Biocatalytic solar fuels for sustainable mobility in Europe

AlgaeEurope 2016

Hilke Heinke
**Outline**

**Overall objective:** Promote the development of high quality, low impact transportation fuels

**Challenges:** Photobiocatalyst development - Synthesis of fuel compounds - Excretion to medium – Direct, continuous separation – Fuel blending – Engine tests
Approach

WP 2 Biocatalyst development
- 2 cyanobacterial strains 1 microalgae
- Target fuels butanol, medium chain alcohols and alkanes, sesquiterpenes.
- Best strain(s) selected and jointly improved in the last 18 month of the project.

WP 3 Production upscaling
- Final volume 5m$^3$
- Outdoor production fuel compounds
- Continuous fuel separation

WP 4 Fuel blending
- Analyse raw products
- Blend on-spec fuels

WP 5 Engine tests
- Analyse raw products
- Blend on-spec fuels

WP6 Assessment
- LCA
- Economic
- Risks

WP7 Business case
- Business case development
Project plan

Management & Dissemination

Biocatalyst development

Cultivation / fuel production

Fuel blending

Engine tests

Assessment

Business case

2016  2017  2018  2019

*Milestone

Biocatalyst selection

Fuel production at... 120 L

5000 L

Assessment criteria agreed


Photofuel – Status Slides
EU-H2020 grant No. 640720
Bielefeld University

Bisabolene synthesis in the green algae

*Chlamydomononas reinhardtii*

Kyle Lauersen, Julian Wichmann, Olaf Kruse
Biocatalyst development (UniBi)

Bisabolene synthase in *C. reinhardtii*

*Abies grandis* bisabolene synthase (*AgBs*)
2451 bp (817 aa)

For *C. reinhardtii* expression: intron addition = 3321 bp

Converts FPP $\rightarrow$ Bisabolene $C_{15}H_{24}$

High specificity

~100% (*E*-α-bisabolene as product
(vs. *PcPs* ~34% patchoulool synth.)
**Strategy**

*Abies grandis* bisabolene synthase = *AgBs* fused to mVenus (YFP) reporter

Constructs transformed and mutants screened for fluorescence.

More fluorescence = more synthase = more product

Farnesyl pyrophosphate (FPP) → **Bisabolene synthase (AgBs) – YFP fusion**
Biocatalyst development (UniBi)

First results: Successful production of Bisabolene!

Simply identifiable in dodecane by GC MS

Fluorescence positive clones produce (E)-α-bisabolene

243 µM standard

AgBs strain

Parental strain
Biocatalyst development (UniBi)

Increase fuel production by shift of C-partitioning
Typically 75% proteins and sugars, 20% lipids, 5% terpenes
→ knock-down starch accumulation
→ ...

20 μl culture
YFP
AgBS linked
Luminescence
amiRNA linked
-N Iodine
Starch stain

WT  Bis  1  2  3  4

Bisabolene μg/g DBM

1xBis  AGPP

19.10.2016

Photofuel – Status Slides
EU-H2020 grant No. 640720
Uppsala University
1-butanol and isobutanol in *Synechocystis*
Pia Lindberg, Rui Miao, Xufeng Liu, Peter Lindblad
Pathways to desired products in *Synechocystis* sp. PCC 6803:

**Pyruvate**

- Glycolysis
- Lactate

**Acetyl-CoA**

- Acetyl-CoA
- Malonyl-CoA
- Butyryl-CoA

**Fatty acid biosynthesis**

- **Red:** native *Synechocystis* genes
- **Green:** heterologously expressed genes
- **X:** Knock-out

**Butyric acid**

**Butyraldehyde**

**Crotonyl-CoA**

**Malonyl-CoA**

**Butyryl-ACP**

**L-Valine**

**2-Ketovalerate**

**L-leucine**

**1-Butanol**

**L-Threonine**

**2-Ketoisovalerate**

**Isobutyraldehyde**

**Isobutanol**

**Butyroldehyde**

**2-Ketobutyrate**

**Acetyl-ACP**

**Acetate**
First results 1-Butanol:
Optimised clostridial- and reverse β-oxidation pathway leads to significant 1-Butanol production rates.
Biocatalyst development (UU)

Knock-out of competing pathways to storage compounds and N-limitation leads to biocatalytic behaviour: Growth and 1-Butanol production are uncoupled.
Imperial College
Medium-chain alcohols and alkanes in cyanobacteria
Patrik Jones, Dipankar Ghosh, Ian Yunus
Biocatalyst development (Imperial)

4 step approach:
1. Increase fatty acid production (+reverse β-oxidase pathway)
2. Set FA-chain length (add thioesterase)
3. Knock-out FFA-recycling (knock-out acyl-ACP synthetase, Aas)
4. Convert free FA to alcohols and alkanes (add reductases)

Cyanobacteria

Key Connecting Enzyme

Photosynthesis
- ATP & NADPH
- CBB Cycle
- Acetyl CoA
- FAS II Cycle
- β_rev Oxidation
- Acyl-CoA

Thioesterases

Acyl-ACP

FFAs (Precursor)

Fatty Aldehydes
Fatty Alcohols
Fatty Alkanes

Free Fatty Acids (FFA) as precursor for biofuels and biochemicals
First results in *Synechococcus 7002* with aas-knockout (no FFA-recycling), different Tes (FFA-chain length) and RBO addition (2\textsuperscript{nd} fatty acid-production pathway) showed high increase in FFA amounts and visible flocks in the broth.
Composition of free fatty acids
- The spectrum of FFAs varied depending on the thioesterase
- The precursors for the Photofuel target fuels 1-Octanol and n-Undekane were produced in considerable shares

→ Next steps:
- FFA reduction to aldehyde and alcohol
- Decarbonylation of fatty-aldehyde to alkane

TesA'

Tes4

TesA' (L109P)

Tes3

C12
WP3: Upscaling & Production

Objectives and approach:

• Optimise cultivation, assess productivity, scale up 1L → 120L → 5000L, batch to continuous, lab to outdoor
• Develop continuous cultivation and product separation system up to pilot scale
• References: Lipid-strains, *B. braunii*
• Fuel compounds for upgrading and testing
• Data for LCA- and economic assessment
University of Florence
Test of wild-type strains of biocatalysts and biofuel reference strains *Botryococcus braunii* and *Nannochloropsis oceanica*
Natascia Biondi, Mario Tredici
Upscaling & Production (UniFi)

**Wild-type Strains to be Tested**

- *Chlamydomonas reinhardtii* CC124; CC400; CC1690; CC1883
- *Synechocystis* PCC 6803
- *Synechococcus* PCC 7002

**Ongoing Activity: Lab & Small Outdoor**

Culture medium optimisation for outdoor cultivation

- preliminary outdoor trials
- indoor cultivation to verify observations emerged during outdoor trials

**Next Steps**

- All strains: Outdoor cultivation at small-scale to verify performances and select the strain for further upscale
Up-scaling & Production (UniFi)

**Synechocystis PCC 6803, Synechococcus PCC 7002**

**Outdoor Growth Experiments**

Maximum temperature reached: **48 °C**

Average daily temperature gradient: **25±7 °C**

*Synechococcus* suffers low night temperature

*Synechocystis* is less sensitive, probably suffering more the high maximum temperatures
Upscaling & Production (UniFi)

**REFERENCE STRAINS TO BE TESTED**

- *Botryococcus braunii* Showa
- *Botryococcus braunii* SAG30.81
- *Nannochloropsis oceanica* F&M-M24

**ONGOING ACTIVITY: OUTDOORD & LABORATORY CULTURES**

- outdoor preliminary trials with both *B. braunii* strains
- laboratory comparison between *B. braunii* Showa and SAG 30.81
- trials of hydrocarbon extraction from *B. braunii* and culture tolerance to solvents

**NEXT STEPS**

- Further extraction tests of *B. braunii* hydrocarbons
- *B. braunii*: outdoor cultivation
- *Nannochloropsis*: cultivation at pilot scale
Imperial College London
Upscaling of biocatalysts and development of a continuous production and fuel separation system
Klaus Hellgardt
GMM-Upscaling (Imperial)

**Batch Process**

- **Nutrient Optimisation**
  - macro and micronutrients

- **Environmental Parameters**
  - Light (Intensity and light-dark cycles)
  - Temperature
  - pH
  - Kinetics

**Continuous Process**

- **Separation Techniques**
  - Efficiency
  - Bio-compatibility
  - Cost

- **Chemostat Operation**
  - Continuous Production
  - Contamination
  - Nutrient control
  - Kinetics

- **Scale-Up**
  - Demonstration
  - Continuous Production
  - Contamination
  - Kinetics
- Kinetics of growth and bisabolene production (shake flask vs 1L photobioreactor)
- Established GC and GC-MS protocol for bisabolene analysis
- Results and observations:
  1. Cultures grow well under standard conditions
  2. Quantitative bisabolene separated via extraction into dodecane
  3. Bisabolene production beyond max biomass concentration indicates biocatalytic or catabolic pathway
  4. Cell density and associated bisabolene productivity increase in flat plate bioreactor due to better control of environomics
5. Fitting of growth curves using logistic function yields maximum growth rate

6. Cultures respond well to increased light intensity, bisabolene productivity increases by 75% between 20 and 100 µEs⁻¹m⁻²

7. Clear evidence of light inhibition at 300 µEs⁻¹m⁻²

8. Optimal growth conditions appear to be 100 µEs⁻¹m⁻² at a temperature of 30 °C

9. Ongoing work on light-dark cycles and optimal pH

10. Ongoing work on macro nutrient uptake
Upscaling & Production (A4F)

A4F
Upscaling of biocatalysts and validation on pilot scale
Diana Fonseca, Vitor Verdelho
**Chlamydomonas reinhardtii - Bisabolene**

### Bubbling effect on growth rate

- 29/08/2016 – 12/09/2016;
- 25°C;
- Air:CO₂ mixture;
- Continuous one-side 100 μmol photons m⁻² d⁻¹.

- **Culture seem to grow very well in both media.**
- **Samples will be sent to UniBi for bisabolene analysis.**

### Average growth rate

<table>
<thead>
<tr>
<th></th>
<th>OD750</th>
<th>CC (Muse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A4F industrial medium</td>
<td>0.36 d⁻¹</td>
<td>4.93E+06 cel mL⁻¹·d⁻¹</td>
</tr>
<tr>
<td>TPNO₃</td>
<td>0.38 d⁻¹</td>
<td>4.72E+06 cel mL⁻¹·d⁻¹</td>
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</table>
Synechocystis sp. PCC 6803 - Butanol

Culture medium trials

- 26/08/2016 – 14/09/2016;
- Orbital Shaker at 80-130 rpm;
- 25°C;
- Gaseous diffusion;
- Continuous 26 µmol photons m⁻² d⁻¹.

Average growth rate (OD730)

<table>
<thead>
<tr>
<th>Culture Condition</th>
<th>Average Growth Rate (d⁻¹)</th>
<th>Standard Deviation</th>
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</thead>
<tbody>
<tr>
<td>BG11</td>
<td>0.12</td>
<td>0.01</td>
</tr>
<tr>
<td>BG11 + 10 g/L NaCl</td>
<td>0.11</td>
<td>0.01</td>
</tr>
<tr>
<td>A4F industrial medium</td>
<td>0.14</td>
<td>0.01</td>
</tr>
<tr>
<td>A4F industrial medium + 10 g/L NaCl</td>
<td>0.12</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Culture seems to grow very well with all media conditions.
**Synechocystis** sp. PCC 6803 - Butanol

**Bubbling effect on growth rate**

- 15/09/2016 – 03/10/2015;
- 25°C;
- Air:CO₂ mixture;
- Continuous one-side 100 µmol photons m⁻² d⁻¹.

**Average growth rate (OD730)**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Average</th>
<th>St Dev</th>
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</thead>
<tbody>
<tr>
<td>A4F industrial medium</td>
<td>0.53 d⁻¹</td>
<td>0.05</td>
</tr>
<tr>
<td>A4F industrial medium + 10 g/L NaCl (PA)</td>
<td>0.60 d⁻¹</td>
<td>0.07</td>
</tr>
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</table>

- Cultures seemed to grow better at 10 g/L
- Samples will be sent to butanol analysis.
Key events:

Workshop on biocatalyst development for direct fuel production. CeBiTec Industrial Biotechnology, Bielefeld September 24-27 2017

Workshop on large scale solar fuel production, Lisboa 2018

Workshop on risk-, economic and environmental assessment (t.b.d.)

Workshop for European stakeholders from policy and industry on key results (t.b.d.)

Collaboration with other projects sought
Thank you!

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