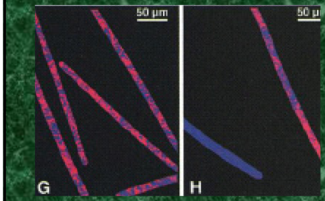


Image courtesy of The Pakrasi Lab

Some unicellular, diazotrophic, marine cyanobacteria separate nitrogen fixation and photosynthesis temporally, fixing nitrogen during the dark cycle

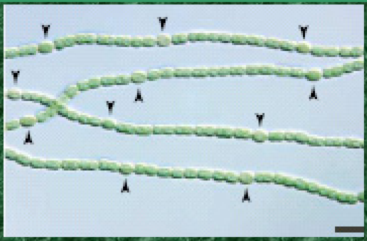
Reddy KJ et al. (1993) J Bacteriol. 1993 175(5):1284-92.



Filamentous cyanobacteria of the genus *Trichodesmium* have evolved ways to separate nitrogen fixation and photosynthesis both temporally and spatially, allowing simultaneous photosynthesis and nitrogen fixation

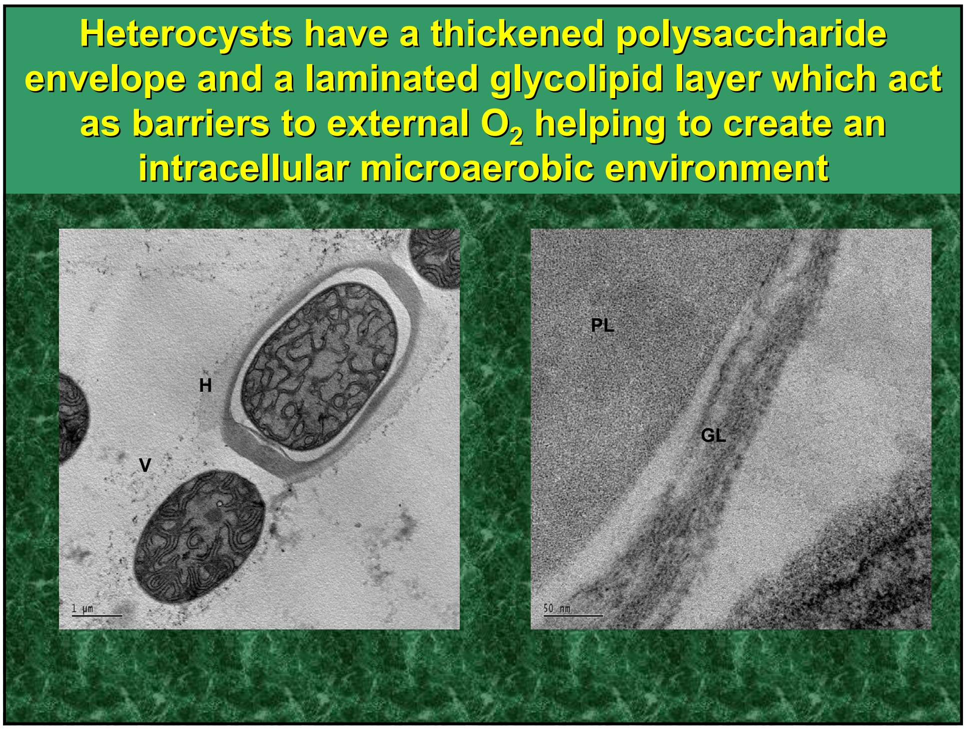
Berman-Frank I. (2001) Science 294(5546): 1534-1537

The most advanced groups of cyanobacteria have evolved specialized, terminally differentiated cells called <u>Heterocysts</u> for nitrogen fixation

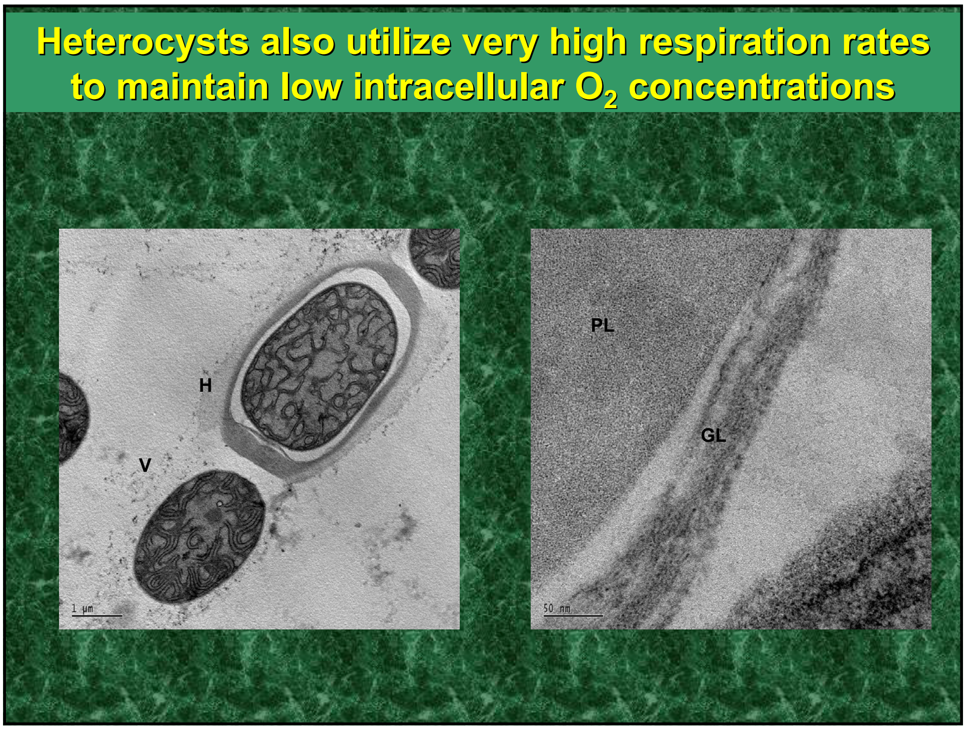


Golden JW, Yoon H. (2003) Heterocyst development in *Anabaena*. Curr Opin Microbiol 6:557-563

- **Heterocyst differentiation occurs at semi-regular intervals along the filament and represents an early, if not the first occurance of multicellular pattern formation**
- **Heterocysts provide a microaerobic environment facilitating nitrogen fixation**
- **Heterocysts maintain photosystem I for ATP generation**
- **Heterocysts lack photosystem II, so they do not evolve O_2, but are also unable to produce reductants for respiration**

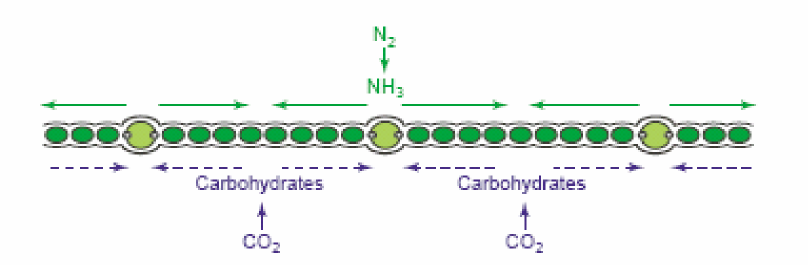


Heterocysts have a thickened polysaccharide envelope and a laminated glycolipid layer which act as barriers to external O_2 helping to create an intracellular microaerobic environment



Heterocysts also utilize very high respiration rates to maintain low intracellular O_2 concentrations

Respiration and nitrogen fixation both require reductants. Since heterocysts lack photosystem II, how do they survive?

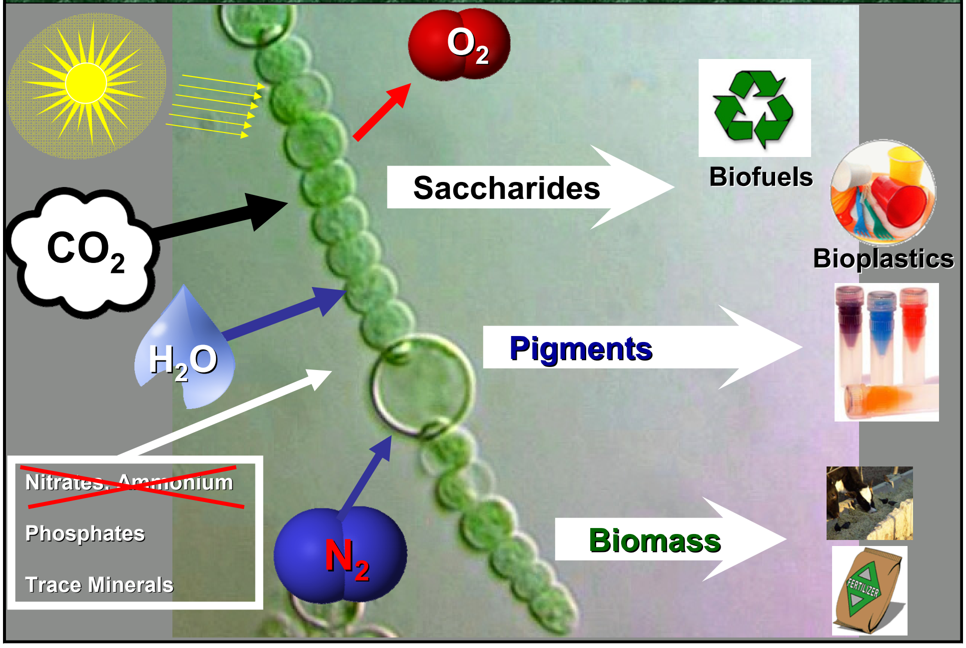


Heterocysts supply vegetative cells with NH_3 and vegetative cells supply heterocysts with reductant (most likely sucrose)

Golden JW, Yoon H. (2003) Heterocyst development in *Anabaena*. Curr Opin Microbiol 6:557-563

This exchange takes place via a channel that bridges heterocysts and vegetative cells

Cyanobacteria are solar micro-factories



O_2

CO_2

H_2O

~~Nitrates, Ammonium~~

Phosphates

Trace Minerals

N_2

Saccharides

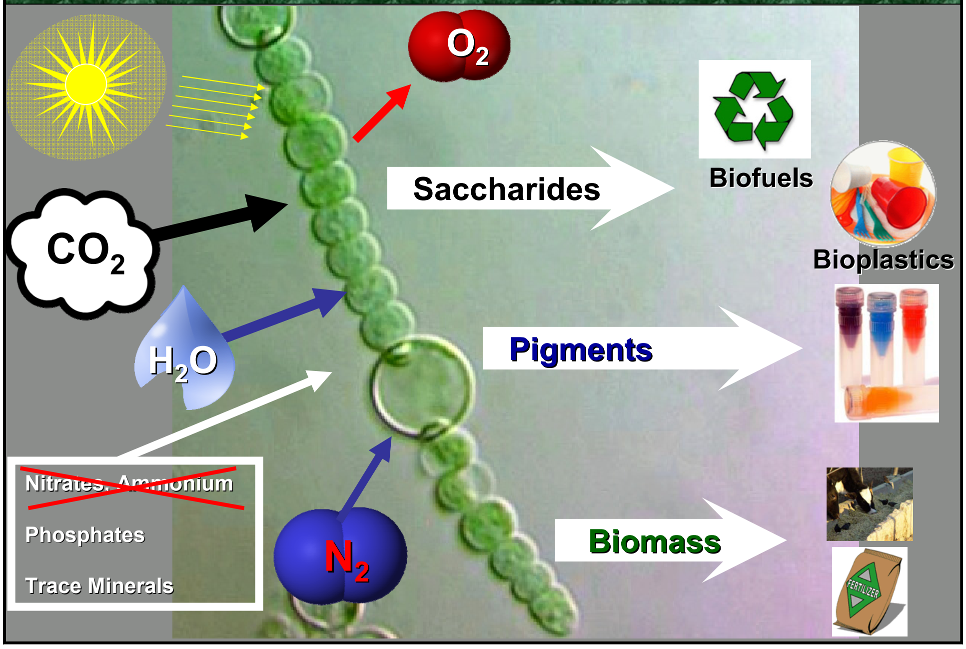
Biofuels

Bioplastics

Pigments

Biomass

With division of Labor!



O_2

CO_2

H_2O

~~Nitrates, Ammonium~~

Phosphates

Trace Minerals

N_2

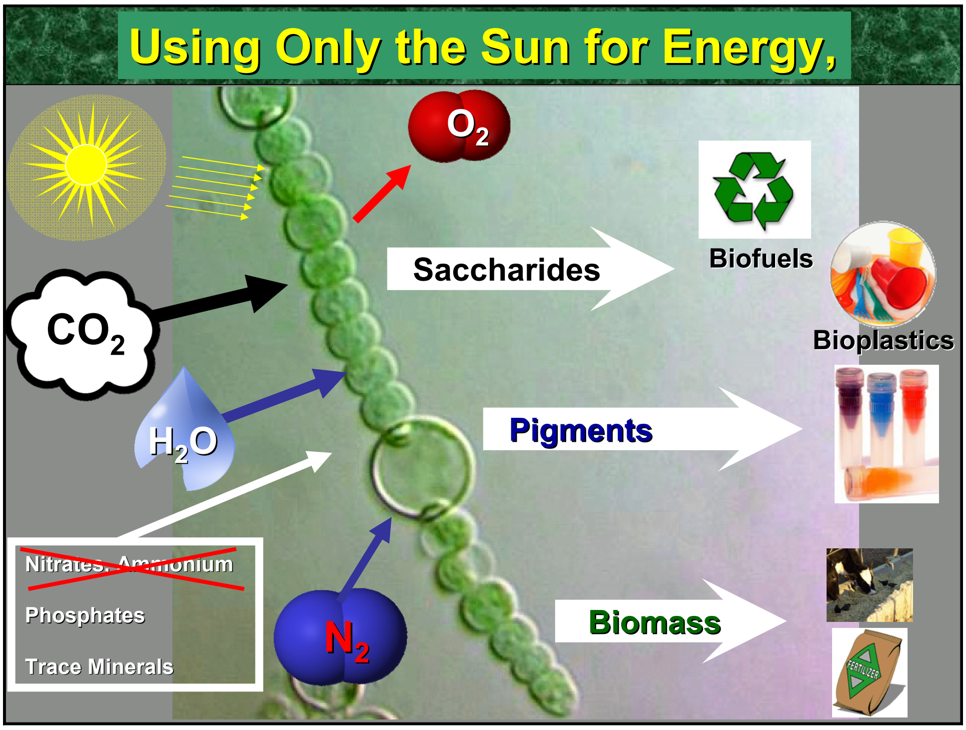
Saccharides

Biofuels

Bioplastics

Pigments

Biomass



Using Only the Sun for Energy,

O_2

Saccharides

Biofuels

Bioplastics

CO_2

H_2O

Pigments

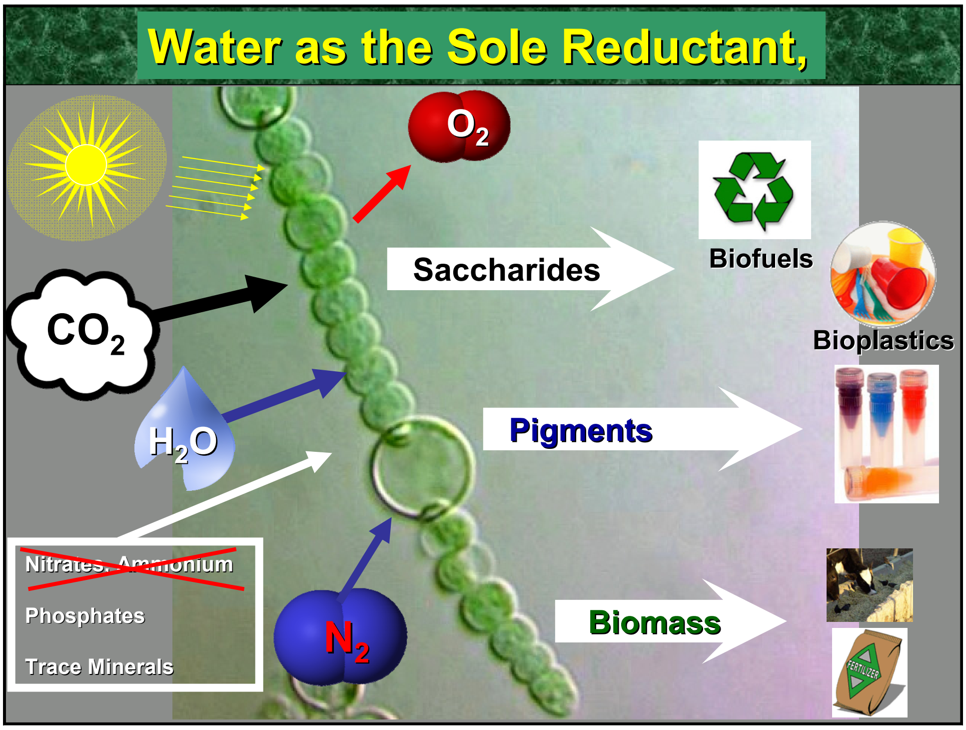
~~Nitrates, Ammonium~~

Phosphates

Trace Minerals

N_2

Biomass



Water as the Sole Reductant,

O_2

Saccharides

Biofuels

Bioplastics

CO_2

H_2O

Pigments

~~Nitrates, Ammonium~~

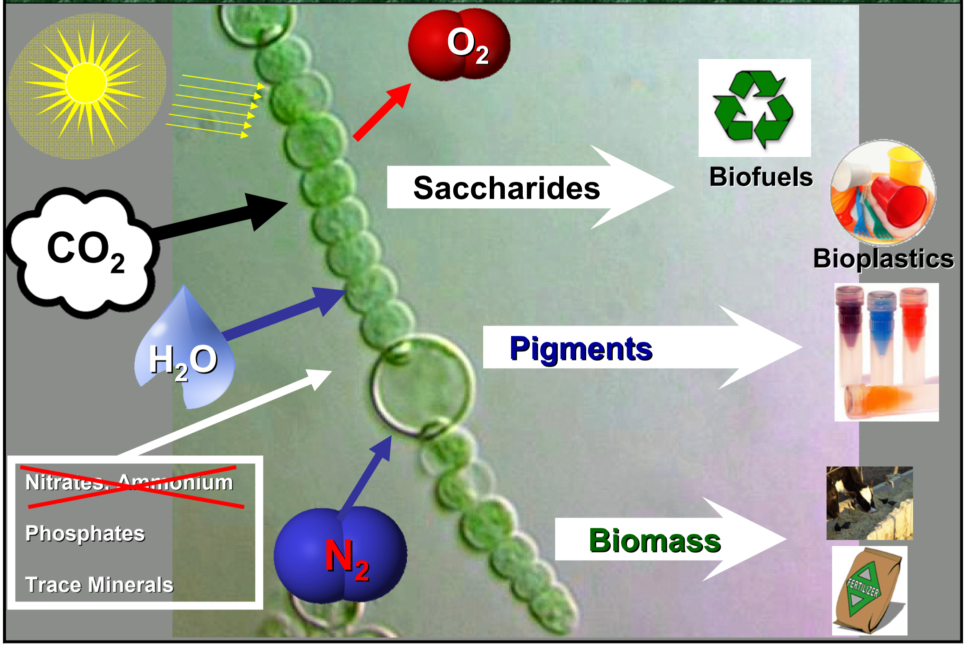
Phosphates

Trace Minerals

N_2

Biomass

and only N_2 as a Nitrogen Source



O_2

Saccharides

Biofuels

Bioplastics

CO_2

H_2O

Pigments

~~Nitrates, Ammonium~~

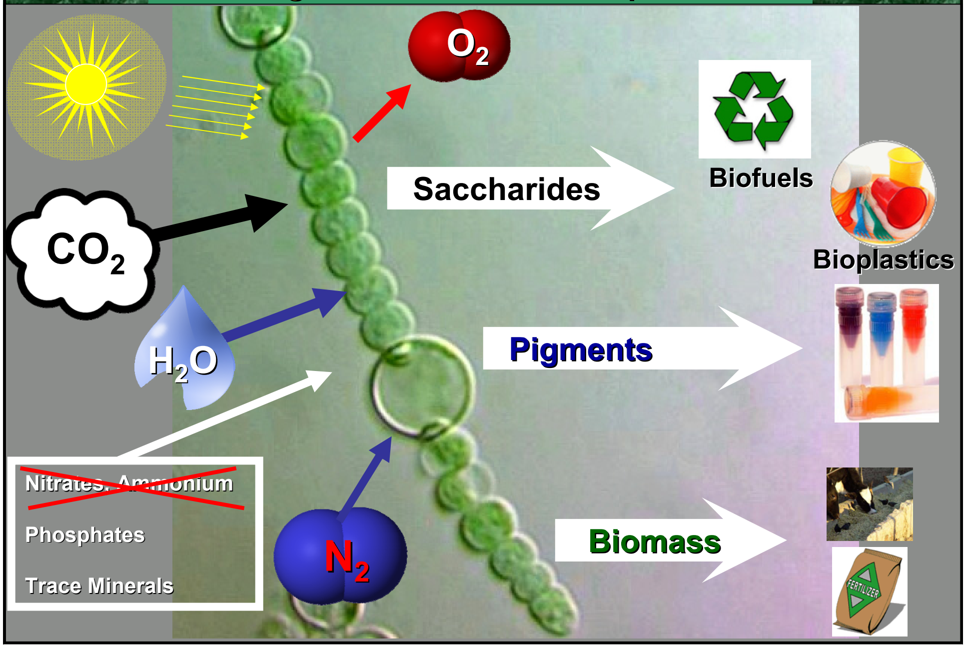
Phosphates

Trace Minerals

N_2

Biomass

Cyanobacteria are the Most Autonomous Organisms in the Photosphere



O_2

Saccharides

Biofuels

Bioplastics

CO_2

H_2O

Pigments

~~Nitrates, Ammonium~~

Phosphates

Trace Minerals

N_2

Biomass

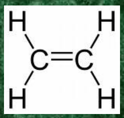
In spite of their regulatory and structural complexity relative to other bacteria, the organizational architecture of cyanobacteria is uncomplicated when compared to that of eukaryotic algae

Because they are bacteria, the basic molecular genetics tools of transformation, conjugation, and electroporation have been successfully utilized for genetic manipulation in a wide range of cyanobacteria

---

Cyanobacteria have been genetically engineered to produce potential biofuels such as:

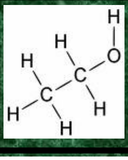
Ethylene



Takahama, K., Matsuoka, M., Nagahama, K., Ogawa, T., (2003) J. Biosci. Bioeng. 95, 302–305.

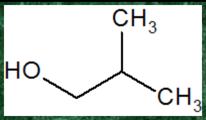
Ethanol

ALGENOL
BIOFUELS
HARNESSING THE SUN TO FUEL THE WORLD™



Deng, M.D., Coleman, J.R., (1999) Appl. Environ. Microbiol. 65, 523–528.

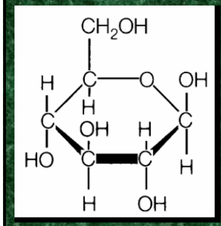
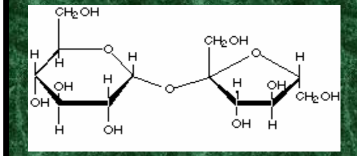
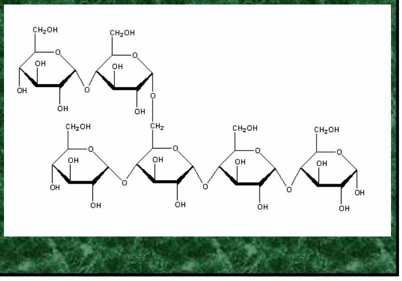
Isobutanol



Service, R.F. (2009) Science 325, 1201.

Cyanobacteria do not naturally produce triacylglycerols nor do they produce other lipids with biofuel potential in quantity

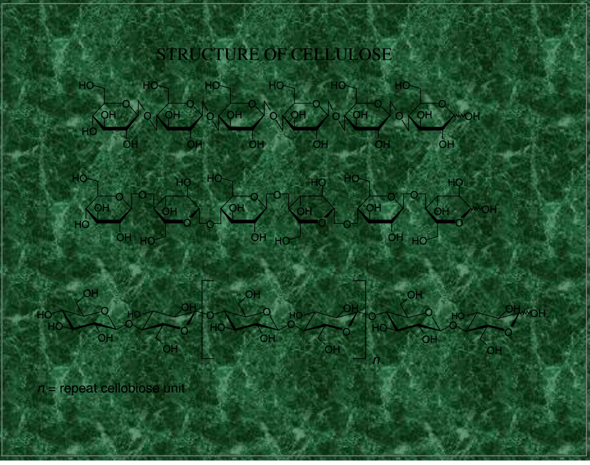
However, they do manufacture large quantities of sugars and polysaccharides

A Bit of Background

Some Key Facts about Cellulose

Cellulose is the most abundant biomacromolecule (10^{11} tons produced annually)


STRUCTURE OF CELLULOSE

n = repeat cellobiose unit

Some Key Facts about Cellulose

Cellulose is produced by:

- **Plants**
- **Algae**
- **Animals (Tunicates)**
- **Bacteria:**
    - ***Escherichia coli***
    - ***Salmonella spp.***
    - ***Agrobacterium spp.***
    - ***Pseudomonas spp.***
    - ***Gluconacetobacter xylinus* (aka *Acetobacter xylinus*)**
    - **Many cyanobacteria**

Some Key Facts about Cellulose

Cellulose occurs in multiple crystalline allomorphs

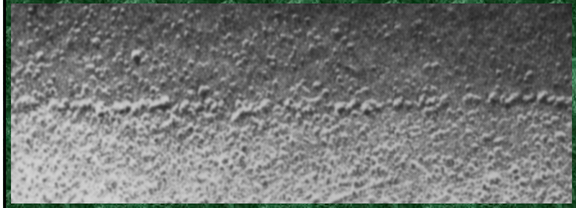
Cellulose I: **Also known as native cellulose, this allomorph is metastable**

Cellulose II: **Also known as regenerated cellulose, this is the most thermodynamically stable allomorph. It is rarely found in nature.**

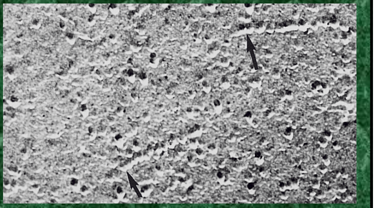
Some Key Facts about Cellulose

Because Cellulose I is metastable, it requires highly organized complexes, called Terminal Complexes (TCs) for crystallization

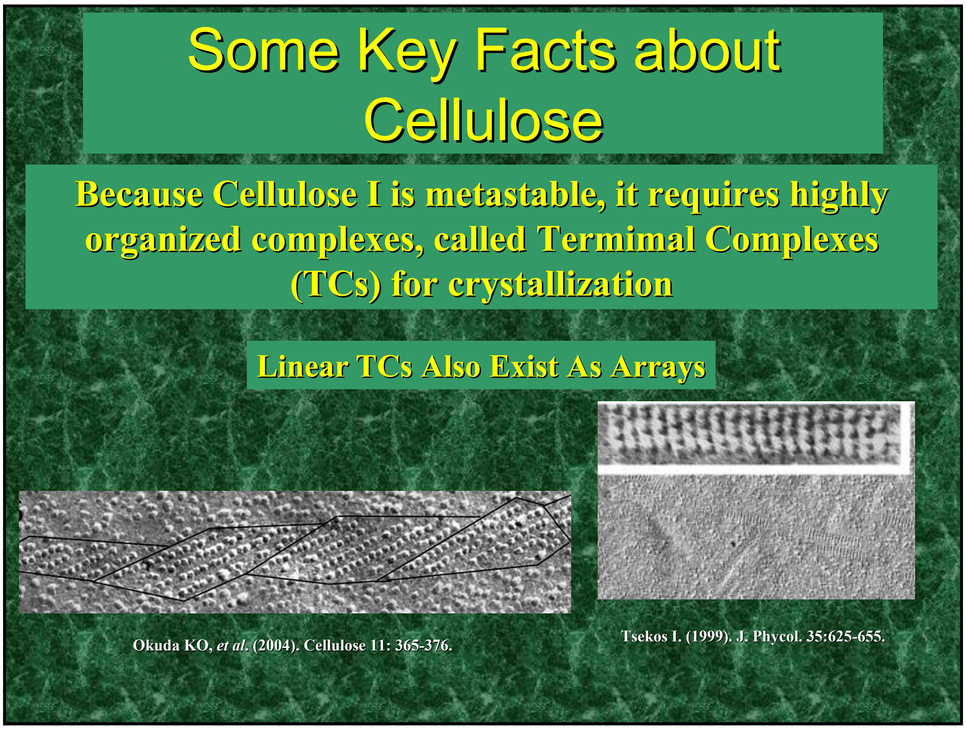
Linear TCs As Discrete Rows



Brown Jr RM, *et al.* (1976). Cellulose biosynthesis in *Acetobacter xylinum*: 1. Visualization of the site of synthesis and direct measurement of the in vivo process. Proc Nat Acad Sci USA 73(12):4565-4569.



Schüßler A, *et al.* (2003). Cellulose synthesizing terminal complexes and morphogenesis in tip-growing of *Syringoderma phinneyi* (Phaeophyceae). Phycol. Res. 51: 35-44.



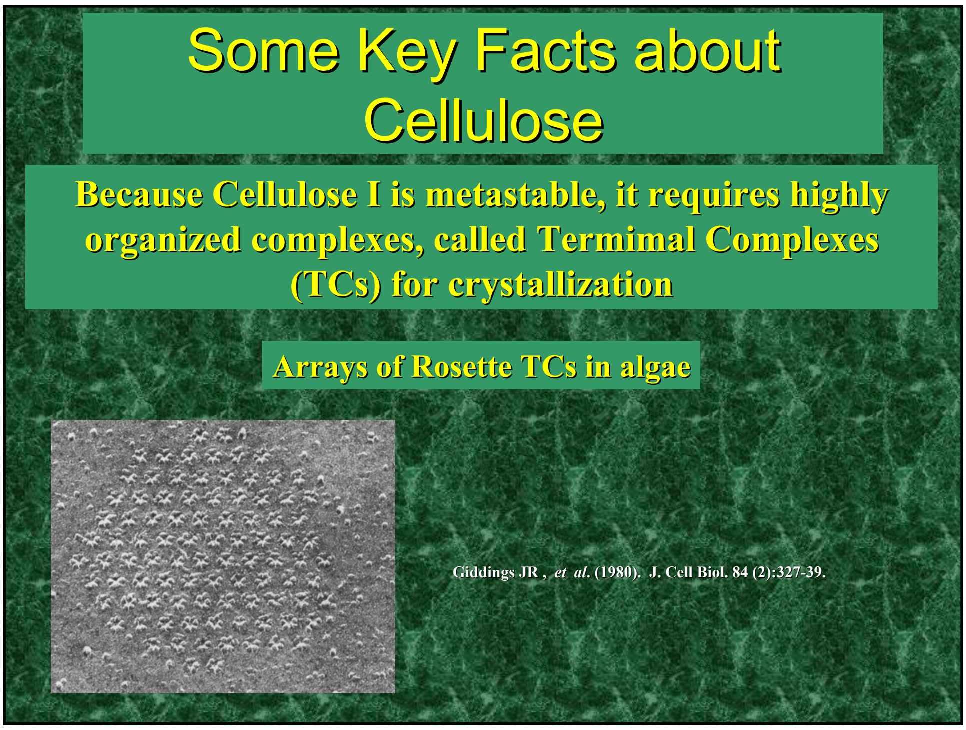
Some Key Facts about Cellulose

Because Cellulose I is metastable, it requires highly organized complexes, called Terminal Complexes (TCs) for crystallization

Linear TCs Also Exist As Arrays

Okuda KO, *et al.* (2004). Cellulose 11: 365-376.

Tsekos I. (1999). J. Phycol. 35:625-655.



Some Key Facts about Cellulose

Because Cellulose I is metastable, it requires highly organized complexes, called Terminal Complexes (TCs) for crystallization

Arrays of Rosette TCs in algae

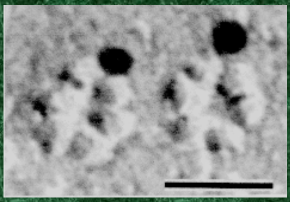
Giddings JR , *et al.* (1980). J. Cell Biol. 84 (2):327-39.

Some Key Facts about Cellulose

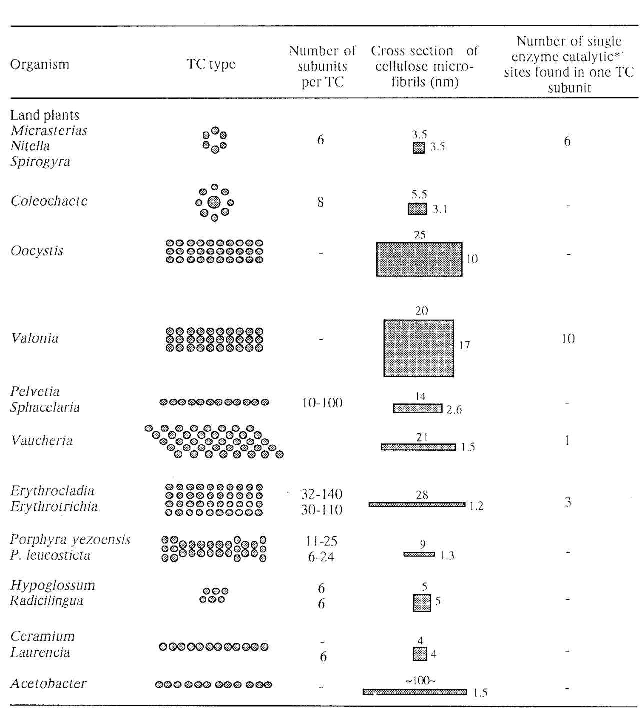
Because Cellulose I is metastable, it requires highly organized complexes, called Terminal Complexes (TCs) for crystallization

Vascular Plants Have Discrete Rosette TCs

Mueller SC, Brown Jr RM. (1980). J Cell Biol 84: 315–326.



Kimura S, *et al.* (1999). Plant Cell 11: 2075–2085.



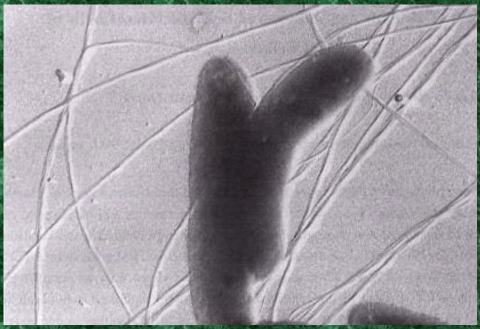
Organism	TC type	Number of subunits per TC	Cross section of cellulose micro-fibrils (nm)	Number of single enzyme catalytic* sites found in one TC subunit
Land plants *Micrasterias* *Nitella* *Spirogyra*		6	3.5 3.5	6
Coleochaete		8	5.5 3.1	-
Oocystis		-	25 10	-
Valonia		-	20 17	10
Pelvetia *Sphacelaria*		10-100	14 2.6	-
Vaucheria			21 1.5	1
Erythrocladia *Erythrotrichia*		32-140 30-110	28 1.2	3
Porphyra yezoensis *P. leucosticta*		11-25 6-24	9 1.3	-
Hypoglossum *Radicilingua*		6 6	5 5	-
Ceramium *Laurencia*		- 6	4 4	-
Acetobacter		-	~100~ 1.5	-

Different TC Architectures Produce Cellulose Crystals of Variable Size and Morphology

Tsekos I. 1999. J. Phycol. 35:625-655.

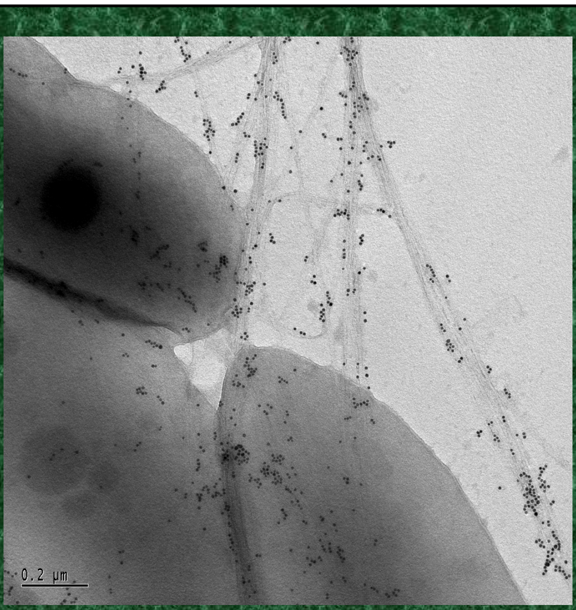
Bacterial cellulose

Gluconcetobacter xylinus is the model system for the study of cellulose biosynthesis



It is the most efficient producer of cellulose on earth!

10^8 glucose molecules polymerized into cellulose/cell/hr translating into 114 MT cellulose ha $^{-1}$ year $^{-1}$!



Cellulose biosynthesis by *Gluconacetobacter xylinus*
The cellulose microfibrils are labeled with CBHI-Gold

The pellicle of cellulose in young and older culture

The cleaned, never-dried cellulose membrane (= pellicle)

The cleaned, dried cellulose membrane

This membrane was dried on a Teflon surface, hence is very smooth

Here is a very thick membrane dried. It is very strong and rigid

Molded objects synthesized by *G. xylinus*

© R. Malcolm Brown, Jr.

Tissue Engineering Studies

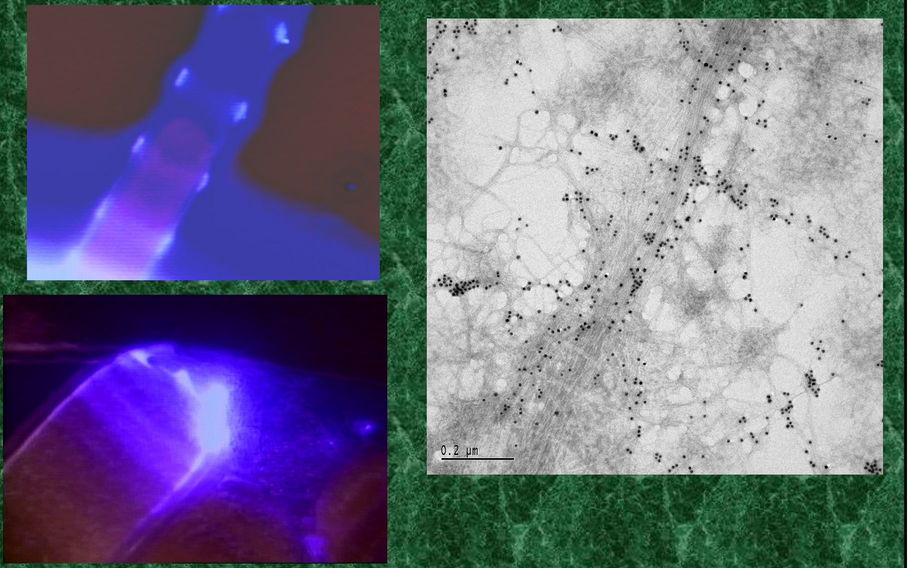
The properties of microbial cellulose make it ideally suited for many biomedical applications such as

- arteries
- burn bandages
- drug delivery agents
- heart valves
- coated stents
- cartilage
- dura mater
- nerve guide elements
- tissue engineering scaffolds for skin and chronic wounds
- pharmaceutical cosmetics

Problem

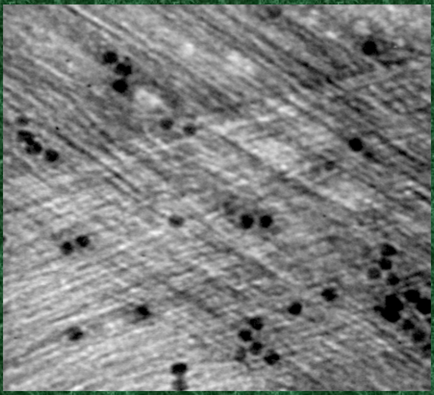
G. xylinus is heterotrophic, therefore cellulose production with this organism is costly. We hoped to produce microbial cellulose with similar properties from cyanobacteria.

Cyanobacteria produce cellulose

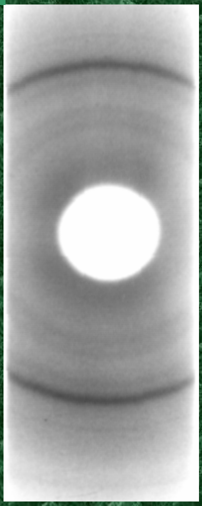


Nobles D R, Romanovicz D K, Brown R M Jr. (2001). *Plant Physiol.*127(2):529-42

Proof for Cellulose in Cyanobacteria



CBH-1-gold labeling



X-ray diffraction

Cyanobacterial Cellulose Production is NOT a Plausible Substitute for *G. xylinus* Cellulose

Although several cyanobacteria from diverse groups synthesize cellulose, most produce very little

Those that produce significant amounts of cellulose grow very slowly – e.g. *Scytonema*

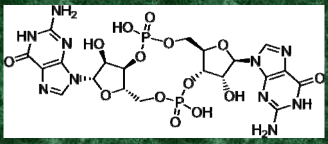
In all cases, cellulose is produced as a component of a complex extracellular matrix that contains other polysaccharides, proteins, etc.…

Nothing is known about the regulation of cellulose synthesis in cyanobacteria, nor the synthesis of other components of the extracellular matrices

More is known about cellulose biosynthesis in *G. xylinus* than in any other organism

The cellulose operon from *G. xylinus* has been cloned, sequenced, and partially characterized

Cellulose synthesis in *G. xylinus* is upregulated by the second messenger, cyclic diguanylate

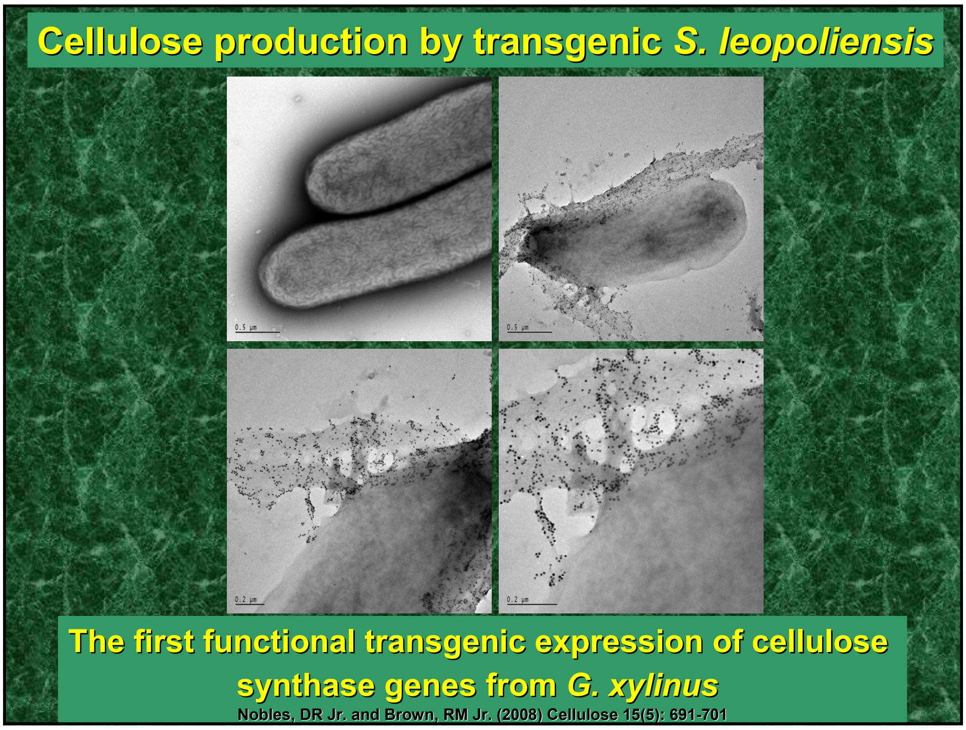


The levels of cyclic diguanylate are regulated by diguanylate cyclase and its cognate phosphodiesterase

---

Solution

Transfer of the cellulose synthase operon from *Gluconacetobacter xylinus* to the chromosome of the non-cellulose producing cyanobacterium *Synechococcus leopoliensis*



Cellulose production by transgenic *S. leopoliensis*

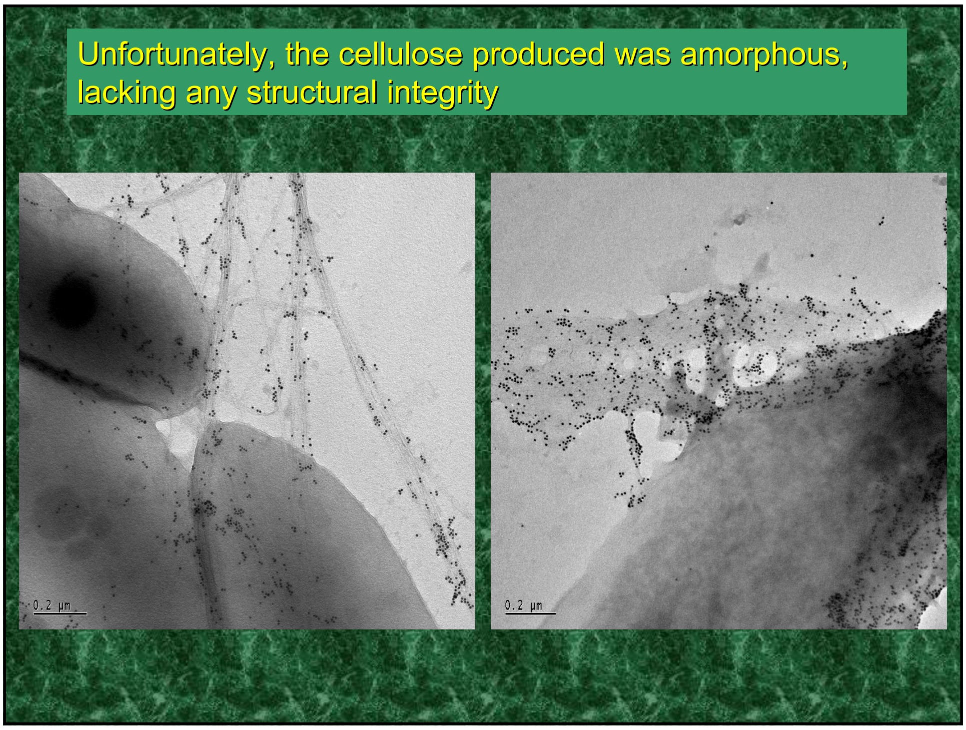
The first functional transgenic expression of cellulose synthase genes from *G. xylinus*

Nobles, DR Jr. and Brown, RM Jr. (2008) Cellulose 15(5): 691-701

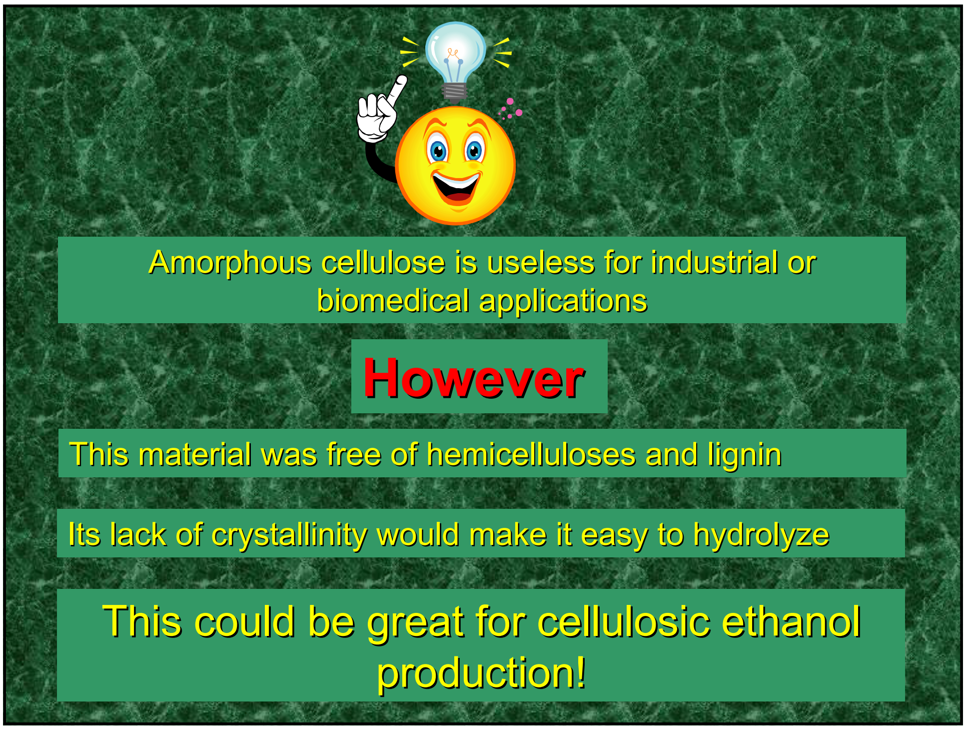
Although significant, initial production levels were modest.

	OD_{750}	Wet Weight (g)	Glucose mg/ml – Sodium Acetate-Only	Total Glucose mg/ml – Celluclast	Glucose mg/ml from cellulose
Wild-type	1.00 +/- 0.18	0.19 +/- 0.08	0.03 +/- 0.04	0.08 +/- 0.03	0.05 +/- 0.03
Transgenic	1.20 +/- 0.19	0.20 +/- 0.07	0.09 +/- 0.06	0.31 +/- 0.012	0.22 +/- 0.06

We have since achieved a 20 fold increase In production levels. Adjusted to scale, equivalent to 16 ha^{-1} $year^{-1}$.



Unfortunately, the cellulose produced was amorphous, lacking any structural integrity



Amorphous cellulose is useless for industrial or biomedical applications

However

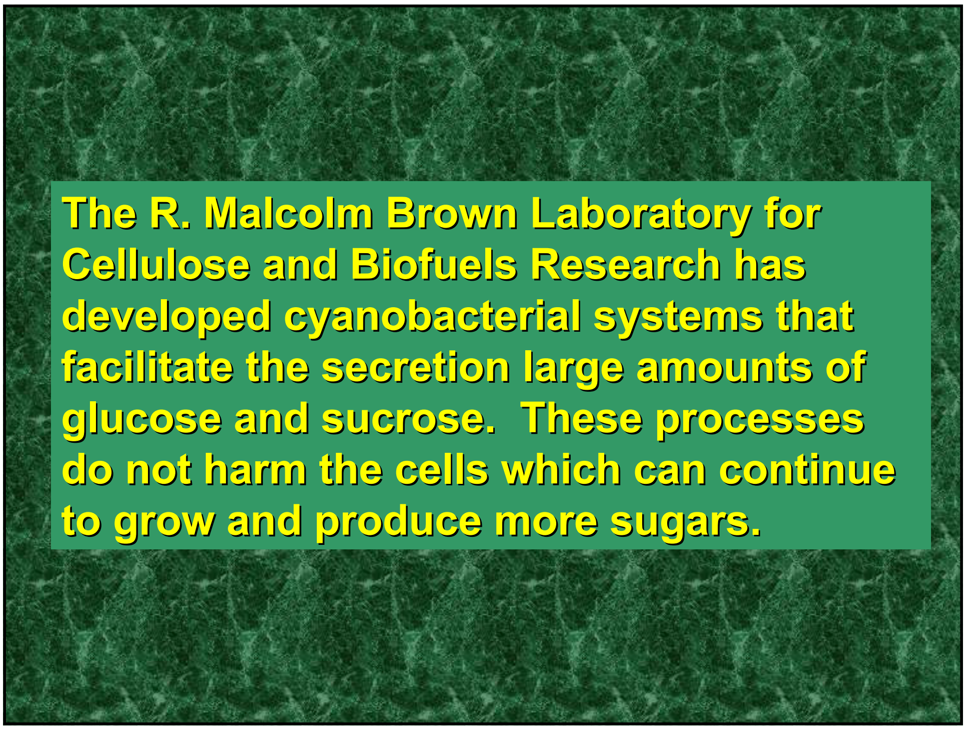
This material was free of hemicelluloses and lignin

Its lack of crystallinity would make it easy to hydrolyze

This could be great for cellulosic ethanol production!

This was our introduction into biofuels research with cyanobacteria

We have since developed more effective strategies



The R. Malcolm Brown Laboratory for Cellulose and Biofuels Research has developed cyanobacterial systems that facilitate the secretion large amounts of glucose and sucrose. These processes do not harm the cells which can continue to grow and produce more sugars.

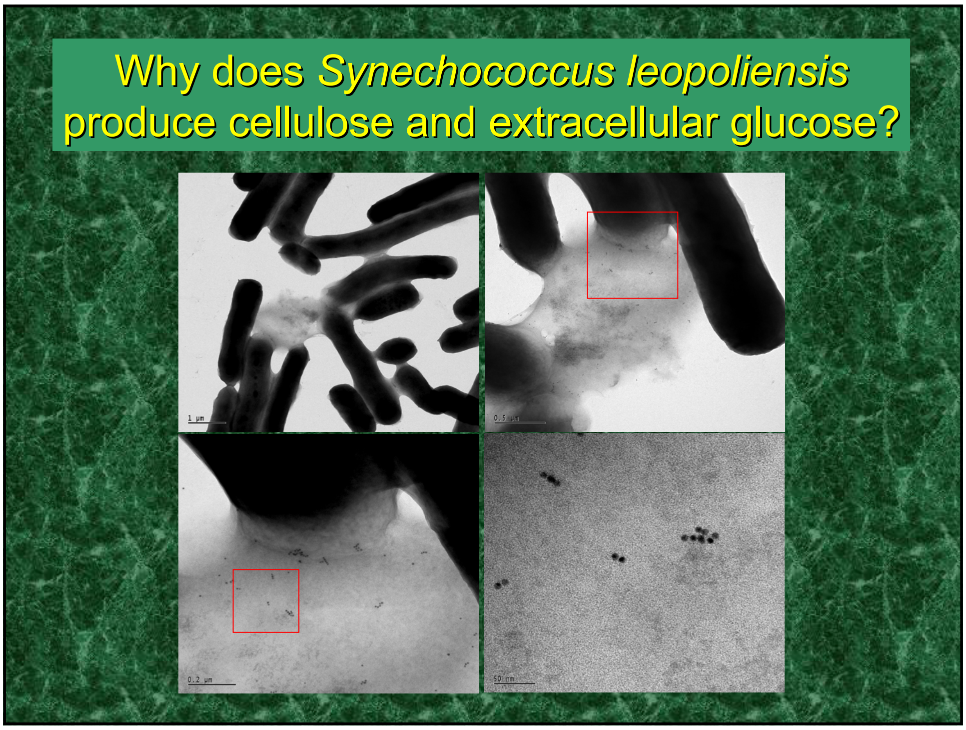
Production of extracellular glucose

The same genetically engineered strain that synthesizes and secretes cellulose, also produces extracellular glucose

Initial results for induction of extracellular glucose production

Strain	OD_{750}	Wet weight (g)	Glucose (mg/ml)	$\frac{\text{mg Glucose}}{\text{g wet weight}}$	$\frac{\text{mg Glucose}}{\text{liter}}$
Wild-type	1.65 +/- 0.13	0.35 +/- 0.10	0.12 +/- 0.06	0.17 +/- 0.25	1.03 +/- 1.40
Transgenic	1.82 +/- 0.19	0.41 +/- 0.15	1.37 +/- 0.06	3.70 +/- 1.55	34.32 +/- 1.62

We have since achieved a more than 3 fold increase in glucose production. Scaled, this is the equivalent of 19 MT ha^{-1} $year^{-1}$!



Why does *Synechococcus leopoliensis* produce cellulose and extracellular glucose?

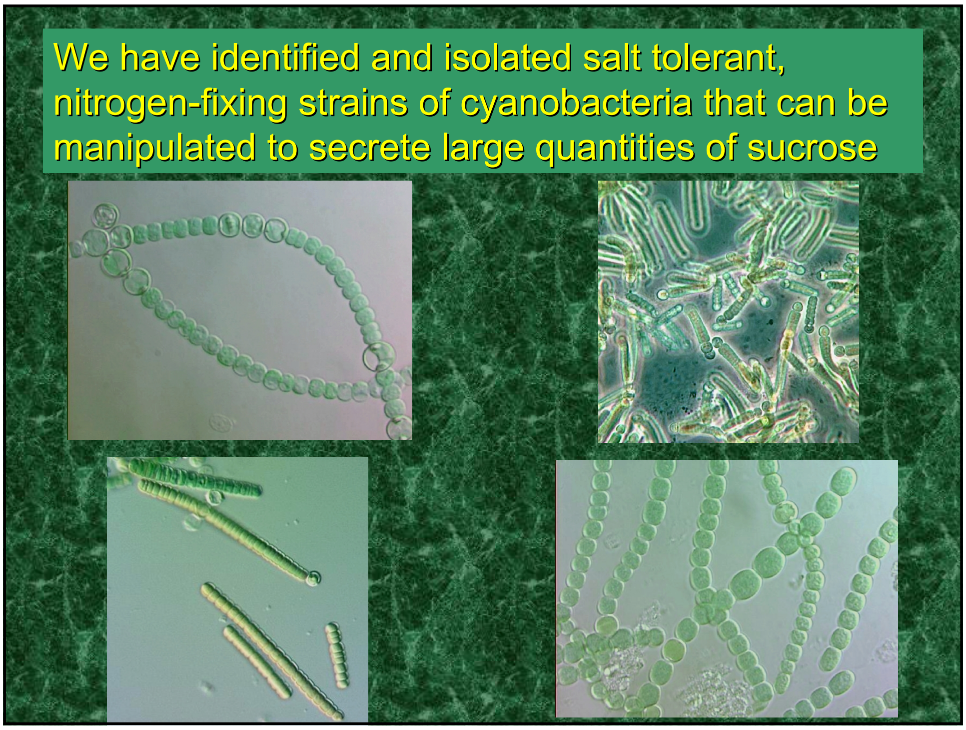
We don’t know…..yet

Its possible that a mechanism exists for secreting glucose directly

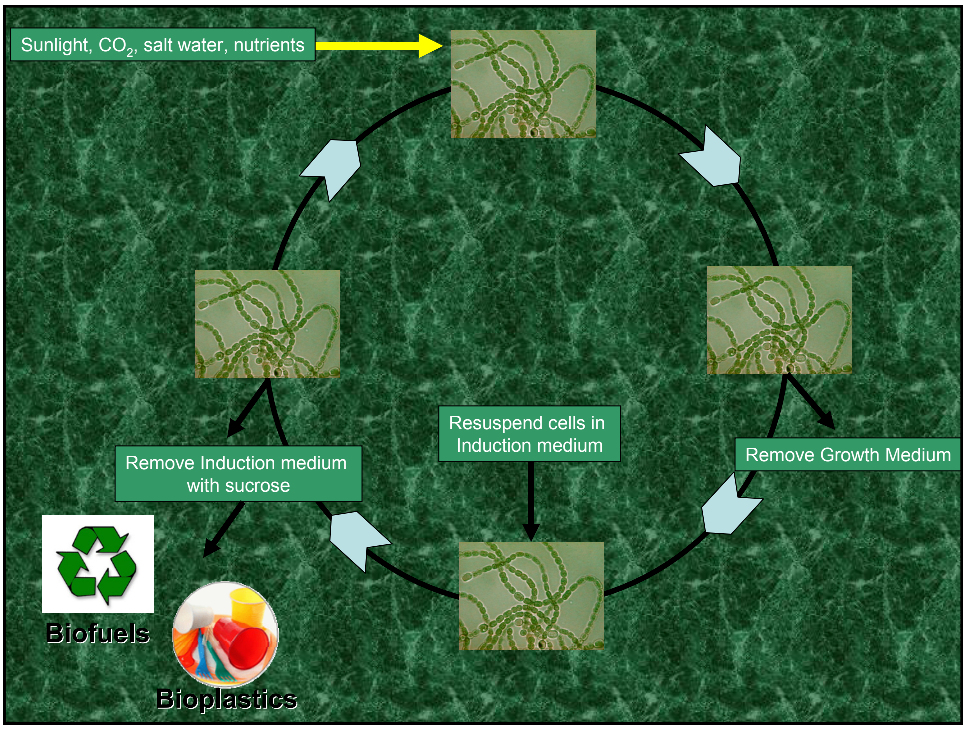
An extracellular or periplasmic glycosylhydrolase releases glucose from cellulosic material

---

Cyanobacterial Sucrose Production



We have identified and isolated salt tolerant, nitrogen-fixing strains of cyanobacteria that can be manipulated to secrete large quantities of sucrose



Sunlight, CO_2, salt water, nutrients

Remove Growth Medium

Resuspend cells in Induction medium

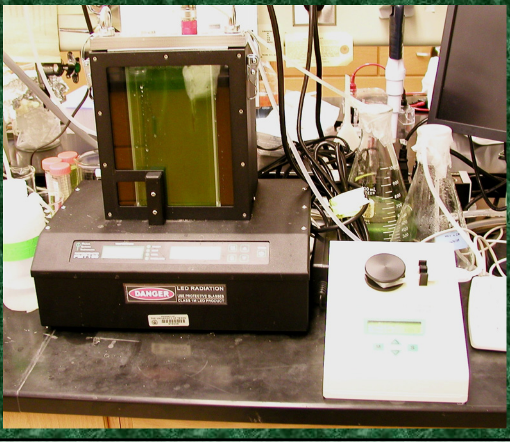
Remove Induction medium with sucrose

Biofuels

Bioplastics

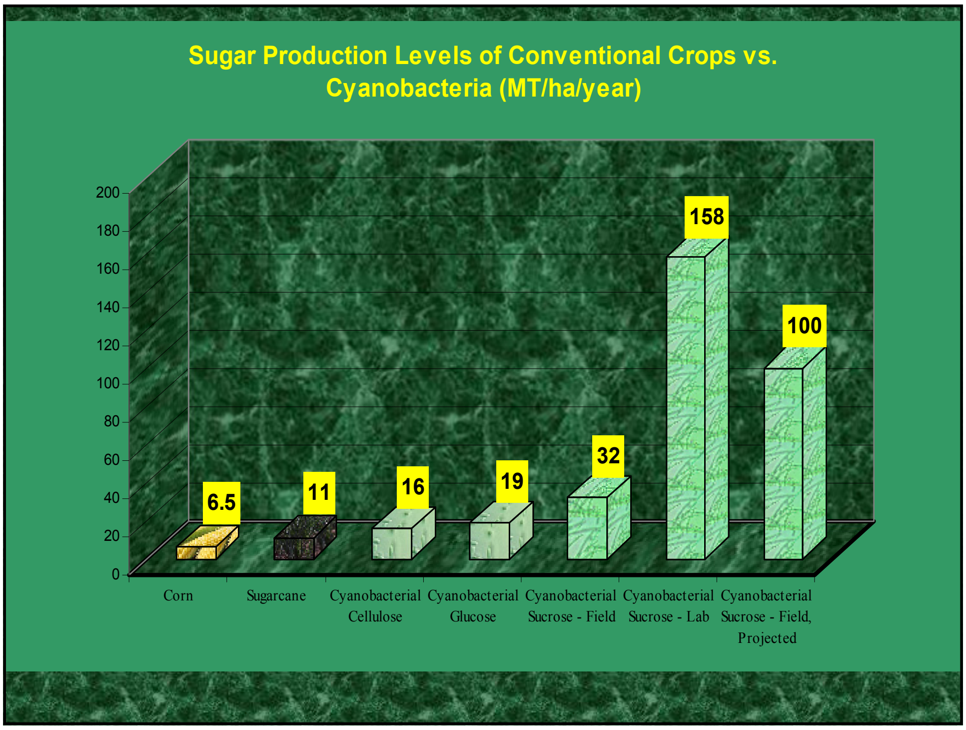
What exactly are “Large Quantities”

In laboratory photobioreactors, we have achieved sucrose production levels of 158 MT hectare^{-1} year $^{-1}$



In preliminary field trials, our salt tolerant cyanobacteria, grown at atmospheric CO_2 levels, without fixed nitrogen, produced the equivalent of 32 MT of sucrose hectare $^{-1}$ year $^{-1}$

How do these production levels compare with conventional sugar crops?



Sugar Production Levels of Conventional Crops vs. Cyanobacteria (MT/ha/year)

Mass production of sugar feedstocks from cyanobacteria will have essentially the same requirements of biodiesel production by eukaryotic algae

Potential advantages in production costs:

- Growth without nitrogen fertilizers
- Secreted products may reduce harvest costs
- Cells are not destroyed during harvest, therefore, energy can be directed to feedstock production rather than cell division

The advent of 4th generation biofuel technologies means that sucrose is not limited to ethanol production

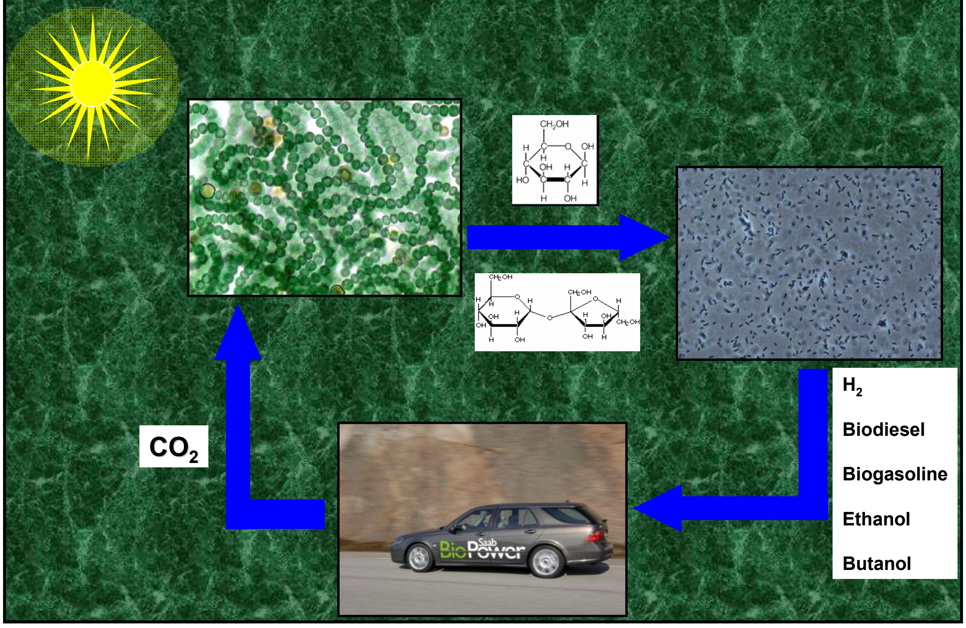
LS9, INC.

VIRENT

AMYRIS BIOTECHNOLOGIES

- Diesel
- Gasoline
- Hydrogen
- Butanol
- Dimethylfuran

The advent of 4th generation biofuel technologies means that sucrose is not limited to ethanol production



CO_2

- H_2
- Biodiesel
- Biogasoline
- Ethanol
- Butanol

Some potential advantages of 4th generation biofuels

- Fundamental changes in vehicle engineering unnecessary
- Flows seamlessly into the transportation infrastructure
- Higher energy density than ethanol
- Increase in net energy production compared to ethanol
- Unlike cellulosic ethanol, has the potential to be viable on a relatively small modular scale

We have applied for four patents related to this research:

Title	Patent Number	Year Filed	Inventors
Expression of Foreign Cellulose Synthase Genes in Photosynthetic Prokaryotes (Cyanobacteria)	20080113413	2007	R. Malcolm Brown, Jr. David R. Nobles, Jr.
Transgenic cyanobacteria: A novel direct secretion of glucose for the production of biofuels	20080085520	2007	R. Malcolm Brown, Jr. David R. Nobles, Jr.
Controlled, direct secretion of sucrose by cyanobacteria for the production of biofuels and plastics	20080124767	2007	R. Malcolm Brown, Jr. David R. Nobles, Jr.
A cellulose producing marine cyanobacterium for ethanol production	20080085536	2007	R. Malcolm Brown, Jr. David R. Nobles, Jr.

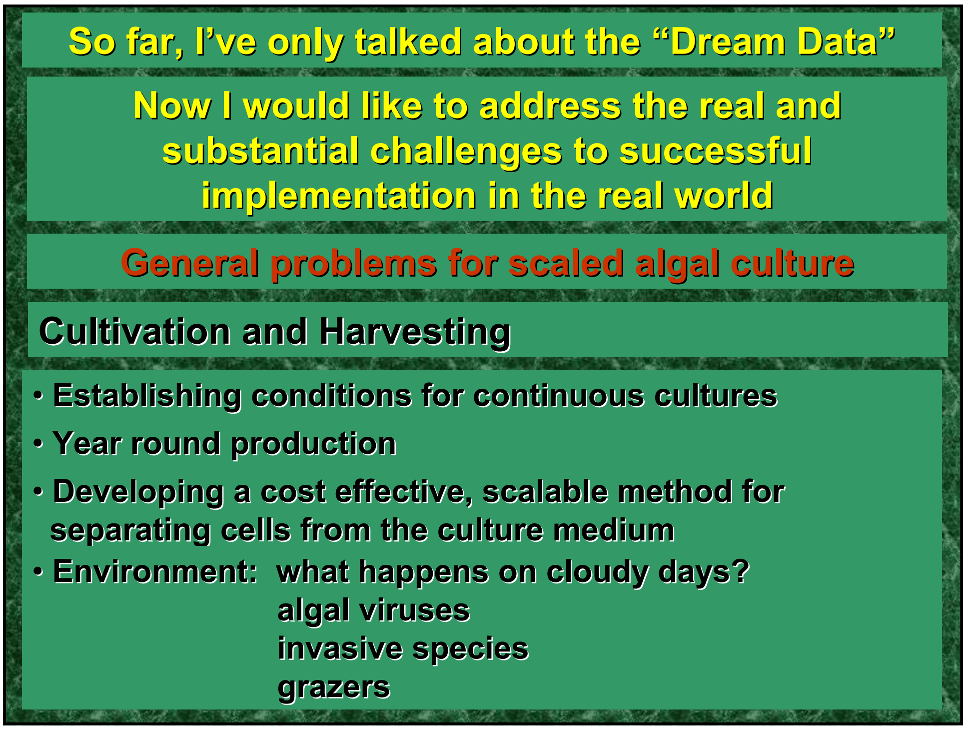
So far, I've only talked about the "Dream Data"

Now I would like to address the real and substantial challenges to successful implementation in the real world

General problems for scaled algal culture

Capital Costs: how does one obtain land and construct open or closed systems for algal cultivation at a cost that will allow investors to recoup their investments in a reasonable time?

- Use land with little monetary value or repurpose
- Reductions in costs of materials and construction
- Increase production levels: culture techniques, GM
- Multiple revenue streams: high value co-products, carbon credits, biomass, wastewater treatment, etc…



So far, I've only talked about the "Dream Data"

Now I would like to address the real and substantial challenges to successful implementation in the real world

General problems for scaled algal culture

Cultivation and Harvesting

- Establishing conditions for continuous cultures
- Year round production
- Developing a cost effective, scalable method for separating cells from the culture medium
- Environment: what happens on cloudy days?
  algal viruses
  invasive species
  grazers

So far, I've only talked about the "Dream Data"

Now I would like to address the real and substantial challenges to successful implementation in the real world

Problems more specific to our project

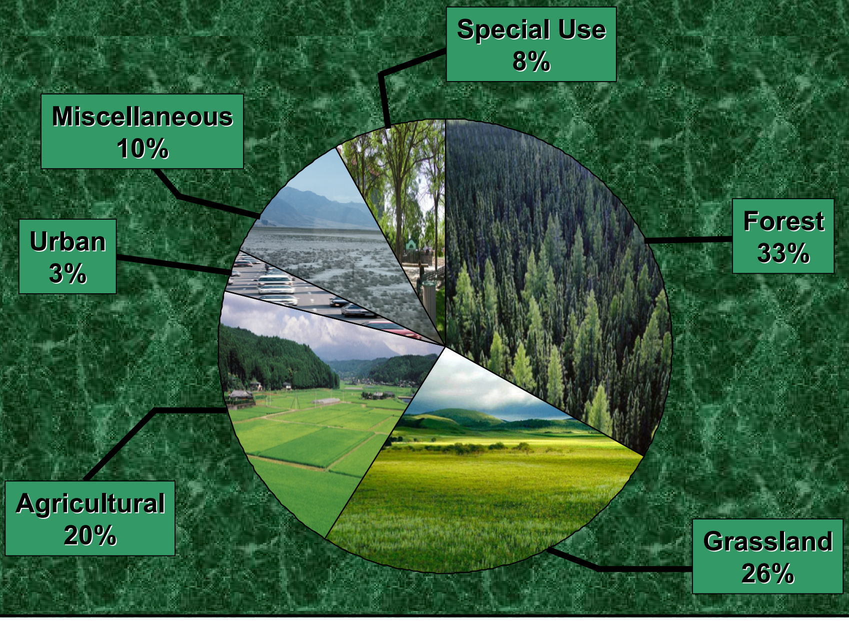
- Nitrogen fixation is not free:

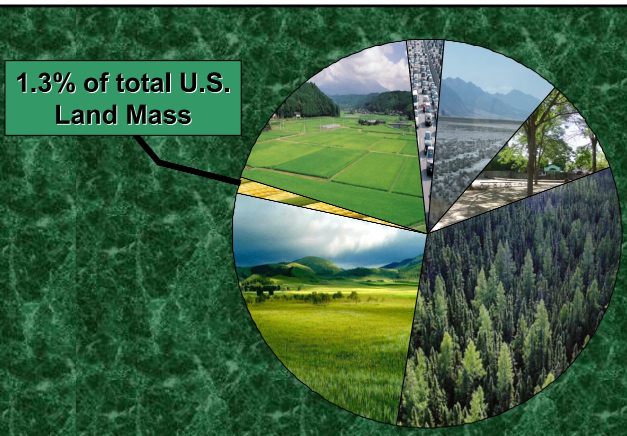
$$N_2 + 8\,H^+ + 8\,e^- + \boxed{16\,\mathrm{ATP}} \rightarrow 2\,NH_3 + H_2 + 16\,\mathrm{ADP} + 16\,\mathrm{Pi}$$

- Our strategy to achieve the production goal of 100 MT ha^{-1} $year^{-1}$ is dependent on genetic modifications: containment issues for large scale production
- Water soluble product

Biofuel Production in the United States

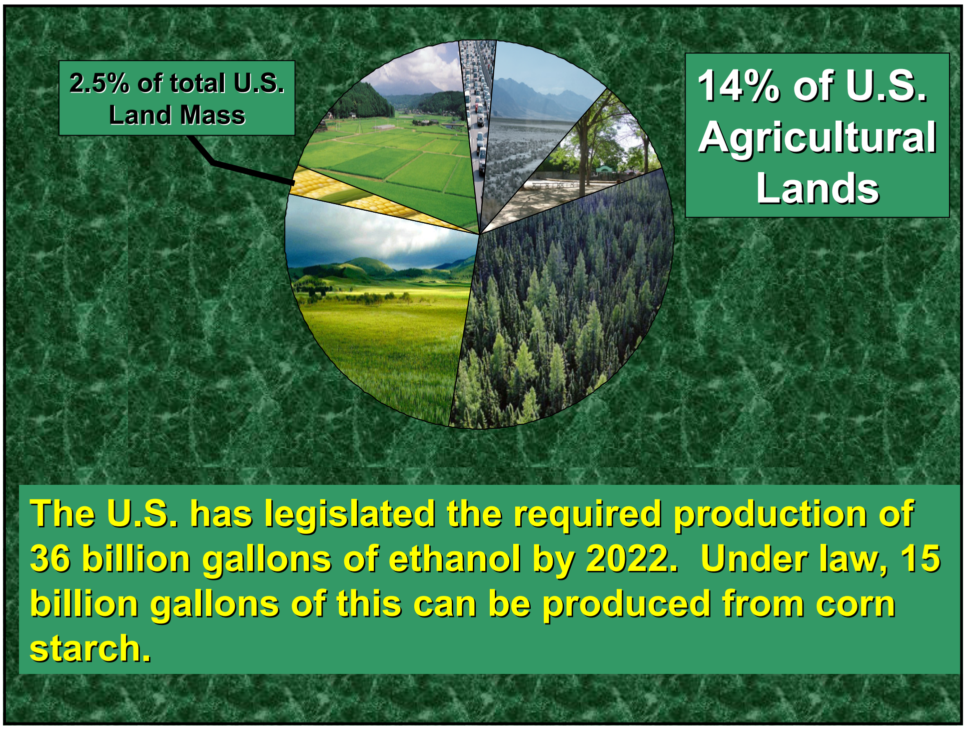
U.S. Land Usage (800 Million Hectares)





7% of U.S. Agricultural Lands

Corn ethanol production in the U.S. now exceeds the 2012 goal of 7.5 billion gallons. Corn cultivation for ethanol requires 10.3 million hectares



2.5% of total U.S. Land Mass

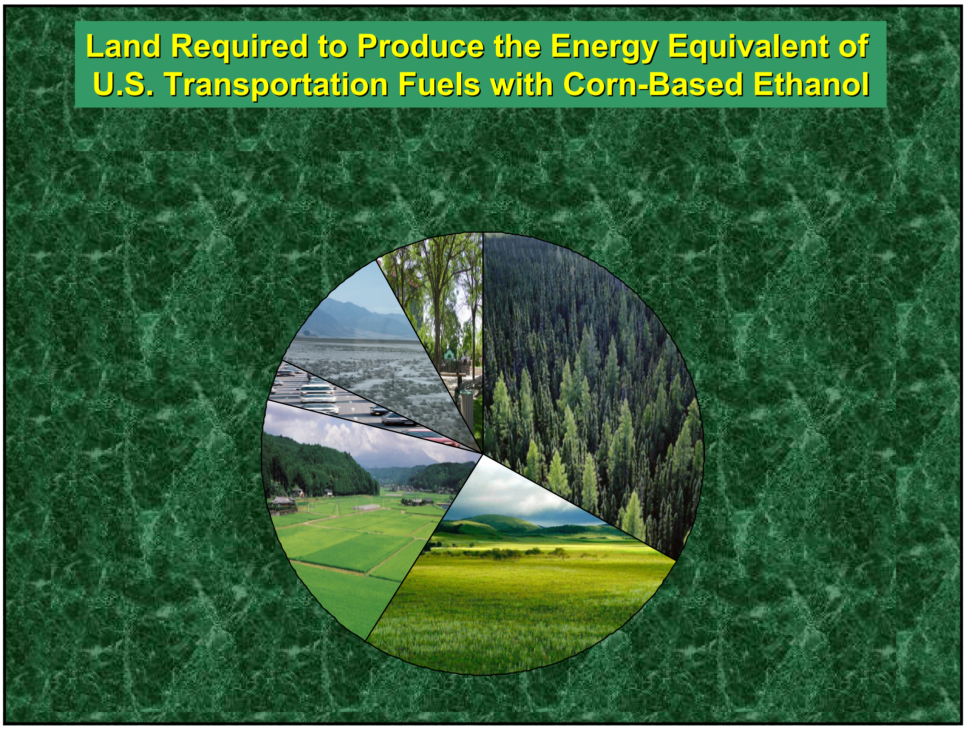
14% of U.S. Agricultural Lands

The U.S. has legislated the required production of 36 billion gallons of ethanol by 2022. Under law, 15 billion gallons of this can be produced from corn starch.

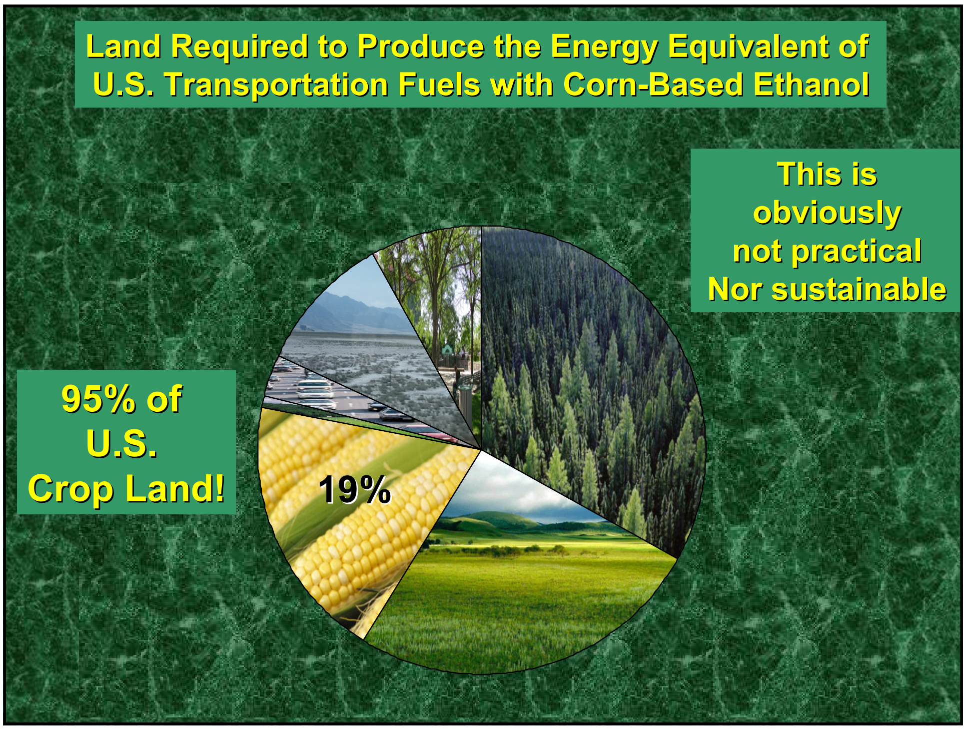
2.5% of total U.S. Land Mass

For the production of the energy equivalent of just over 8% of U.S. transportation fuels

The U.S. has legislated the required production of 36 billion gallons of ethanol by 2022. Under law, 15 billion gallons of this can be produced from corn starch.



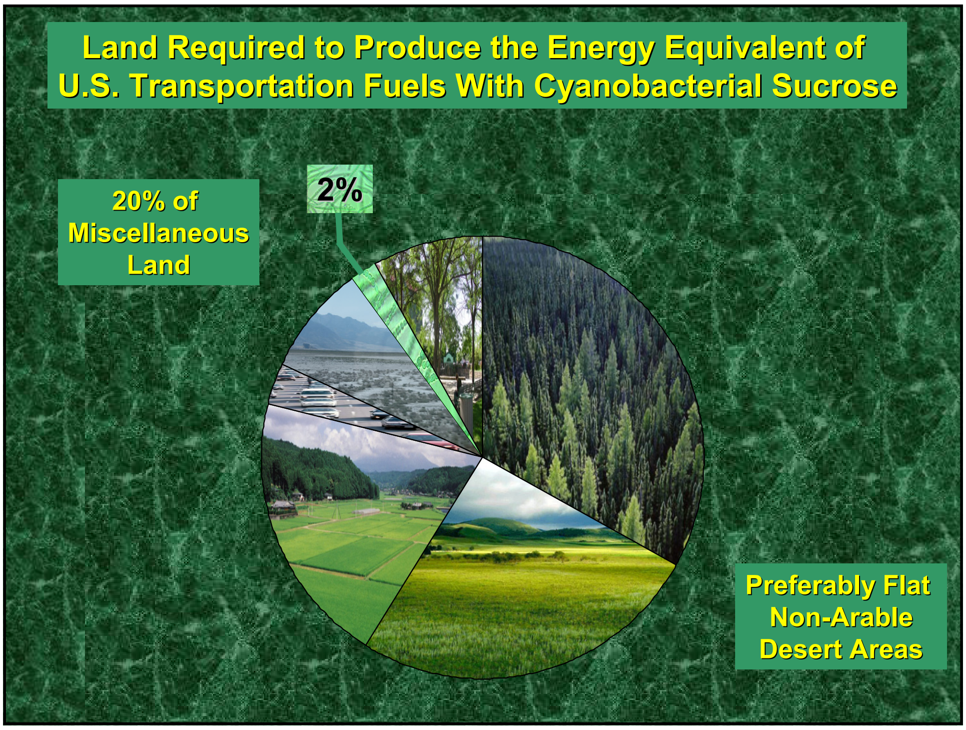
Land Required to Produce the Energy Equivalent of U.S. Transportation Fuels with Corn-Based Ethanol



Land Required to Produce the Energy Equivalent of U.S. Transportation Fuels with Corn-Based Ethanol

This is
obviously
not practical
Nor sustainable

95% of
U.S.
Crop Land!



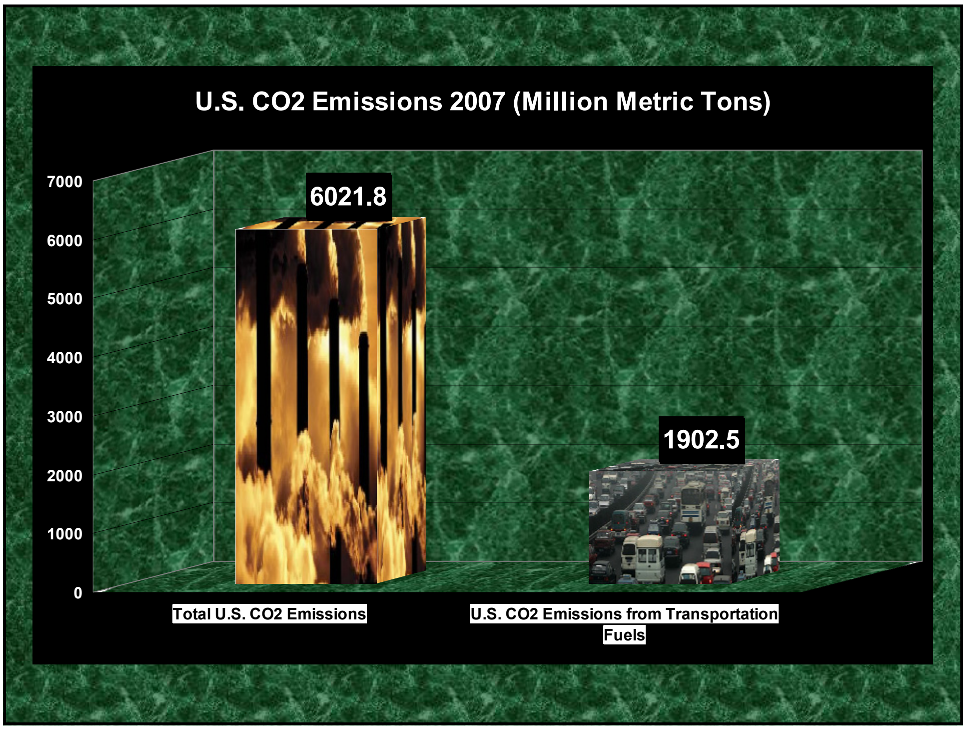
Land Required to Produce the Energy Equivalent of U.S. Transportation Fuels With Cyanobacterial Sucrose

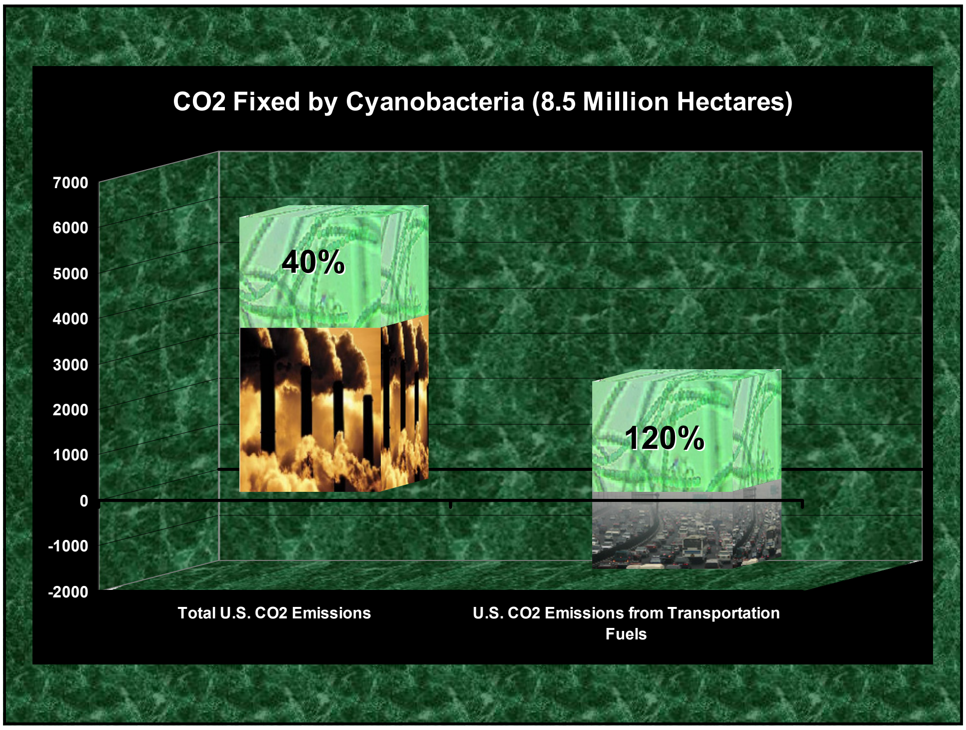
20% of Miscellaneous Land

2%

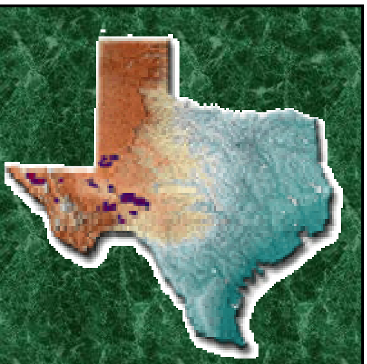
Preferably Flat Non-Arable Desert Areas

Cultivation of cyanobacteria on this scale could have a positive impact on CO_2 emissions


U.S. CO2 Emissions 2007 (Million Metric Tons)


CO2 Fixed by Cyanobacteria (8.5 Million Hectares)

Texas System Lands
850,000 hectares = 0.1% of U.S. land



Current Scenario-Projected productivity would be more than 4.2 billion gallons of ethanol per year (based on the present lab production rate of 2000 gallons of ethanol/acre/year)

Future Production – 100 MT ha^{-1} year^{-1}
At this level, the 850,000 hectares of UT lands would yield 85 MMT per year : **5% of the total US annual transportation fuel!**

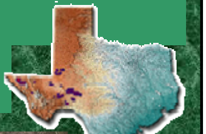
Role of the University of Texas
In RENEWABLE ENERGY & BIOFUELS RESEARCH
UT Oil Lands for the production of Biofuel Feedstocks

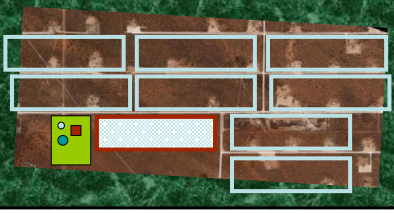
We plan to use rejuvinated oil fields!

The perfect solution!

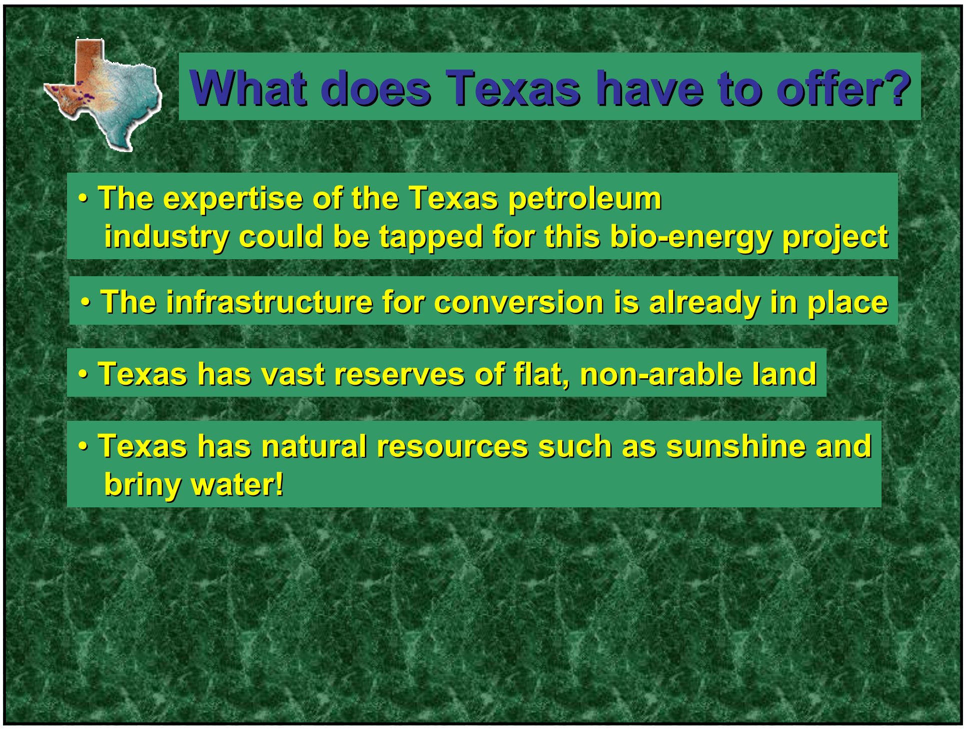
1. Briney water
2. CO$_2$ available
3. Energy infrastructure already in place
4. On non-arable lands
5. Flat land

2.1 Million acres of UT LANDS



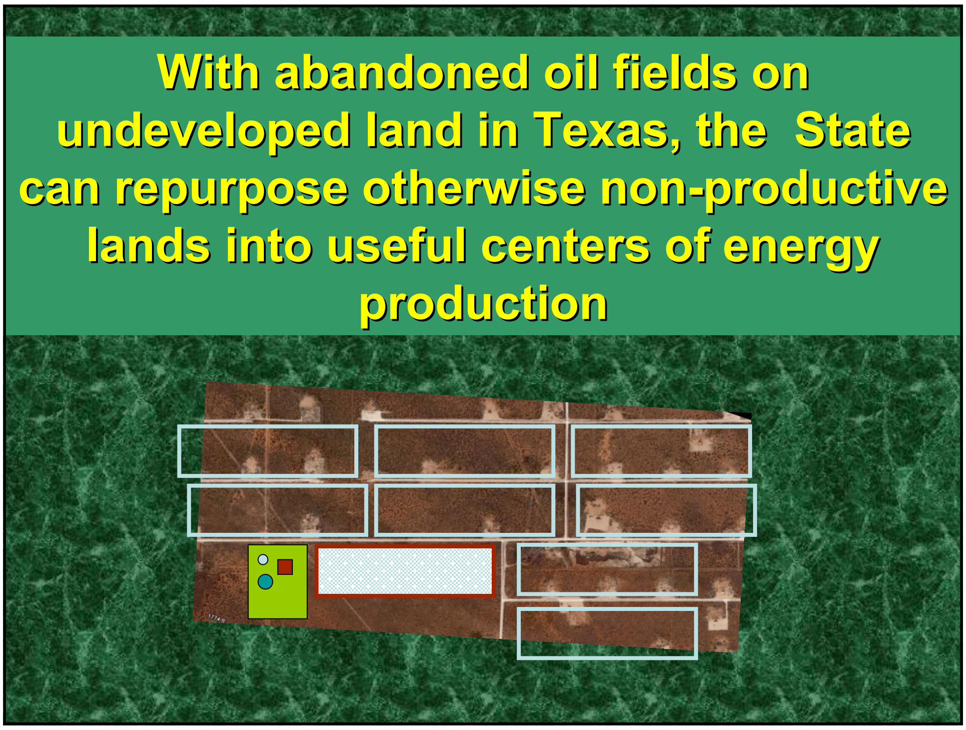


Scaleup plans for cyanobacterial ponds



What does Texas have to offer?

- The expertise of the Texas petroleum industry could be tapped for this bio-energy project
- The infrastructure for conversion is already in place
- Texas has vast reserves of flat, non-arable land
- Texas has natural resources such as sunshine and briny water!



With abandoned oil fields on undeveloped land in Texas, the State can repurpose otherwise non-productive lands into useful centers of energy production

What is Needed?

A **"Manhattan"** type of project is urgently needed to stay ahead of the competition and to put the USA on the road to a sustainable recovery in biofuels and to reduce global climate change.

The replacement of fossil fuels with renewable fuels from algae will require the dedicated efforts of many scientists and engineers

BUT ALSO

Business leaders and venture capitalists

In concert with

Politicians, diplomats, and policymakers

The challenges on the path to renewable fuels from cyanobacteria are tremendous

But not as tremendous as the opportunities!

Thank You For
Your Time Today