From electricity towards biodiesel production in yeast

Project 16-655
Final report

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Introduction

The aim of this project has been to investigate the opportunities to engineer the yeast *Yarrowia lipolytica* for usage as a biocatalyst in a process where renewable electricity is converted into biodiesel (**Figure 1**). This process involves the production of acetate from CO_2 and electrolysed water via microbial electrosynthesis (MES), and the subsequent conversion of acetate to biodiesel by *Y. lipolytica*. Within this project the production of biodiesel from acetate will be improved by metabolic engineering of *Y. lipolytica*. This will result in a more efficient production of biodiesel and will make the production of biodiesel from electricity more feasible process.

We have approached this challenge from multiple angles, through:

- Studying growth of *Y. lipolytica* during cultivation in bioreactors on acetate.
- Establishing efficient CRISPR/Cas9 protocols in our laboratory.
- Evolving *Y. lipolytica* to against industrial environmental conditions.
- Abolition of filamentous growth in *Y. lipolytica*.
- Developing methods for computational modelling of metabolism.

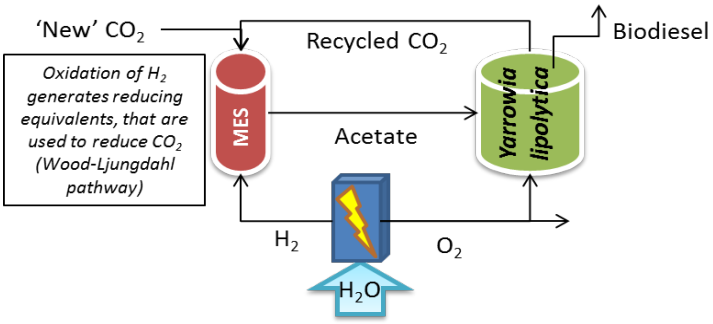

Figure 1. Schematic overview of the process, from electrolysis of water, acetate production via microbial electrosynthesis (MES) to biodiesel production by *Y. lipolytica*. The green reactor is the subject of investigation of this project.

Results

Establishing a baseline for future research: high lipid accumulation on acetate

To initiate this project and set a baseline for future research in acetate-assimilating *Y. lipolytica* for biodiesel production, we characterized chemostat cultivations of *Y. lipolytica* during growth on acetate using standardized protocols to facilitate comparison with previous and future datasets.

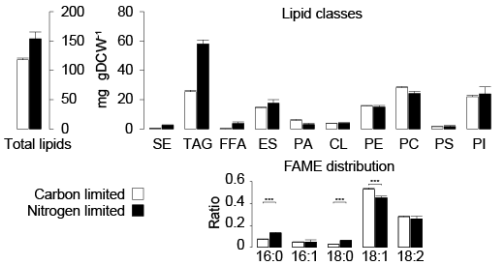

Figure 2. Lipid classes and fatty acid methyl ester (FAME) distributions. The preferred lipid is triacylglycerol (TAG).

Nitrogen limitation invoked a moderate increase in lipid accumulation, from 118 (±2.9) to 154 (±11.6) mg gDW^{-1} (**Figure 2**). In the acetate limited cultures, the total lipid content was 2.5-fold higher than during glucose limitation, likely due to acetate being more readily available as precursor to lipid biosynthesis. The lipid accumulation levels reached upon nitrogen limitation is similar for both carbon sources (two-tailed *t*-test, t = 2.01999, df = 4, p = 0.1135), suggesting that a maximum lipid accumulating capacity is reached in these chemostat conditions. Combined, this indicates the strong potential of cultivation of *Y. lipolytica* on acetate for lipid production.

Transcriptomics analysis indicates targets for genetic engineering

From the chemostat fermentations, samples were taken for RNA sequencing. Differential expression and gene-set analysis indicates a typical transcriptional response to nitrogen limitation, enriched for a readjustment of transporter activities, upregulation of ribosomal process and downregulation of beta-oxidation, represented as acyl-CoA oxidase activity (**Figure 3**). From this analysis, we can infer that high lipid accumulation coincides with down-regulation of beta-oxidation and upregulation of glutamine metabolism. Consequently, from these differentially expressed proteins we have generated a detailed list of targets for genetic engineering.

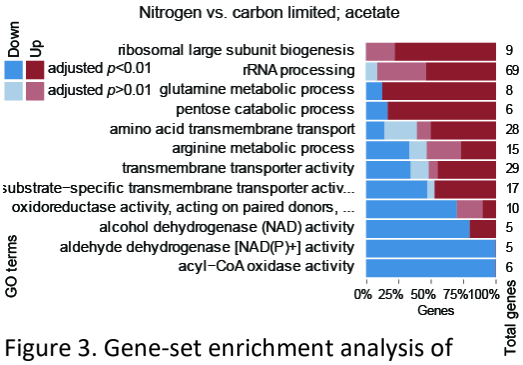

Figure 3. Gene-set enrichment analysis of mRNA changes. Highest lipid levels, observed upon nitrogen limitation, coincide with upregulation of glutamine metabolism and downregulation of acyl-CoA oxidases (i.e. beta-oxidation).

Establishing a CRISPR/Cas9 genetic toolbox for *Y. lipolytica*

After identifying genetic targets, the following challenge has been the implementation of the genetic engineering. While some efforts have been made previously to develop a genetic toolbox for *Y. lipolytica*, with plasmids and constructs that facilitate overexpression and deletion of genes, these toolboxes have been unsatisfiable for a number of reasons, e.g.: (i) low efficiency; (ii) random integration into the genome; (iii) dependency on leucine as a marker (while previously it was shown that leucine is involved in regulating lipid metabolism). Therefore, we decided to leverage on the recent advances in CRISPR/Cas9 and implement these methods in our laboratory. To not reinvent the wheel, we performed a review of the current field to establish what are the current most promising developed CRISPR/Cas9 tools for *Y. lipolytica*, which we subsequently published (Shi et al. 2018).

We selected the EasyCloneYALI approach for all our future genetic engineering of *Y. lipolytica*, and in close collaboration with the original authors we have managed to successfully establish the methods and protocols in our lab. This paves the way for significantly improved precision and speed in our genetic engineering efforts. After implementing some proof-of-principles mutations, we are currently in the process of systematically implementing mutations that were suggested by the acetate cultivations.

Evolve tolerance against industrial conditions

Besides sufficiently high productivity, successful industrial application of *Y. lipolytica* is impeded by further challenges. One of these is the tolerance against the environmental conditions it encounters during industrial cultivations. This includes low pH (especially for acetic acid in contrast to glucose), elevated temperatures and osmolarity. To address these issues, we have exposed *Y. lipolytica* for three months to sub-lethal levels of these three environmental stresses, which acted as selection pressure, and during this time *Y. lipolytica* has naturally evolved tolerance through spontaneous random mutations. This approach has yielded a library of 42 strains with improved robustness (**Figure 4**), and we have performed whole-genome sequencing of all strains to catalogue all genetic mutations that have occurred during the evolution.

Deconvolution of the whole-genome data has prioritized a set of mutations that are potentially conferring the observed robustness. The next step in this project is to perform genome editing to validate the causal mutations of increased tolerance.

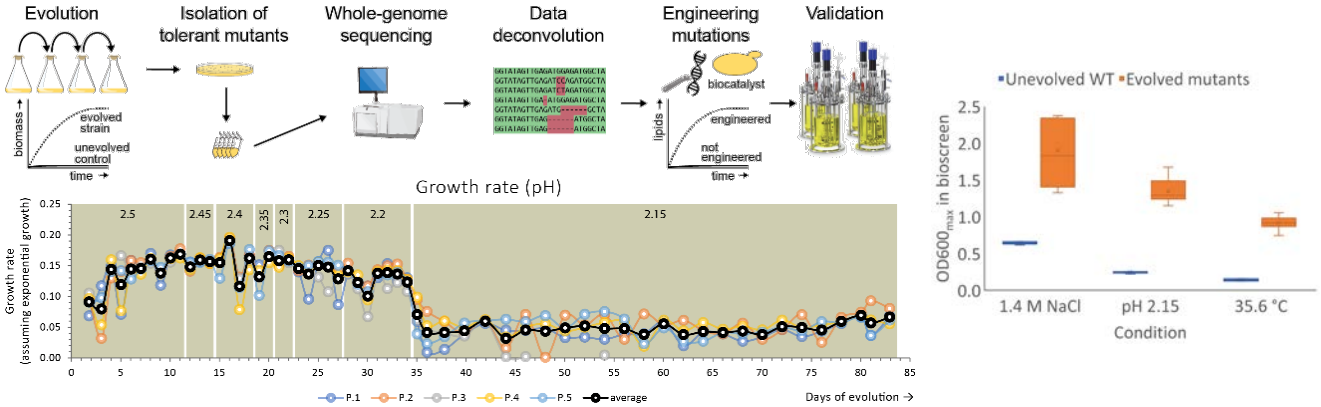

Figure 4. Schematic overview of the evolution of stress tolerance in *Y. lipolytica* (above). Curve showing evolution of growth rate at decreasing pH levels (from 2.5 to 2.15, below). Boxplots of growth potential of unevolved and evolved mutants on three stresses (right).

Engineer *Y. lipolytica* for reduced hyphal growth

Another challenge for industrial usage of *Y. lipolytica* is its dimorphic nature, where *Y. lipolytica* can form hyphae under stressful conditions. This phenotype is not preferred, as it reduces nutrient uptake, increases sheer-stress and overall reduces productivity. To address this, we have genetically engineering *Y. lipolytica* for reduced hyphal growth.

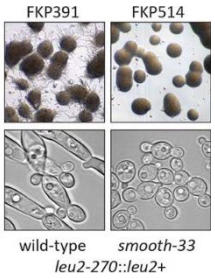

Figure 5. Micrographs showing wild-type (left) and engineered smooth mutant (right), both on colony (top) and cell level (bottom).

Non-hyphal, or *smooth* mutants were obtained by screening approximately 100,000 plated colonies for a lack of pseudo-hyphal phenotype, followed by strict selection to result in 6 non-hyphal strains (Pomraning et al. 2018). Genome- and RNA-sequencing of these strains aided in identifying genetic targets that are underlying the observed phenotype, and deletion of the *msn2* gene abolishes this detrimental phenotype (**Figure 5**).

Developing methods and tools for modelling lipid metabolism

Studies and engineering of microbial metabolism can be greatly aided by the use of computational models of metabolism. Previously, we have generated a genome-scale constraint-based model of *Y. lipolytica* and this has been proven useful in e.g. unravelling regulation of lipid metabolism. While significant interest in *Y. lipolytica* and other oleaginous yeasts is focused on their lipid metabolism, the previous modelling approach had difficulties to accurately represent the large variety of lipid species and acyl-chain distributions. To address this, we have developed the SLIMEr formalism, where we Split Lipids In Measurable Entities (Sánchez et al. 2019). This approach now allows us to directly incorporate measurements of lipid classes and acyl-chain distributions (**Figure 2**), which allows us to more accurately estimate flux distributions through lipid metabolism in particular. We demonstrated wide applicability of this formalism by applying it during the reconstruction of the first genome-scale model of *Rhodotorula toruloides*, another promising oleaginous yeast (Tiukova et al. 2019).

Summary of accomplishments

During this project we have reach a number of valuable accomplishments. We have …

1) obtained a baseline data-set of *Y. lipolytica* growing on acetate, demonstrating the feasibility of the original research proposal and providing a background to compare our future results to;
2) established efficient and precise CRISPR/Cas9 methods in our lab, for all our future *Y. lipolytica* genetic engineering needs;
3) identified a detailed list of targets for genetic engineering for stabile lipid accumulation on acetate;
4) evolved *Y. lipolytica* for increased tolerance against industrially relevant stresses and prioritized a list of possible causative mutations;
5) abolished hyphal growth in *Y. lipolytica*, which otherwise would be detrimental to efficient usage of *Y. lipolytica* in industrial scale cultivations;
6) defined a new model formalism to describe lipid metabolism, which allows direct integration of lipid class and acyl-chain distribution data, and demonstrated its use by reconstructing a genome-scale model of another oleaginous yeast *R. toruloides;*
7) published 5 papers kindly acknowledging support from Åforsk; of which 4 peer-reviewed (Pomraning et al. 2018; Shi et al. 2018; Sánchez et al. 2019; Zhou, Kerkhoven, and Nielsen 2018) and 1 currently in pre-print (Tiukova et al. 2019); with another 1-2 manuscripts in preparation stage.

Future plans

While this project has been very productive, a number of open ends have remained. Most prominently, we are currently in the process of reverse engineering the mutations identified from the evolution experiment, to confer tolerance to environmental conditions in the parental strain. With CRISPR/Cas9 methods now established in our lab, this is progressing well. In addition, targets that have been identified from the bioreactor cultivations and transcriptomics analysis remain to be genetically engineered. To support the continuing research in this direction we have received a *Chalmers Energy Area of Advance* grant for small projects.

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