Adaptive evolution of *Chlamydomonas reinhardtii* strains resulted high lipid content cells

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Adaptive evolution is a widely practiced strategy for selecting and engineering economically valuable traits.

Adaptive evolution typically starts from an environmental change and results in genetically inheritable adaptation.

The initial cellular response, which usually includes a transient reprogramming of cellular activities, is termed "shock", while the subsequent cellular state that involves inheritable traits resulted from long-term exposure and selection (i.e., after generations) is termed "adaptation".

The vast numbers of genetic variables (e.g., strains used) and environmental variable (e.g., culture conditions) across the studies hampered meaningful comparisons between shock- and adaptation-responses.
Development of biotechnological processing of microalgae by flow cytometry

- Applications of FCM analyses for biotechnological processes with microalgae
- Separation of microalgae from bacteria

Evaluation of intracellular lipid bodies in green microalgae *Chlamydomonas reinhardtii* strains by flow cytometry

- **BODIPY 505/515 staining**

- **NileRed staining**

**Evaluation of intracellular lipid bodies in green microalgae**

*Chlamydomonas reinhardtii* strains by flow cytometry


<table>
<thead>
<tr>
<th>Strains and growth phase</th>
<th>Mean fluorescence intensity (a.u.)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>FL5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unstained FL2&lt;sup&gt;u&lt;/sup&gt;</td>
<td>Nile Red stained FL2</td>
</tr>
<tr>
<td><strong>CC124</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exponential phase (48h)</td>
<td>3.06</td>
<td>25.52</td>
</tr>
<tr>
<td>Mid-stationary phase (96h)</td>
<td>3.20</td>
<td>49.73</td>
</tr>
<tr>
<td>Late-stationary phase (120h)</td>
<td>4.19</td>
<td>47.18</td>
</tr>
<tr>
<td><strong>sta6</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exponential phase (48h)</td>
<td>1.71</td>
<td>4.41</td>
</tr>
<tr>
<td>Mid-stationary phase (96h)</td>
<td>1.87</td>
<td>13.78</td>
</tr>
<tr>
<td>Late-stationary phase (120h)</td>
<td>1.84</td>
<td>17.92</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean fluorescence intensity (a.u.) calculated using the following formula:

\[
\text{Mean fluorescence intensity (a.u.)} = \frac{\text{Unstained fluorescence}}{\text{Stained fluorescence}}
\]

Exponential phase:
- CC124: 3.06
- sta6: 1.71

Early-stationary phase:
- CC124: 3.20
- sta6: 1.87

Late-stationary phase:
- CC124: 4.19
- sta6: 1.84
Flow cytometry sorted single algal cells were able to grow on 96-well plates containing TAP broth (a). Contamination check – TAP agar plates – algal growth before (b) and after (c) cell sorting.

Comparison of lipid content of *Aurantiochytrium* sp. strain by BODIPY 505/515 and Nile Red staining

- BODIPY 505/515 staining

- Nile Red staining
Evaluation of intracellular lipid bodies in *Aurantiochytrium* sp. strain by flow cytometry

- **BODIPY 505/515 staining (530/40 nm)**

- **Nile Red staining (580/30 nm)**

<table>
<thead>
<tr>
<th>Strains and growth phase</th>
<th>Mean fluorescence intensity (a.u.)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nile Red stained</td>
</tr>
<tr>
<td>Exponential phase (12h)</td>
<td>83.93</td>
</tr>
<tr>
<td>Early-stationary phase (72h)</td>
<td>761.47</td>
</tr>
<tr>
<td>Late-stationary phase (96h)</td>
<td>1091.54</td>
</tr>
</tbody>
</table>

<sup>a</sup> FL2: FL1
Flow cytometry sorted single *Aurantiochytrium* cells were able to grow on 96-well plates containing yeast extract based nutrient broth.
Strain development by adaptive evolution using flow cytometry

Two dimensional plot

CC124

Flow cytograms

CC124

25000 Cells from Top 2% were sorted and regenerated in N (+) or N (-) TAP medium
Strain development by adaptive evolution using flow cytometry

**CC124**

Generation

Cell count 10^6 cells/ml

**sta6-1**

Generation

Cell count 10^6 cells/ml
Strain development by adaptive evolution using flow cytometry

CC124

Unstained

Bodipy 505/515 stained

Gen 1

Gen 4

Gen 1

Gen 4

sta6-1

Unstained

Bodipy 505/515 stained

Gen 1

Gen 3

Gen 1

Gen 3
### GC quantification of FAME content of evolutionary adapted strains of CC124 and sta6-1 at different stages

<table>
<thead>
<tr>
<th>Strain</th>
<th>Different stages (Generation)</th>
<th>Nitrogen-repletion condition</th>
<th>Nitrogen-depletion condition</th>
<th>Fatty acid (% of total fatty acids in nitrogen-depletion condition)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Lipid (%)</td>
<td>Total Lipid (%)</td>
<td>C14:0</td>
<td>C14:1</td>
</tr>
<tr>
<td>CC124</td>
<td></td>
<td></td>
<td>6.58</td>
<td>10.52</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>6.58</td>
<td>10.52</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5.88</td>
<td>13.52</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5.29</td>
<td>11.35</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6.99</td>
<td>15.86</td>
<td>0.15</td>
</tr>
<tr>
<td>sta6-1</td>
<td></td>
<td></td>
<td>5.84</td>
<td>5.94</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5.84</td>
<td>5.94</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6.67</td>
<td>8.10</td>
<td>1.14</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7.42</td>
<td>14.04</td>
<td>0.15</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Strain</th>
<th>Different stages (Generation)</th>
<th>Biochemical elements</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C</td>
</tr>
<tr>
<td>CC124</td>
<td></td>
<td>47.783</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>49.170</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>50.271</td>
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<td></td>
<td>3</td>
<td>51.003</td>
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<tr>
<td></td>
<td>4</td>
<td>49.164</td>
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<tr>
<td></td>
<td>2</td>
<td>47.240</td>
</tr>
</tbody>
</table>

- **Elemental analysis**
- **C:N ratio**
- **CC124**
  - 9.5:1 to 11:1
- **sta6-1**
  - 10.2:1 to 11.75:1
Number of intracellular lipid bodies are massively increased at the end of adaptive evolution.
Chloroplast content of cells are gradually decreased at the end of adaptive evolution.
Proteomics analysis of evolutionary adapted cells

pH3-10

CC124-G-1  CC124-G-2  CC124-G-3  CC124-G-4
sta6-G-1  sta6-G-2  sta6-G-3
Proteomics analysis revealed that number of protein spots in evolutionary adapted cells gradually increased at the indicated time period at pH range 4-7.

**pH 4-7**

<table>
<thead>
<tr>
<th>No. of Spots</th>
<th>CC124-G-1</th>
<th>CC124-G-2</th>
<th>CC124-G-3</th>
<th>CC124-G-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC124-G-1</td>
<td>578</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC124-G-2</td>
<td>785</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC124-G-3</td>
<td>805</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>CC124-G-4</td>
<td>879</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No. of Spots</th>
<th>sta6-G-1</th>
<th>sta6-G-2</th>
<th>sta6-G-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>sta6-G-1</td>
<td>598</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sta6-G-2</td>
<td>759</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sta6-G-3</td>
<td>969</td>
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<td></td>
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</tbody>
</table>
Regulation of protein levels for some of the key enzymes

- Pyrroline-5-carboxylate reductase
- Stress-related chlorophyll a/b binding protein 2
- Dehydroascorbate reductase
- Periplasmic L-amino acid oxidase

- Rubisco activase
- Phosphoribulokinase
- RubisCo activase

- Isocitrate lyase

- Triose-phosphate isomerase
- RubisCo
Proteomic response mechanism for evolutionary adaptation

- Chloroplast
  - Calvin cycle
  - Ru5P (C5)
  - RuBP (C5)
  - Oxidative Pentose Phosphate pathway (OPP)
  - 6PGDH
  - DHAP (C3)
  - TPI
  - G6P
  - Gluconeogenesis
  - ALD
  - G3P
- Mitochondria
  - TCA cycle
  - Citrate
  - isocitrate
  - malate
  - Fumarate
- Periplasmic L-amino acid oxidase
- TAG
  - Stress related chlorophyll a/b binding proteins/thylakoid membrane proteins
Conclusions

- A high-throughput screening methodology developed for screening of marine microalgal strains, thraustochytrids and green algae *Chlamydomonas reinhardtii* using FACS in combination with fluorescent dye BODIPY 505/515

- The adaptive evolution under nitrogen starvation through sorting and regeneration of high-lipid content cells led to the artificial generation of lipid overproducing *C. reinhardtii* CC124 and sta6-1 populations

- Systematically programmed evolutionary adaptation revealed the potential role of carbon and nitrogen pathways during lipid accumulation in *C. reinhardtii* strains

- The results could demonstrate that adaptive evolution of microalgae can be a new tools to develop *C. reinhardtii* strains with desired phenotypes such as high lipid accumulation
Acknowledgement

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Thanks for your attention