Photosynthetic production of biofuels from CO$_2$ by cyanobacteria using Algenol’s Direct to Ethanol® process – Strain development aspects

Paul Roessler - Oct 1, 2014 – Algal Biomass Summit
### Algenol Overview

**Advanced Industrial Biotechnology Company**
- Started up in 2006
- Headquartered in Fort Myers, Florida
- Research Labs in Florida and Berlin, Germany
- ~200 employees including >70 with advanced degrees

**Research and Development Facilities**
- 60,000 ft² of Research and Development lab space in Fort Myers and Berlin, Germany
- 4 acre Process Development Unit (PDU)
- 36 acre Integrated Bio-Refinery (IBR)

**Commercializing Direct To Ethanol® Technology**
- >$200M equity capital
- $25M Department of Energy Integrated Biorefinery grant
- $10M economic development grant from Lee County, FL

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Fort Myers Research Labs

Process Development Unit

Integrated Biorefinery [Paul Woods & Ed Legere]
$250 million investment
200 dedicated people
100 scientists
8 buildings
8 years
#### Disruptive Core Technology

Algenol's Direct to Ethanol® process has three main components:

<table>
<thead>
<tr>
<th>Highly Productive Algae Platform</th>
<th>Specialized VIPER™ Photobioreactors (PBRs)</th>
<th>Energy Efficient Downstream Processing</th>
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</thead>
</table>
| Proprietary enhanced algae make ethanol and biomass directly from CO₂, water, and sunlight.  
  - **8,000 gallons per acre per year**  
  - 85% of the CO₂ is converted into products | Algae are grown in saltwater contained in proprietary PBRs that are exposed to the sun and fed CO₂ and nutrients.  
  - After production phase, the spent algae are separated from the water-ethanol mixture | Water-ethanol mixture is sent to proprietary downstream processing equipment, which separates and concentrates it to fuel grade ethanol.  
  - Spent algae are processed into a high grade bio-crude that can be refined into diesel, gasoline, and jet fuel |
Commercial Vision

- Closed photobioreactors (seawater)
- Very low freshwater consumption
- Non-arable land

CO₂ can be sourced from:
- Power Plant
- Refinery or Chemical Plant
- Cement Plant
- Natural Gas Well
### Core Technology: Enhanced Cyanobacteria

<table>
<thead>
<tr>
<th>Algenol’s Direct to Ethanol® process uses enhanced cyanobacteria to produce ethanol</th>
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<tbody>
<tr>
<td>▪ 2,300 strains collected globally and screened as candidates for development.</td>
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<td>▪ Fermentation pathway enzymes are over-expressed to enhance ethanol production.</td>
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<td>▪ Commercial strains have been selected and are being optimized.</td>
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<td>▪ Main product is ethanol, but also convert biomass to hydrocarbons in the gasoline, diesel, and jet fuel ranges.</td>
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</table>

![Diagram showing the process of photosynthesis, carbon fixation, intracellular fermentation, and the chemical reaction for ethanol production from CO₂ and H₂O.](image_url)

\[
2 \text{ CO}_2 + 3 \text{ H}_2\text{O} \rightarrow \text{C}_2\text{H}_5\text{OH} + 3 \text{ O}_2
\]
Metabolic Pathway for Ethanol Production

- Direct linkage of ethanol synthesis to carbon fixation via five enzymatic steps

**Simplified network of central carbohydrate metabolism in cyanobacteria**

- **CO₂** → RuBisCo → Calvin cycle
- **RuBP** → **Ru5P** → **3PGA** → **1,3bisPGA** → **PGM** → **2PGA** → **PEP** → **PYK** → **pyruvate** → **PDC** → **acetaldehyde** → **EtOH**

**Calvin cycle**
- **RuBP** → **Ru5P** → **3PGA** → **1,3bisPGA** → **PGM** → **2PGA** → **PEP** → **PYK** → **pyruvate** → **PDC** → **acetaldehyde** → **EtOH**

**TCA cycle**
- **malate** → **fumarate** → **succinate** → **isocitrate** → **citrate** → **OAA** → **acetyl CoA** → **acetate** → **EtOH**

**Glycolysis**
- **G6P** → **F6P** → **FBP** → **ADP** → **ATP** → **2PGA** → **2OG** → **Glu** → **pHB** → **CO₂** → **NADH + H⁺** → **NAD⁺** → **Pi**

**OPPP**
- **gluconate-6P** → **H₂O** → **NADPH + H⁺** → **ADP** → **G1P** → **G6P** → **ADP-Glc** → **glycogen**

**PDC** = Pyruvate decarboxylase
**ADH** = Alcohol dehydrogenase
Application of Systems Biology Tools

• Algenol utilizes state-of-the-art ‘omics and other systems biology tools to identify and prioritize strain optimization strategies
  • coupled with biochemical, physiological, and photobiological assays
• Independent technologies utilized to confirm findings or provide complementary experimental results
  • genomics
  • transcriptomics
  • proteomics
  • metabolomics and flux modeling
Genomics

- Top candidate strains were sequenced by several methods
- Provided phylogenetic information
- Revealed presence of potential restriction enzymes
- Established the presence and sequence of endogenous plasmids
  - leveraged for transformation system developed
- Enabled evaluation of metabolic pathways present in commercial host strains, including competing pathways
  - potential knockout and overexpression targets identified
- Codon usage calculated to optimize synthetic gene design
Key considerations for ethanol cassette design:

- High PDC and ADH activities lead to increased partitioning of fixed carbon into ethanol
  - Solution: strong promoters, optimized genes
- Growth of ethanol-producing cells is slower than non-producing cells
  - Fast culture growth is *desirable* during scale up phase, but *undesirable* during ethanol production phase
  - Solution: use an inducible promoter for ethanol genes (esp. *pdc*)
Transcriptomics

- **Identification of Promoters via RNA-Seq**

  - Both inducible and constitutive promoters of different strengths are desirable for strain optimization
  - RNA-Seq used to assess mRNA levels under different growth conditions or stages of cultivation

- Example: metal-inducible promoters
  - nickel, zinc, copper, cobalt
  - mRNA levels measured before and after addition of metals
  - results confirmed by qRT-PCR
Metal-Inducible Promoters ID’d by RNA-Seq

- Bioinformatics-based identification of metal-inducible promoters is often not possible

<table>
<thead>
<tr>
<th>ORF No.</th>
<th>Description</th>
<th>Inducer</th>
</tr>
</thead>
<tbody>
<tr>
<td>0128</td>
<td>Hypothetical protein</td>
<td>Ni</td>
</tr>
<tr>
<td>1486</td>
<td>Putative Ni-containing Superoxide Dismutase</td>
<td>Ni</td>
</tr>
<tr>
<td>3621</td>
<td>Hypothetical protein</td>
<td>Ni</td>
</tr>
<tr>
<td>1071</td>
<td>Mn transporter</td>
<td>Zn</td>
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<tr>
<td>1542</td>
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<tr>
<td>1824</td>
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<td>Zn</td>
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<tr>
<td>3126</td>
<td>Metallothionein</td>
<td>Zn</td>
</tr>
<tr>
<td>0221</td>
<td>Copper resistance protein</td>
<td>Cu</td>
</tr>
<tr>
<td>0316</td>
<td>Hypothetical protein</td>
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<tr>
<td>3232</td>
<td>Cation transporting ATPase</td>
<td>Cu</td>
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<tr>
<td>3749</td>
<td>Hypothetical protein</td>
<td>Co</td>
</tr>
</tbody>
</table>
Validation of Metal-Inducible Promoters

- PDC activity vs copper concentration:
Patterns of Induction by Metal Salts

- Multiple additions of inducer (CuSO₄) maintain high PDC activity and ethanol productivity
Chelated Metal Induction

- One-time addition of Cu-EDTA simplifies the induction protocol

![Graph showing PDC activity vs. time for different Cu-EDTA concentrations.](image-url)
Modification of a Zinc-Inducible Promoter

Porf3126 (PsmtA) native

Porf3126* optimized (modified nucleotides indicated in red)

![Graph showing PDC Activity](chart.png)
Optimization of the *nirA* Promoter

- Modification of various *nirA* promoter structural elements has provided a spectrum of expression cassettes
  - *ntcA* and *ntcB* binding sites, RBS, and TATA box modifications

<table>
<thead>
<tr>
<th>PnirA</th>
<th>PnirA*1</th>
<th>PnirA*2</th>
<th>PnirA*3</th>
<th>PnirA*4</th>
</tr>
</thead>
<tbody>
<tr>
<td>12x</td>
<td>10x</td>
<td>14x</td>
<td>8x</td>
<td>7x</td>
</tr>
</tbody>
</table>

- **PDC activity [µmol/min*mg protein]**

- **Samples:**
  - 1578-NO3
  - 1578+NO3
  - 1701-NO3
  - 1701+NO3
  - 1658-NO3
  - 1658+NO3
  - 1697-NO3
  - 1697+NO3
  - 1663-NO3
  - 1663+NO3
Carbon Partitioning into Ethanol

- 70-90% of the stably fixed carbon in Algenol’s production strains is partitioned into ethanol
Many Potential Gene Targets Identified

- Transcriptomics, metabolomics, and $^{13}$C flux analyses are conducted to identify:
  - bottlenecks for ethanol biosynthesis
  - metabolic imbalances
  - stress responses that result from expression of ethanol synthesis genes
  - regulation of photosynthesis
Summary

• Algenol has developed world-class production host strains suitable for commercial deployment
• Patented methods and tools for the genetic enhancement of Algenol’s strains have been established and successfully used to produce strains with high ethanol productivity
• We are gaining a greater understanding of the impacts of ethanol production on the biochemistry and physiology of the production strains in order to prioritize new strain improvement strategies