Adaptive evolution of *Yarrowia lipolytica* for osmotic and saline tolerance

Improving tolerance towards osmotic and saline stress for sustainable production of biodiesel

Master's thesis within the Biotechnology Master Program

JOHN HELLGREN
Adaptive evolution of *Yarrowia lipolytica* for osmotic and saline tolerance

Improving tolerance towards osmotic and saline stress for sustainable production of biodiesel

John Hellgren
Adaptive evolution of *Yarrowia lipolytica* for osmotic and saline tolerance
Improving tolerance towards osmotic and saline stress for sustainable production of biodiesel
JOHN HELLGREN

© JOHN HELLGREN, 2017.

Supervisors: Joakim Norbeck and Eduard Kerkhoven
Department of Biology and Biological Engineering
Division of Systems and Synthetic Biology
Chalmers University of Technology

Examiner: Joakim Norbeck
Department of Biology and Biological Engineering
Division of Systems and Synthetic Biology
Chalmers University of Technology

Master’s Thesis 2017
Department of Biology and Biological Engineering
Division of Systems and Synthetic Biology
Chalmers University of Technology
SE-412 96 Gothenburg
Telephone +46 31 772 1000

Cover: Fluorescent microscope picture of lipid accumulating *Yarrowia lipolytica* stained with Nile Red.

Typeset in \LaTeX
Gothenburg, Sweden 2017
Abstract

We need renewable resources to allow sustainable production of fuels. By using lipid accumulating yeast, biodiesel can be produced in a sustainable way from resources that were previously not used, for example lignocellulose-based sources such as agricultural waste. However, for this process to be profitable, the tolerance of the yeast needs to be improved. This project aims to improve the tolerance of the oleaginous yeast *Yarrowia lipolytica* towards osmotic and saline stress by using the method adaptive laboratory evolution. This method has previously been shown to be efficient in constructing strains to tolerate new conditions without the need of prior knowledge. After evolving *Y. lipolytica* for 220 generations in minimal medium containing 1.4 M NaCl, an improved performance in the same medium was observed, along with evolved cross-tolerance towards low pH. This indicates that this adaptive evolution of *Y. lipolytica* resulted in improved ionic tolerance rather than pure osmotic tolerance. The evolved strains were sent for whole genomic sequencing to find out which mutations that caused this phenotype. During this project, two previous CRISPR/Cas9 strategies were combined and adapted for efficient markerless reverse engineering. When genome data arrives, this strategy will be used for reconstruction of candidate mutations to find out which mutations are important for the observed phenotype. The gained knowledge from this evolution experiment can later be used for constructing a robust industrial strain that efficiently converts lignocellulose-based material to biodiesel, allowing sustainable production of fuels.

Keywords: *Yarrowia lipolytica*, adaptive laboratory evolution, osmotic, saline, tolerance, biodiesel.
Acknowledgements

After spending two summers in a row at Sysbio participating in the iGEM competition, I knew that this was the place where I’d want to continue working. Therefore, I chose to conduct my 10-month master’s thesis work here at Sysbio. I want to thank my supervisor and examiner Joakim Norbeck and my secondary supervisor Eduard Kerkhoven for allowing me to do this master’s thesis project and for always being there to provide me with useful advice and guidance when I needed it.

When I was not in the lab, I spend a lot of time in the master’s office, called the dungeon. I’d like to thank all the other members of the dungeon, along with all the master students of Sysbio and Indbio for great discussions, tips and tricks and fika, especially Dóra Vitay and Gustaf Edman.

I would also like to thank all the research engineers at Sysbio for their incredible work to maintain a functional lab and their problem-solving skills.

Finally, I would like to thank everyone working at Sysbio for providing this great and friendly atmosphere, where you could always ask questions to anyone without hesitation.

John Hellgren, Gothenburg, June 2017
# Contents

1 Introduction  
1.1 Aims ........................................ 1

2 Theory  
2.1 Yarrowia lipolytica  
2.1.1 Lipid accumulation ................................... 2
2.2 Osmotic stress  
2.2.1 Compatible solute .................................... 3
2.2.2 High osmolarity glycerol pathway .................... 5
2.2.3 Ionic stress ........................................ 7
2.2.4 Tolerance ......................................... 7
2.3 Adaptive laboratory evolution  
2.3.1 Temperature ....................................... 8
2.3.2 pH .............................................. 8
2.3.3 Saline ........................................... 9
2.4 Genetic tools ....................................... 9
2.4.1 CRISPR/Cas9 ..................................... 9

3 Methods  
3.1 Strains, media and culture conditions .................. 11
3.2 Pilot cultivation ..................................... 12
3.3 Adaptive laboratory evolution  
3.4 Evaluation of strains .................................. 12
3.4.1 Bioscreen ......................................... 13
3.4.2 Shake flask characterization  
3.4.3 Lipid accumulation .................................. 13
3.5 Whole genome sequencing ................................ 14
3.6 Plasmid construction .................................. 14
3.6.1 pCAS2yl-erg3 .................................... 15
3.6.2 pCAS3yl ......................................... 15
3.6.3 pCAS4yl-hph .................................... 16
3.6.4 E. coli transformation ................................ 16
3.6.5 Yeast transformation ................................ 16

4 Results  
4.1 Pilot cultivation ..................................... 17
4.2 Adaptive laboratory evolution  
4.3 Bioscreen ......................................... 19
4.3.1 Round one ....................................... 19
4.3.2 Round two ............................... 20
4.4 Shake flasks characterization .......................... 22
  4.4.1 NaCl ........................................ 22
  4.4.2 Sorbitol ..................................... 24
  4.4.3 Low pH .................................... 25
  4.4.4 Lithium and high temperature ............................ 28
4.5 Lipid accumulation ........................................ 28
4.6 Whole genome sequencing .............................. 30
  4.6.1 Genomic extractions ............................. 30
4.7 Reverse engineering ...................................... 31
  4.7.1 pCAS2yl-erg3 .................................. 31
  4.7.2 pCAS4yl-hph .................................. 32

5 Discussion .............................................. 33

6 Conclusion ............................................... 35

Bibliography .................................................. 36

A Appendix 1 ................................................... I
  A.1 Primers .............................................. I
  A.2 CRISPR guides ....................................... I
  A.3 Plasmids ............................................. II
    A.3.1 pCAS4yl-hph .................................. II
  A.4 Bioscreen sample outline ............................. VI

B Appendix 2 ................................................. VIII
  B.1 Genomic extraction for colony PCR ..................... VIII
  B.2 Phenol:chloroform genomic extraction .................. VIII
  B.3 Yeast electroporation ................................ IX
One option for sustainable production of fuels is to produce biodiesel from vegetable oils. However, the profit of this process is heavily dependent on several factors, such as seasons, climate and the current situation of the market [1]. As a better alternative, microbes can be used to produce biodiesel from plants. This microbial solution has the advantages of being seasonal independent, have a fast growth rate and does not take up as much arable land [1]. However, one important factor is not to use a sugar-based feedstock, as that competes with the food production industry, which could lead to increasing prices and decreased availability of food in poor regions [2].

The competition with the food production could be avoided if the feedstock were based on lignocellulose instead, for example agricultural waste [2]. The problem with using a feedstock based on lignocellulose is that it requires extensive pre-treatments before the sugars can be utilized by microbes through fermentation. The treatments often cause the release of various inhibitors from the wood material, which negatively effects the fermentation process [3].

A promising candidate for biodiesel production is the oleaginous yeast Yarrowia lipolytica. The wild type can accumulate up to 36 % of dry cell weigh as lipids when growing on glucose. This production can be increased to 77 % (30.8 g/l lipids) in batch cultivation and 73 % (85 g/l lipids) in fed-batch cultivation through metabolic engineering and optimization of cultivation conditions [4]. However, the tolerance of Y. lipolytica needs to be improved to achieve these levels in lignocellulose-derived media [5].

1.1 Aims

The main goal of this study is to evolve Y. lipolytica to increase its tolerance towards high osmotic and saline stress using adaptive laboratory evolution. The final evolved strain will be characterized and the mechanism of adaption discovered by connecting the improved phenotype to genome data obtained through whole genome sequencing. The findings of this study can then be transferred to an industrial strain, contributing to the construction of a robust strain capable of efficient conversion of lignocellulose waste to biodiesel.
2 Theory

2.1 Yarrowia lipolytica

*Y. lipolytica* is a dimorphic yeast that can either grow as yeast single cells or as filamentous hyphae depending on environmental conditions [6]. An example of the different morphologies can be seen in Figure 2.1.

![Morphology of Y. lipolytica in complex medium (left) and minimal medium (right)](image)

*Figure 2.1:* Morphology of *Y. lipolytica* in complex medium (left) and minimal medium (right)

Wild type *Y. lipolytica* can utilize a wide range of substrates, including glucose, fructose, glycerol and hydrophobic substrates such as alkenes, fatty acids, fats and oils [7, 8], in contrast with *Saccharomyces cerevisiae*, which prefers glucose as the carbon source [9]. *Y. lipolytica* even grows faster on glycerol (0.3 h\(^{-1}\)) than on glucose (0.24 h\(^{-1}\)) [9]. The ability to utilize hydrocarbons is of special interest for use of *Y. lipolytica* for bioremediation of soil polluted with oil or industrial waste containing oils [10]. In the industry, *Y. lipolytica* has been used in large-scale production of single cell protein and citric acid [11]. Furthermore, with its native ability to secrete proteins, mainly proteases and lipases, *Y. lipolytica*’s efficient secretion system has been exploited for heterologous protein production [11].

In contrast to *S. cerevisiae*, which both respire and ferment, *Y. lipolytica* has an obligatory respiratory metabolism [12] and requires oxygen to survive [13]. The respiratory chain of *Y. lipolytica* is more similar to mammalian cells than *S. cerevisiae*, and presence of the mitochondrial complex I makes *Y. lipolytica* an attractive model of mitochondrial complex I studies [14].

2.1.1 Lipid accumulation

Important for biodiesel production is the ability of the yeast to produce a large amount of lipids [15]. *Y. lipolytica* is an oleaginous yeast, which means that it accumulates lipids
to a level of more than 20% of its biomass [4]. Wild type *Y. lipolytica* can accumulate typically up to 36% lipids, where for example the non-oleaginous yeast *S. cerevisiae* can accumulate less than 15% [16]. The lipid accumulation of *Y. lipolytica* is extensively studied and is therefore used as a model oleaginous yeast [16]. Lipid accumulation is often induced by cultivating the cells in nitrogen limiting conditions, which diverts the carbon flux towards lipid synthesis [16]. The large amounts of lipids produced are stored mainly as triacylglycerols (TAG) in lipid droplets [17], as free fatty acids could be toxic for the cell [4].

The assimilation of TAGs can be seen in Figure 2.2. It begins with acylation of glycerol-3-phosphate (G-3-P), first by G-3-P acyltransferase (*SCT1*) into lysophosphatidic acid (LPA) and secondly by lysophosphatidic acid acyltransferase (*SLC1*) into phosphatidic acid (PA). PA is dephosphorylated by phosphatidic acid phosphohydrolase (*PAP, PAH1* in *S. cerevisiae*), forming diacylglycerol (DAG). The last acyl is added either by an acyl-CoA-dependent pathway, where addition of acyl-CoA is catalyzed by diacylglycerol acyltransferase (*DGA1,2*) and steryl ester synthase (*ARE1,2*), or through an acyl-CoA-independent pathway, where addition of glycerophospholipid (PL) is catalyzed by phospholipid diacylglycerol acyltransferase (*LRO1*) [16].

Lipid accumulation can be verified *in vivo* by staining of lipid droplets with e.g. Nile red.

![Figure 2.2](image.png)

**Figure 2.2**: Formation of TAG in *Y. lipolytica* from G-3-P. G-3-P: glycerol-3-phosphate; LPA: lysophosphatidic acid; PA: phosphatidic acid; DAG: diacylglycerol; PL: glycerophospholipid; TAG: triacylglycerols; *SCT1*: G-3-P acyltransferase; *SLC1*: lysophosphatidic acid acyltransferase; *PAP*: phosphatidic acid phosphohydrolase; *LRO1*: phospholipid diacylglycerol acyltransferase; *DGA1,2*: diacylglycerol acyltransferase; *ARE1,2*: steryl ester synthase. Picture adapted from [4, 16]

### 2.2 Osmotic stress

One kind of stress that the cells experience in lignocellulose-derived medium is osmotic stress [18]. Osmotic stress is caused by changes in external water activity and is a stress factor that cells need to cope with to be able to survive when conditions change. Decreased water activity is caused by an increase of osmolarity outside the cell, which could be the result of a high concentration of salts or sugars. This kind of stress is called hyper-osmotic stress and cellular water is lost due to passive diffusion, which leads to cell shrinking, arrest of cellular functions and denaturation of biomolecules [19]. The loss of cell volume can be as high as 50% when *S. cerevisiae* are exposed to 1 M sodium chloride (NaCl) [20].

In contrast, hypo-osmotic stress is the result of high water activity, i.e. low concentration of salts, which leads to an influx of water that causes cell swelling and an increase of turgor pressure. This can result in bursting of cells; however, the cell wall of a yeast cell prevents
2. Theory

that from happening [19].

2.2.1 Compatible solute

A common way to handle hyper-osmotic stress is for the cell to produce organic osmolytes which keeps the osmotic pressure inside the cell equal to the outside environment and thus helps the cell to retain its water [21]. In fungi, polyols are commonly used as an osmolyte, where the extensively studied yeast *S. cerevisiae* uses glycerol as their osmolyte (or compatible solute as it does not interfere with cellular functions) when growing on glucose [22]. The information of how *Y. lipolytica* copes with osmotic stress is limited, but observations in *S. cerevisiae* are relevant, as many pathways are possible conserved in yeast.

Glycerol is produced from dihydroxyacetone phosphate (DHAP) in two steps in *S. cerevisiae*, see Figure 2.3. In the first step DHAP is converted into glycerol-3-phosphate (G-3-P) by glycerol-3-phosphate dehydrogenase, which has two isozymes (Gpd1 and Gpd2). Deletion of *GPD1* results in moderate sensitivity to osmotic stress, while *GPD2* deletion does not cause any increase in sensitivity, showing that Gpd1 plays the most important role during osmotic stress [22]. However, deletion of both isozymes is required to obtain strong sensitivity to osmotic stress [22]. The second step in glycerol production is conversion of G-3-P into glycerol by glycerol-3-phosphatase (Gpp1,2). The expression of *GPD1*, *GPP1* and *GPP2* is induced by osmotic stress [22]. There is also another route to glycerol through dihydroxyacetone (DHA), but it is not contributing to the glycerol production during osmotic stress [22]. Interestingly, *S. cerevisiae* does not produce glycerol when respiring on ethanol, but instead produces trehalose to combat osmotic stress, showing that the response is carbon source dependent [23].

![Figure 2.3: Glycerol production from DHAP in *S. cerevisiae*. DHAP: dihydroxyacetone phosphate; G-3-P: glycerol-3-phosphate; *GPD*: glycerol-3-phosphate dehydrogenase; *GPP*: glycerol-3-phosphatase](image)

*Y. lipolytica* has a unique metabolism of glycerol, where G-3-P production competes with glycerol production, possible contributing to its high levels of TAG formed from G-3-P [15]. In contrast to *S. cerevisiae*, *Y. lipolytica* only possess one gene coding for Gpd and is lacking the *GPP* gene [15]. This could indicate that *Y. lipolytica* uses another route for glycerol production, or is using another compatible solute for protection against hyperosmotic stress. The use of a different compatible solute is supported by the observation that erythritol production can be increased by exposing *Y. lipolytica* to osmotic stress when growing on glycerol, indicating that erythritol is being used as a compatible solute in *Y. lipolytica* under these conditions [24].
2. Theory

2.2.2 High osmolarity glycerol pathway

The pathway which utilizes the most control over glycerol accumulation in *S. cerevisiae* is the high osmolarity glycerol (HOG) mitogen-activated protein kinase (MAPK) pathway, and is summarized by Hohmann (2015) \[22\]. Briefly, it consists of two branches: the Sln1 and the Sho1 branch, see Figure 2.4. Sln1 is a sensor histidine kinase, located in the plasma membrane, that acts as a negative regulator. Sln1 is active and autophosphorylated during normal conditions and keeps Ssk1 inactive by phosphorylation via the phosphotransfer protein Ypd1. During osmotic stress, Sln1 is inactivated and the inhibition of Ssk1 is relieved, causing Ssk1 to activate Hog1 through a phosphorylation cascade involving Ssk2, Ssk22 and Pbs2. *Sln1Δ* or *ypd1Δ* is lethal as the HOG pathway then never turns off \[22\].

The Sho1 branch is a positive regulator of HOG-pathway and is thought to sense osmotic changes through the membrane proteins Hkr1 and Msb2. During osmotic stress, Hkr1 and Msb2 interact with the membrane scaffold protein Sho1 and the G-protein Cdc42, causing activation of Hog1 through a phosphorylation cascade involving Ste20-Cla4, Ste11 and Pbs2 \[22\].
2. Theory

Figure 2.4: Sln1 and Sho1 branch of the HOG-pathway in *S. cerevisiae*. The activity of Sln1 is inhibited by osmotic stress, resulting in Hog1 activation through a phosphorylation cascade, which results in osmoadaptation through increased glycerol production and uptake, and reduced water loss. The Sho1 branch is activated by osmotic stress and leads to phosphorylation cascade, which activates Hog1 in the end. Both branches are connected through Pbs2. Picture adapted from [22]

Activated Hog1 in glucose conditions has targets both in the cytosol and the nucleus, resulting in up-regulation of *GPD1* and *GPP2* for increased glycerol production and up-regulation of *STL1* for increased glycerol uptake. Hog1 also increases the activity of Gpd1 and Pfk26 for increased glycolytic flux towards glycerol. Activated Hog1 also limits the glycerol efflux by inhibiting the glycerol transporter Fps1. Furthermore, water loss in limited by down-regulation of *AQY2*, coding for an aquaporin [22]. Hog1 is still activated during osmotic stress when grown on ethanol, even though *S. cerevisiae* does not accumulation glycerol in those conditions [23].

*Y. lipolytica* has predicted homologues for both the Sln1 and Sho1 branch, however, Hkr1 and Msb2 is not yet predicted to exist in *Y. lipolytica* [25]. *Ste11Δ* in *Y. lipolytica* can still handle osmotic stress, indicating that other pathways might active during osmotic stress, e.g. the Sln1 branch [26].
2. Theory

2.2.3 Ionic stress

Apart from causing osmotic stress, NaCl also induces an ionic stress as Na$^+$ ions replace K$^+$ ions in biomolecules, inducing toxicity [19]. To cope with this, cells living in environments with high levels of NaCl must spend a lot of energy to maintain low levels of cytosolic Na$^+$, while keeping sufficient amounts of K$^+$ [27]. This is controlled by restricting uptake of Na$^+$, high efflux of surplus Na$^+$ and compartmentalization of ions into organelles [27].

The efflux of Na$^+$ in *S. cerevisiae* is mostly achieved by two different systems in the plasma membrane: the Ena Na$^+$ - ATPases and the Nha1 Na$^+$/$H^+$ antiporter [27]. The ENA genes are transcribed from the ENA/PMR2 locus, which contains five copies for *S. cerevisiae* S288C (ENA1-5) and only one single allele (ENA6) for *S. cerevisiae* CEN.PK stains [28]. This difference is responsible for the hypersensitivity of CEN.PK stains towards Na$^+$, potentially caused by low transcript levels and/or impaired kinetic properties of ENA6, resulting in insufficient efflux of Na$^+$ [28]. All sequenced yeast species contain ENA homologues [29].

While the Ena transporters are the most important for detoxification of Na$^+$ during higher pH, the Nha1 Na$^+$/$H^+$ antiporter is responsible detoxification at acidic pH [27]. *Y. lipolytica* possesses two different Nha transporters, where Ylnha1 is responsible for efflux of K$^+$ and Ylnha2 for efflux of Na$^+$ [30]. The HOG-pathway is coupled to ion stress resistance, as Hog1 controls the activity of Nha1 and induces the expression of ENA1 through downstream targets [27].

2.2.4 Tolerance

*Y. lipolytica* has previously been shown to tolerate concentrations of NaCl up to 2 M [30] compared to *S. cerevisiae*, which tolerates concentrations up to 1.5 M [27]. The difference could partly be explained by the overrepresentation of ion transporter in genome of *Y. lipolytica* compared to other yeast species [31].

2.3 Adaptive laboratory evolution

Even though yeast cells are evolved to adapt and survive stressful environments [32], it would be interesting to find out if this capability can be improved for better performance at high osmotic pressure, using NaCl as the stressor. This could be achieved through adaptive laboratory evolution (ALE). In an ALE of microorganisms, the course of evolution is guided by the scientist by the choice of selective pressure. The cells are grown in a condition that is either stressful or contains nutrients that the organisms cannot utilize fully, resulting in a slower growth. By prolonged exposure to these conditions, there is a chance a cell acquires a beneficial mutation, which could result in a higher growth rate than the other cells in the population. The increased growth rate will allow the mutant to outcompete the cells lacking the beneficial mutation and take over the population [33].

At the end of the ALE, genomic DNA of the evolved strains are isolated and whole genomes sequenced using next-generation DNA sequencing (NGS) techniques. By evolving several populations in parallel, shared deviations in genomic sequence from the wild type could be potential candidate mutations that cause the improved phenotype. To verify the genotype-phenotype connection, the identified mutations are reconstructed through
genetic engineering in wild type strains and its phenotype compared with evolved strains. Potential findings could possible include improvements to the mechanisms of osmotic and ion stress tolerance.

In section 2.3.1 - 2.3.3 three previous studies using ALE for adaptation of *S. cerevisiae* to environmental stress are summarized to demonstrate what can be achieved using ALE.

2.3.1 Temperature

Three populations of *S. cerevisiae* CEN.PK113-7D were evolved by Caspeta et al. (2014) for >300 generations (90 days) at 39.5 ± 0.3 °C in minimal media with daily dilutions (after 4-7 generations) into fresh medium. The cultures were diluted 6-10 times to obtain an optical density (OD$_{600}$) of 0.2. The final evolved strains showed an average 1.57 ± 0.11 times increase in specific growth rate ($\mu$) at 40 °C compared to wild type [34].

After whole genome sequencing, apart from a lot of different mutations, one nonsense mutation in *ERG3* was found in all lineages. This mutation created one premature stop codon for each strain at position Gln$^{50}$, Tyr$^{66}$ or Tyr$^{185}$ respectively. This caused the membrane sterol composition to change from ergosterol to fecosterol, which is a more bent sterol. This could have an important role for membrane fluidity at elevated temperatures, which also have been seen in thermophiles [34]. Reconstruction of the stop codon at Tyr$^{185}$ in wild type showed up to 86 % of the specific growth rate compared to evolved strains [34].

2.3.2 pH

Fletcher et al. (2017) evolved *S. cerevisiae* CEN.PK113-7D in three different setups for three different kinds of low pH stress, using five replicate shake flasks for each. The ALE was conducted in minimal medium, with glucose as the carbon source, using either the inorganic acid HCl (Glu-HCl) or the organic acid lactic acid (Glu-LA) for the low pH stress. A third setup also contained lactic acid, but used raffinose as the carbon source (Raf-LA) to be able to isolate adaptations specific to lactic acid by comparing the results of the two evolved cell lines [35].

The three different ALE started at pH 4, with 88 mM lactic acid for Glu-LA and Raf-LA, and was conducted for 10 weeks. One colony from each population was isolated and characterized further. After 281 generations, the five Glu-HCl strains were down to pH 2.8 and showed 31.3 % increase in maximum average growth rate. Glu-LA evolved for 312 generations and the 5 strains showed 200 % increase in maximum average growth rate at pH 2.8 with 0.3 M lactic acid. The five strains of Raf-LA showed 11.5 % increase in maximum average growth rate at pH 3.2 with 0.2 M lactic acid after 277 generations of ALE [35].

Whole genomic sequencing showed that disruption of ergosterol biosynthesis (*ERG5*) and iron update (*FRE1*) was share between all five isolated strains from Glu-HCl. Construction of erg$^{5\Delta}$ and fre$^{1\Delta}$ resulted in an improved growth rate of 20 % and 34 % respectively compared to the parental strain during batch fermentation in bioreactors. However, the best performing evolved strain showed an increase of approximate 56 % in growth rate, indicating that other factors contribute to the phenotype of the evolved strain [35].
Four of the lactic acid evolution (two for each carbon source) showed adaptation by formation of clumps. This was a result from defect transcriptional activator (Ace2), which activates the expression of genes involved in separation of mother and daughter cell during cell division. Construction of \( ace2\Delta \) resulted in 5.3-fold increase in growth rate compared to wild type during batch fermentation in bioreactors. The evolved strain had growth rate of roughly three times higher than the \( ace2\Delta \) strain, also indicating that other factors are important for the evolved phenotype [35].

2.3.3 Saline

\textit{S. cerevisiae} BY4741 was evolved as 3 replicate cell lines in complex medium containing 2 % peptone, 1 % yeast extract, 2 % galactose and 0.5 M NaCl (YPGN) by Dhar et al. (2011) for 300 generations. For each serial transfer the culture was diluted 1000 times into fresh medium. The transfers were performed every 24 hours (10 generations) and was repeated 30 times, resulting in an ALE of 30 days [36].

The evolved lines showed 8-12 % increased growth rate in YPGN, along with a smaller increase in growth rate in YPG (without NaCl). After whole genome sequencing of the three populations and the ancestral strain, the only common change was found to be a mutation in population two that caused the amino acid change G230D in \( MOT2 \). \( MOT2 \) is involved in transcriptional regulation and post-translational modifications. However, reconstruction of this mutation only explained 25.3 % of the increased fitness for population two. Furthermore, reconstruction of G230D in \( MOT2 \) also resulted in a fitness increase without NaCl [36].

2.4 Genetic tools

To be able to reconstruct and validate the genetic changes found in the evolved strains, genetic tools are required. The downside with using \textit{Y. lipolytica} is the high frequency of non-homologous end-joining (NHEJ) mechanism for repair of double stranded breaks (DSB) of DNA, resulting in random integration of exogenous DNA rather than site specific integrations through homologous recombination (HR) repair [37]. Furthermore, the use of NHEJ for DSB DNA repair results in nonspecific insertions or deletions [38]. This can, however, be avoided by knocking out \( KU70 \), which is important for NHEJ mediated DNA repair, and thus forcing the cells to use HR instead of NHEJ [39]. Knocking out \( KU70 \) did not result in any growth effects on \textit{Y. lipolytica} [40], but have been reported to cause negative growth effects and sensitivity to UV-light in \textit{Pichia pastoris} [41]. The strains used in this study have previously been made \( KU70 \) deficient by integration of the hygromycin B (hph) resistance cassette into the \( KU70 \) locus [42].

2.4.1 CRISPR/Cas9

An efficient genetic tool to construct genetic changes is the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system. CRISPR/Cas9 is a two-component system, based on a system used by bacteria and archaea for immunity against viral DNA, that has been exploited to be used in genetic engineering for precise modifications [43]. The endonuclease Cas9 is guided by a synthetic single guide RNA (sgRNA) to a 20 bp region of the chromosome complementary to a part of the sgRNA sequence and makes a double stranded cut [43]. Furthermore, the 20 bp region needs to be followed by a protospacer adjacent motif (PAM) for Cas9 to be able to
cut [43]. This cut needs to be repaired so the cells can survive and outcome can be guided by the design of the construct. If no homologous DNA fragments exists, the cell will choose NHEJ for the DNA repair, which could result in gene disruption by the mutation that arise in the process [44]. Another possibility is to repair the cut by HR, which is assisted by providing the cells with homologous DNA fragments, resulting in either deletions or insertions depending on the design [44].

For efficient gene editing, the intracellular levels of sgRNA needs to be high enough and properly folded [45]. If the sgRNA is incorrectly processed from the primary transcript, the Cas9 can be targeted to the wrong site as the recognition site for Cas9 is $5'$-N20-NGG-$3'$, where N20 matched the 5' end of the sgRNA and NGG is the PAM [44]. This can be avoided by, for example, using promoters with known transcription start sites [46], flanking sgRNA with ribozymes (catalytic RNA) [47] or exploiting the endogenous tRNA processing machinery [48].

Transformed *Y. lipolytica* should be allowed 2-4 days of outgrowth in selective medium to allow efficient expression and utilization of the CRISPR system [44]. The disruption efficiency is very low without the outgrowth step [38]. Due to limits of usable markers in *Y. lipolytica*, the one plasmid design from Gao et al. (2016) was chosen instead of the two-plasmid design by Schwartz et al. (2016).
Methods

3.1 Strains, media and culture conditions

The *Y. lipolytica* strains used in this study are shown in Table 3.1 and are all derived from *Y. lipolytica* W29 [42]. FKP355 is a leucine auxotroph and will be used for reverse engineering. The ALE will be conducted on the strain FKP391, which is a prototrophic strain, to avoid the need to add leucine to the medium during the ALE. T67C is a delft adapted control kindly provided by Xiaojun Ji. JHYl001 is constructed during this study to be sensitive towards hygromycin B, later to be used as a marker for further genetic engineering. All strains were cryopreserved in 25 % sterile glycerol at -80 °C, using a cooling unit for slow freezing.

Table 3.1: Strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FKP355</td>
<td><em>matA, xpr2-332, axp-2, ku70::hph+, leu2-270</em></td>
<td>[42]</td>
</tr>
<tr>
<td>FKP391</td>
<td><em>matA, xpr2-332, axp-2, ku70::hph+, leu2-270::leu2+</em></td>
<td>[42]</td>
</tr>
<tr>
<td>T67C</td>
<td>Delft adapted FKP391</td>
<td>Xiaojun Ji (unpublished)</td>
</tr>
<tr>
<td>JHYl001</td>
<td><em>matA, xpr2-332, axp-2, ku70Δ, leu2-270</em></td>
<td>This work</td>
</tr>
</tbody>
</table>

The minimal (Delft) medium used in this study contained 7.5 g (NH$_4$)$_2$SO$_4$, 14.4 g KH$_2$PO$_4$, 0.5 g MgSO$_4$•7H$_2$O, 20 g glucose, 1 mL vitamin stock (50 mg/L D-Biotin, 1 g/L D-Pantothenic acid hemicalcium salt, 1 g/L Thiamin-HCl, 1 g/L Pyridoxin-HCl, 1 g/L, Nicotinic acid, 200 mg/L 4-aminobenzoic acid, 25 g/L m-Inositol, pH = 6.5) and 1 mL trace metal solution (3 g/L FeSO$_4$•7H$_2$O, 4.5 g/L ZnSO$_4$•7H$_2$O, 4.5 g/L CaCl$_2$•2H$_2$O, 1 g/L MnCl$_2$•4H$_2$O, 300 mg/L CoCl$_2$•6H$_2$O, 300 mg/L CuSO$_4$•5H$_2$O, 400 mg/L Na$_2$MoO$_4$•2H$_2$O, 1 g/L H$_3$BO$_3$, 100 mg/L KI, 19 g/L Na$_2$EDTA•2H$_2$O, pH = 4) per liter.

The complex medium used for yeast cultivation was yeast-peptone-dextrose (YPD) medium, which contains 10 g/L yeast extract, 20 g/L peptone from meat and 20 g/L glucose. *E. coli* DH5α was grown in lysogeny broth (LB), containing 10 g/L peptone from casein (tryptone), 10 g/L NaCl and 5 g/L yeast extract. YPD and LB plates contained 20 and 16 g/L agar, respectively.

Transformed *E. coli* was selected on LB with 100 µg/mL ampicillin (amp) plates. Synthetic complete (SC) dropout plates without leucine was used for selection of *Y. lipolytica* and contained 6.9 g/L yeast nitrogen base (YNB) without amino acids, 0.69 g/L complete supplement mix (CSM) drop out without leucine, 20 g/L glucose and 20 g/L agar. Agar and glucose was autoclaved separately and the rest sterile filtered.
3. Methods

All cultivations were performed, unless specified, in 100 mL shake flasks containing 20 mL medium on rotary shakers at 200 revolutions per minute (rpm) at 30 °C. OD\textsubscript{600} was measured using an GENESYS™ 20 Visible Spectrophotometer (Thermo Ficher Scientific) and samples diluted to match the linear range (0.05 - 0.3) when necessary. Technical replicates in this study are from the same preculture and biological replicates from different precultures.

3.2 Pilot cultivation

Several cultivation trials were performed to decide the starting stress level for the ALE. The preculture for all pilot trials were grown overnight in 50 mL falcon tubes containing 5 mL YPD medium before washed and resuspended in the final medium and diluted to OD\textsubscript{600} of 0.1. The concentrations used in the pilot trial ranged from 0.6 - 2 M NaCl.

3.3 Adaptive laboratory evolution

One colony of FKP391 was grown overnight in a 50 mL falcon tube containing 5 mL YPD medium. The overnight culture was washed twice with Delft medium containing 1.2 M NaCl before diluted to an OD\textsubscript{600} of 0.1 in 5 shake flasks with the final culture volume of 20 mL Delft medium containing 1.2 M NaCl. The cultures were diluted every 3-4 generation (1-1.5 days) to an OD\textsubscript{600} of 0.05-0.1 in fresh medium. The old set of populations was kept on shaker as a backup until next dilution, in case the newly diluted culture did not grow. The number of generations was calculated as log\textsubscript{2}(final OD\textsubscript{600}/initial OD\textsubscript{600}), assuming exponential growth between every dilution. The stress level was increased to 1.4 M NaCl after 10 generations.

The evolution was paused after 100 generations, with the populations cryopreserved in -80 °C for 2 weeks. The populations were recovered in Delft medium containing 0.7 M NaCl for a few generations before the stress level was increased back to 1.4 M NaCl. The evolution continued in Delft medium containing 1.4 M NaCl until the total number of generations reached 220 and all 5 populations cryopreserved at -80 °C.

The purity of the populations during the ALE was ensured by weekly microscopically checks, colony PCR (cPCR) of Internal Transcribed Sequence (ITS) region [49] (after genomic extraction according to section B.1) and cryopreservation of half the population. The ITS PCR products of the final evolved populations were sent for sequencing for additional verification.

3.4 Evaluation of strains

From each evolved population, 10 colonies were isolated by plating on YPD with 1.2 M NaCl plates. These 50 colonies were characterized in the Bioscreen (section 3.4.1) to select the 3 best performing colonies from each population. 5 colonies from each population was cryopreserved at -80 °C after overnight growth in YPD. Some of the best performing strains were also characterized in shake flasks (section 3.4.2) and checked for lipid accumulation (section 3.4.3).
3. Methods

3.4.1 Bioscreen

The Microbiology Reader Bioscreen C (Oy Growth Curves Ab Ltd) was used for high throughput screening of growth characteristics in multiwell plates with the following shaking settings: continuous shaking, maximum amplitude and fast speed. The temperature was kept at 30 °C and automatic measurements taken every 15 minutes. All raw data was blank corrected (mean blank of that time point) and corrected according to equation 3.1 from Warringer et al. (2003) to adjust for the non-linearity at higher cell densities [50]

\[
OD_{\text{cor}} = OD_{\text{obs}} + OD_{\text{obs}}^2 \times 0.449 + OD_{\text{obs}}^3 \times 0.191
\]  

where \( OD_{\text{cor}} \) is the corrected optical density and \( OD_{\text{obs}} \) the observed optical density.

\( OD_{\text{cor}} \) was converted to \( OD_{600} \) with the equation 3.2 by adjusting for the path length inside the bioscreen wells

\[
OD_{600} = \frac{OD_{\text{cor}}}{1.32 \times \frac{V}{r^2}}
\]  

where \( V \) is the volume of the well (mL) and \( r \) is the radius of the well (cm).

In the first round of screening, 10 colonies from each population and wild type FKP391 was inoculated from YPD with 1.2 M NaCl plates into the multiwell plate containing 145 µL Delft medium containing 1.4 M NaCl, using 2 replicates, according to Table A.3 - A.4. The first screening was conducted for 135 hours (≈ 5.5 days).

The five best performing colonies of each population was selected for the second round bioscreen. This time precultures were made in Delft medium containing 1.4 M NaCl and grown for 2 days before spun down, resuspended in fresh medium, diluted to an \( OD_{600} \) of 0.1 and loaded 145 µL into the multiwell plates as 5 technical replicates, according to Table A.5 - A.6. Furthermore, two biological replicates were used for the FKP391 control.

3.4.2 Shake flask characterization

Some of the best performing strains was also characterized in shake flasks as the aeration and stirring is not optimal in multiwell cultivation settings. Colonies from YPD plates were inoculated on 1 day in Delft medium, spun down (3000 g, 5 min) and resuspended in medium appropriate for that trial. The conditions tested is shown in Table 3.2.

Table 3.2: Concentrations of the 5 different stressors tested during the shake flask characterization

<table>
<thead>
<tr>
<th>NaCl (M)</th>
<th>Sorbitol (M)</th>
<th>LiOAc (mM)</th>
<th>Low pH</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.4</td>
<td>2.5</td>
<td>50, 200</td>
<td>2.15, 2.27</td>
<td>35.6</td>
</tr>
</tbody>
</table>

The pH and temperature levels were chosen from the end point levels of Xiaojun Ji’s adaptive evolution experiment of \( Y. \) lipolytica FKP391 for low pH (pH = 2.15) and high temperature (35.6 °C) tolerance. The pH = 2.27 medium was kindly provided by Xiaojun Ji. 50 mM glycine was added to the low pH medium for increased buffering capacities.
3.4.3 Lipid accumulation

To make sure that the increased performance of the evolved strains in high salt medium was not gained at the cost of lost lipid accumulation ability, the evolved strains were tested for lipid accumulation with and without NaCl. This was done in nitrogen limiting (3.75 g/L (NH₄)₂SO₄) Delft medium to promote lipid accumulation. Precultures were grown in Delft medium for 1 day, spun down, resuspended in N-limited Delft medium, diluted to an OD₆₀₀ of 0.1 in shake flasks and let grow until stationary phase (roughly 3 days). FKP391 grown in normal Delft medium for 1 day was used as negative control, as these conditions should not induce lipid accumulation.

100 - 300 µL samples (depending on OD₆₀₀) were washed with phosphate-buffered saline (PBS) and stained with 0.2 µL Nile Red for 15 min in the dark to allow measurement of lipid accumulation. The cells were spun down and resuspended in PBS and lipid accumulation was measured using a Fluorescent microscope by overlaying bright field pictures (TL-DIC, exposure 30 ms, gain 3.1, intensity 56) with pictures taken with YFP filter (FLUO, excitation filter 510/20 nm (500–520 nm), barrier filter 560/40 nm (540–580 nm), 30 ms exposure, 3.1 gain, intensity 1).

3.5 Whole genome sequencing

Two different methods for genomic extraction were used in this study: phenol:chloroform extraction for the strains and the Amresco provided kit for the populations. The genomic DNA of all 15 strains and wild type FKP391 was extracted by growing overnight in 50 mL falcon tubes containing 5 mL YPD, made into spheroplasts by zymolyase treatment, cell lysis with SDS and extraction of the polar phase twice with phenol:chloroform:isoamyl alcohol and once with chloroform. Extracted DNA was precipitated with 2.5 volumes of pure Ethanol, DNA pellet washed twice with cold 70 % ethanol and resuspended in 1/10 TE-buffer. A more detailed protocol can be found in section B.2.

Genomic DNA from the populations was extracted by inoculation of the cryostocks into shake flasks containing 20 mL Delft medium and cultivated for 36 hours before genomic DNA extraction using the yeast genomic DNA purification kit (Amresco, Solon, OH).

DNA concentration was determined with Qubit 3.0 Flurometer (Thermo fisher Scientific), DNA purity with NanoDrop 2000 (Thermo Fisher Scientific) and DNA quality by electrophoresis (0.7 % agarose gel, GelRed, 1X TAE buffer, GeneRuler 1 kb DNA Ladder) and imaging with Gel Doc XR+ (Bio-Rad).

Genomic DNA was sent to Joint Genome Institute (Walnut Creek, CA, USA) for whole genome sequencing using Illumina sequencing.

3.6 Plasmid construction

Plasmids used in this study are shown in Table 3.3. All plasmids used are replicative in Y. lipolytica and express Cas9 and the sgRNA. LEU2 is used for selection in Y. lipolytica and AmpR for E. coli. The purpose of these plasmids is set up a markerless genome editing tool, which will be utilized for the reverse engineering when genome data of the evolved strain arrives.
3. Methods

Table 3.3: CRISPR/Cas9 plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCAS1yl-trp</td>
<td>Pol II expressed guide, ribozyme processing, targets TRP1, NHEJ repair</td>
<td>[38]</td>
</tr>
<tr>
<td>pCRISPRyl</td>
<td>Pol III expressed guide, tRNA processing, no target</td>
<td>[44]</td>
</tr>
<tr>
<td>pCAS2yl-erg3</td>
<td>Pol II expressed guide, ribozyme processing, targets ERG3, HR repair</td>
<td>This work</td>
</tr>
<tr>
<td>pCAS3yl</td>
<td>Pol III expressed guide, tRNA processing, no target</td>
<td>This work</td>
</tr>
<tr>
<td>pCAS4yl-hph</td>
<td>Pol III expressed guide, tRNA processing, targets HPH, HR repair</td>
<td>This work</td>
</tr>
</tbody>
</table>

All primers used are shown in Table A.1 and was ordered from Eurofins Genomics. The CRISPR guides used are shown in Table A.2 and was generated with the Benching (Benchling, Inc.), were high specificity scores was considered most important to avoid off-targets. Gibson Assembly design generated with NEBuilder (web tool, New England Biolabs) with overlaps of 30 bp between all fragments. The annealing part of the all primers was optimized by use of Tm Calculator (web tool, Thermo Fisher Scientific). Plasmids and their sequences were obtained from Addgene. The genome of Y. lipolytica W29 (GenBank: LJBI00000000.1) was used for design of primers for amplification of genomic DNA.

Plasmids were extracted using GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific). DNA fragments for the constructs were generated through polymerase chain reaction (PCR) using Phusion High-Fidelity DNA Polymerase. Both PCR products and restriction digested plasmids was verified on 1 % agarose gels in 1X Tris-acetate-EDTA (TAE) buffer with GeneRuler 1 kb DNA Ladder as reference. All fragments were purified with GeneJET Gel Extraction Kit (Thermo Fisher Scientific) prior the Gibson Assembly reaction. Enzymes and buffers was purchased from Thermo Fisher Scientific.

3.6.1 pCAS2yl-erg3

To test the CRISPR/Cas9 system from Gao et al. (2016) [38], it was designed to knock out ERG3, which might lead to increased temperature tolerance as it did in S. cerevisiae [34]. The sgRNA of pCAS1yl-trp was modified to target ERG3, along with the addition of 500 bp repair fragments to the plasmid, which was homologous to upstream and downstream of ERG3, to promote knock-out by HR. pCAS1yl-trp was cut with MssI and SalI to remove the old guide targeting TRP1. The guide was exchanged through two PCR reactions of the sgRNA cassette: gRNA-erg3-up (upstream of the old guide) and gRNA-erg3-dw (downstream of the old guide), where the new guide was added as an overlap between both fragments, using pCAS1yl-trp as template. The repair fragments, erg3-up and erg3-dw, was amplified from genomic DNA of Y. lipolytica extracted according to section B.1. The four fragments were generated with corresponding primers in Table A.1 and were assembled with the cut vector with Gibson Assembly for 2 hours to generate pCAS2yl-erg3 (12 951 bp).

3.6.2 pCAS3yl

The cassette expressing the sgRNA from a polymerase II promoter was exchanged to the hybrid SCR1-tRNA<sup>Gly</sup> polymerase III promoter system used in the pCRISPRyl plasmid created by Schwartz et al. (2016). pCAS1yl-trp was cut with MssI and SalI to remove the old guide targeting TRP1 and the hybrid polymerase III promoter extracted from pCRISPRyl through PCR with pol3 FW/RV primers (Table A.1). After Gibson Assembly
3. Methods

of the two fragments, the intermediate plasmid pCAS3yl was generated. This plasmid does not direct the expressed Cas9, but will be the basis of all future constructs.

3.6.3 pCAS4yl-hph

pCAS3yl was modified to knock out hygromycin B resistance gene (HPH) to be able to reuse that marker for further genetic engineering. This was done by exchanging the sgRNA and adding 1000 bp repair fragments homologous to upstream and downstream of HPH, using the same strategy as for pCAS2yl-erg3. pCAS3yl was cut with MssI and SalI. Four PCR fragments, hph-up and hph-dw (amplified from genomic DNA of Y. lipolytica), gRNA-hph-up (amplified from pCRISPRyl) and gRNA-hph-dw (amplified from pCAS3yl), were generated with corresponding primers in Table A.1. The four fragments were assembled with the cut vector with Gibson Assembly for 2 hours to generate pCAS4yl-hph (13 153 bp).

3.6.4 *E. coli* transformation

Assembled plasmids were transformed to chemically competent *E. coli* DH5α by heat shock. Frozen competent cells were thawed 20-30 min on ice, mixed with 1-5 µL plasmid DNA (>10 ng), placed on ice 20-30 more minutes, heat shocked 42 °C for 45 seconds, placed on ice for 3 minutes, addition of 250 µL LB medium, recovered for 45-100 min in a 37 °C air incubator at 200 rpm, plated at LB+amp plates and grown overnight at 37 °C. Transformants were isolated and plasmids extracted. The plasmids were verified by restriction digestion and sequencing (Eurofins Genomics) before transformation into Y. lipolytica.

3.6.5 Yeast transformation

The verified plasmids were transformed to *Y. lipolytica* FKP355 by electroporation according to the protocol in section B.3. Briefly, overnight cultures in YPD was diluted 10X in YPD, grown for a few hours and made competent by lithium acetate (LiOAc) and dithiothreitol (DTT) treatment. Electroporation was carried out at 1.5 kV with a pulse duration between 4-6 ms. The protocol was adapted to *Y. lipolytica* at the last step: the cells was resuspended in 200 µL after centrifugation, half plated on SC -LEU plates and the rest inoculated into 14 mL cultivation tubes containing 2 mL of SC -LEU liquid medium. The liquid medium was allowed 4 days of outgrowth before plating on SC -LEU plates and colony isolation.

Genomic DNA for colony PCR was extracted by resuspending a colony in 25 µL of 20 mM NaOH supplemented with a small spoon of 425-600 µm glass beads (Sigma G-8772), heated at 98 °C for 20 minutes, vortexed 15 seconds and cell debris spun down for 1 min with a table top centrifuge. Colony PCR was performed with DreamTaq polymerase with corresponding cPCR primers in Table A.1. Verified colonies were inoculated in YPD medium for 1 day outgrowth to remove the plasmid, allowing recycling of the marker for later genetic modifications. Outgrowth culture was plated on YPD plates, colonies isolated and verified for plasmid removal by streak out on SC -LEU plates, where they should not be able to grow without the plasmid.
4

Results

4.1 Pilot cultivation

Pilot cultivations were performed to test the performance of *Y. lipolytica* and decide which starting level of NaCl to use in the adaptive evolution experiment. The results of the pilot cultivation of *Y. lipolytica* FKP391 in Delft medium with different concentrations of NaCl are shown in Figure 4.1 and 4.2. Trials with 0.6, 0.7 and 0.8 M NaCl did not show strong effect on the growth: the final OD$_{600}$ was 9.1, 7.7 and 5.9 respectively. The final OD$_{600}$ for 1 and 1.2 M NaCl was 3.3 and 2.4 respectively. The higher concentrations resulted in a final OD$_{600}$ of 2 (1.4 M), 1.5 (1.6 M), 0.82 (1.8 M) and 0.2 (2 M). Cultivation without stress reached a final OD$_{600}$ of 10.6. The starting level of the evolution was chosen to be 1.2 M NaCl, as higher concentrations were thought to result in a too low growth rate for daily dilutions. After a few transfers (10 generations), the selective pressure of 1.2 M NaCl was decided to be too low and was increased to 1.4 M.

![Graph](image1)

*Figure 4.1:* Growth of *Y. lipolytica* FKP391 in Delft medium supplied with 0, 1.0 and 1.2 M NaCl
4. Results

Figure 4.2: Growth of *Y. lipolytica* FKP391 in Delft medium supplied with 0, 1.4, 1.6, 1.8 or 2 M NaCl

4.2 Adaptive laboratory evolution

The course of the evolution in 1.4 M NaCl can be followed in Figure 4.3, which shows the changes in average growth rate as number of generations accumulates.

Figure 4.3: Changes in average growth rate for all five cultures during the adaptive evolution experiment in Delft + 1.4 M NaCl

The total improvements are summarized in Figure 4.4, which shows the increase of average growth rate for each of the five populations.
4. Results

Figure 4.4: Improvement of average growth rate of each of the five populations after 220 generations of evolution in Delft + 1.4 M NaCl

4.3 Bioscreen

Ten colonies from each population were isolated and screened for performance in the Bioscreen to select the best performing ones for whole genome sequencing and further characterization. The results of the two rounds of Bioscreen are summarized in this section. The nomenclature of the strains in this report is the following: S.1.1 stands for saline evolved strain, population 1, colony 1. The strains were chosen both based on growth rate and final OD$_{600}$.

4.3.1 Round one

The results from the first round of bioscreen are summarized in Figure 4.5, where the final OD$_{600}$ of the 10 strains from each population are shown. The highlighted strains were chosen for the second round of screening.
4. Results

Figure 4.5: Final OD$_{600}$ of each evolved strain in the first round of bioscreen, with two replicates (blue and orange). Highlighted bars correspond to colonies chosen for the second round of bioscreen. The final OD$_{600}$ of the best wild type is marked with a line.

The inconsistency of some of the biological replicates could be due to that the outer wells were used in the multiwell plate. Apparently, these wells evaporate a lot of liquid, which the inner wells do not because they are surrounded by wells containing liquid. This results in a higher concentration of NaCl in the outer well and thus higher inhibition of growth. By comparing the low performing strains with their location in the plate (Table A.3), one can see that most of them are located in the outer wells. This problem was uncovered long after the two bioscreen runs and there is a risk that a few high performing colonies located in the outer wells were discarded because of this.

4.3.2 Round two

The growth of some of the top performing strains in the second round of bioscreen are shown in Figure 4.6, with precultures done in Delft medium containing 1.4 M NaCl, with much better performance than the wild type. The upper and lower rows of wells yielded lower OD values than corresponding technical replicates. These wells were removed from the data analysis, resulting in four technical replicates instead of five.
4. Results

Figure 4.6: Growth of S.1.8, S.2.2, S.3.10 and wild type FKP391 in Delft + 1.4 M NaCl during the second round of bioscreen, using precultures in the same medium.

The final $OD_{600}$ for all 25 evolved strains and wild type FKP391 are shown in Figure 4.7. All tested strains performed better than the wild type. The best three colonies of each population were chosen for whole genome sequencing.

Figure 4.7: Final $OD_{600}$ of each evolved strain in the second round bioscreen. Strains with black bars were chosen for genomic sequencing. The final $OD_{600}$ of the best wild type is shown with a line. Error bars represent standard deviation from four technical replicates.
4. Results

4.4 Shake flasks characterization

In this section, the results from the shake flasks characterization in five different stress conditions (NaCl, sorbitol, low pH, lithium or high temperature) are summarized. The last four are tested to check for cross-tolerance, which could have evolved during the ALE. Shake flask cultivation results in a more representative characterization of the performance of the strains, as the drawbacks of the Bioscreen are sedimentation and reduced aeration.

4.4.1 NaCl

The growth of S.1.8 and wild type FKP391 can be followed in Figure 4.8 after precultured in Delft medium. There is no clear difference between the strains during the first 24 hours in stressful medium, however, the growth of the wild type is slowed after 40 hours, while the evolved strain continues to grow. In the end, the evolved strain obtained a 47 % higher OD$_{600}$ than the wild type.

![Growth S.1.8 and wild type FKP391 in Delft + 1.4 M NaCl. Error bars represent standard deviation from three biological replicates](image)

The difference in morphology of the two strains in this trial are shown in Figure 4.9. S.1.8 shows more pseudohyphae formation, while wild type FKP391 shows hyphae and pseudohyphae formation.
4. Results

Figure 4.9: Microscope pictures of S.1.8 (left) and wild type FKP391 (right) after 3 days of growth in Delft + 1.4 M NaCl.

The trial was repeated with two more evolved strains included. Furthermore, the Delft adapted strain T67C was included to exclude the possibility that the improved phenotype is due to Delft adaptation and not NaCl adaption. The results, shown in Figure 4.10, clearly indicate that the improved performance of the evolved strains is not only due to Delft adaptation, as the Delft adapted strain performed worse than the wild type.

Figure 4.10: Growth of S.1.8, S.2.2, S.3.10, wild type FKP391 and Delft adapted T67C in Delft + 1.4 M NaCl. Error bars represent standard deviation from two technical replicates.

The morphology of the strains in this trial can be seen in Figure 4.11, where all saline evolved strains show similar pseudohyphae morphology. The Delft adapted strain shows a more yeast-like phenotype.
4. Results

Figure 4.11: Microscope pictures of S.1.8, S.2.2, S.3.10, T67C and WT after 1 day of growth in Delft + 1.4 M NaCl

4.4.2 Sorbitol

NaCl induces both osmotic and ionic stress [19]. Therefore, it would be interesting to test the performance of the evolved strains in pure osmotic stress medium, with sorbitol as the stressor. The growth of three evolved strains and wild type FKP391 can be followed in Figure 4.12, after preculutred in Delft medium. It was clear that the strains evolved in 1.4 M NaCl did not show an improved phenotype towards pure osmotic stress compared to wild type.
4. Results

Figure 4.12: Growth of S.1.8, S.2.2, S.3.10 and wild type FKP391 in Delft + 2.5 M sorbitol. Error bars represent standard deviation from two technical replicates

4.4.3 Low pH

The next cross-tolerance tested of the evolved strain was tolerance towards low pH, which could have arisen during the evolution. Figure 4.13 shows the growth of three salt tolerant evolved strains and wild type FKP391 in low pH Delft medium provided by Xiaojun Ji. Interestingly, strain S.3.10 shows good performance at low pH. The purity of the cultures was verified by colony PCR with ITS primers.

Figure 4.13: Growth of S.1.8, S.2.2, S.3.10 and wild type FKP391 in Delft (pH = 2.27). Error bars represent standard deviation from two technical replicates
The morphology of the strains during this trial is shown in Figure 4.14. All evolved strains formed clumps (some of them insoluble during OD$_{600}$ measurements), which also was visible in the shake flasks during the cultivation. Some of the cells were stuck to the wall when the shake flasks were taken from the shaker, but they could be detached by rotary shaking of the flasks by hand.

New medium was made and the trial repeated to verify the observed phenotype. The result of this growth trial can be seen in Figure 4.15. All strains showed lower performance, which could be due to the lower pH of this trial compared to the previous one, even though the difference of the pH is very small. The cultures formed even more insoluble clumps in this trial, which made OD measurements hard, resulting in the high standard deviation between the replicates. However, strain S.3.10 still shows a clear improved phenotype compared to the other strains in low pH medium.
4. Results

Figure 4.15: Growth of S.1.8, S.2.2, S.3.10 and wild type FKP391 in Delft (pH = 2.15). Error bars represent standard deviation from two technical replicates.

The morphology of the tested strains is shown in Figure 4.16. Some of the S.3.10 clumps covered the entire screen, while wild type FKP391 did not seem to form any clumps at all (maybe due to very low number of cells).

Figure 4.16: Microscope pictures of S.1.8, S.2.2, S.3.10 and wild type FKP391 in Delft (pH = 2.15) after 140 hours of cultivation.
4. Results

4.4.4 Lithium and high temperature

Cross-tolerance towards Li$^+$ was tested to investigate if evolved strains showed increased tolerance towards pure ionic stress. However, the trials with 0.2 M and 50 mM LiOAc did not result in any growth at all for S.1.8, S.2.2, S.3.10 or the wild type FKP391, as the concentration was too high. The trials were discontinued after one week each. Further experiments are needed to evaluate the ion specific stress resistance of the evolved strains. Cross-tolerance towards high temperature was also tested, but the high temperature trial did not show any growth for the same evaluated strains, indicating no evolved cross tolerance towards elevated temperatures.

4.5 Lipid accumulation

The lipid accumulation of the evolved strains was tested to make sure that the improved performance towards high concentrations of NaCl did not evolve at the cost of lipid accumulation ability. Microscope pictures from the lipid accumulation trial are shown in Figure 4.17, where the cells are stained with Nile red after cultivation in nitrogen limiting medium. All strains show lipid accumulation capabilities after evolution. It looks like S.3.10, S.4.4 and S.5.7 give a weak signal from the Nile red, but if looking closely one can see that there are droplets inside those cells, probably lipid droplets, which somehow have not been stained by Nile red, indicating lipid accumulation capabilities.

It can be noted that some of the strains differ in morphology, even within a population. For example, both S.3.10 and S.4.4 show a more yeast like phenotype, while the other two strains from the same population show a more pseudohyphae and hyphae phenotype.

Lipid accumulation ability was also checked in N-limiting Delft medium supplemented with 1.4 M NaCl for S.1.8, S.2.2 and S.3.10. Nile red staining showed that all 3 evolved strains accumulate lipids at salt stressed conditions as well, see Figure 4.18.
Figure 4.17: Fluorescent microscope pictures of evolved strains stained with Nile red after cultivation in N-limiting Delft medium for 3 days. The negative control (N) is wild type FKP391 cultivated in normal Delft medium.
4. Results

Figure 4.18: Fluorescent microscope pictures of evolved strains stained with Nile red after cultivation in N-limiting Delft medium supplemented with 1.4 M NaCl for 3 days

4.6 Whole genome sequencing

The best three strains from each population (section 4.3.2) were selected for whole genomic sequencing to connect the improved phenotype with changes in the genome. Common changes between the population are potential candidate mutations, which later can be reconstructed using the CRISPR/Cas9 technique.

4.6.1 Genomic extractions

Before whole genome sequencing, the genomic DNA needs to be extracted and purified. The results of the quality control of extracted genomic DNA are summarized in Table 4.1 and Figure 4.19. A_{260/280} should be between 1.6 - 2.0 and A_{260/230} should be between 1.8 - 2.2. Lower ratios indicate presence of contaminants which absorbs at 280 or 230 nm. Furthermore, the genomic DNA should be of high molecular weight and at least 500 ng in amounts. The results from the whole genome sequencing are expected to be received in late 2017.
Table 4.1: Concentrations and purity for the genomic extractions done with phenol:chloroform extraction (P) or with Amresco kit (A).

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>S.1.6 (P)</td>
<td>376.9</td>
<td>1.84</td>
<td>2.02</td>
<td>373.9</td>
<td>1.84</td>
<td>2.02</td>
<td>48.4</td>
<td>2.42</td>
</tr>
<tr>
<td>S.1.7 (P)</td>
<td>1909.3</td>
<td>1.88</td>
<td>2.03</td>
<td>1730.9</td>
<td>1.9</td>
<td>2.13</td>
<td>85.2</td>
<td>4.26</td>
</tr>
<tr>
<td>S.1.8 (P)</td>
<td>528.3</td>
<td>1.82</td>
<td>1.99</td>
<td>577.1</td>
<td>1.81</td>
<td>2.08</td>
<td>73.4</td>
<td>3.67</td>
</tr>
<tr>
<td>S.2.2 (P)</td>
<td>1977.9</td>
<td>1.9</td>
<td>2.01</td>
<td>1868.4</td>
<td>1.9</td>
<td>2.03</td>
<td>89</td>
<td>4.45</td>
</tr>
<tr>
<td>S.2.3 (P)</td>
<td>772.9</td>
<td>1.88</td>
<td>2.26</td>
<td>689.5</td>
<td>1.88</td>
<td>2.2</td>
<td>69</td>
<td>3.45</td>
</tr>
<tr>
<td>S.2.5 (P)</td>
<td>260.6</td>
<td>1.84</td>
<td>1.95</td>
<td>316.1</td>
<td>1.83</td>
<td>1.79</td>
<td>69.2</td>
<td>3.46</td>
</tr>
<tr>
<td>S.3.1 (P)</td>
<td>889.7</td>
<td>1.83</td>
<td>2.08</td>
<td>806.4</td>
<td>1.83</td>
<td>2.15</td>
<td>63.2</td>
<td>3.16</td>
</tr>
<tr>
<td>S.3.7 (P)</td>
<td>227</td>
<td>1.8</td>
<td>1.77</td>
<td>227</td>
<td>1.81</td>
<td>1.78</td>
<td>36.8</td>
<td>1.84</td>
</tr>
<tr>
<td>S.3.10 (A)</td>
<td>756.4</td>
<td>2.01</td>
<td>2.16</td>
<td>741.5</td>
<td>2</td>
<td>2.18</td>
<td>44.2</td>
<td>2.21</td>
</tr>
<tr>
<td>S.4.3 (P)</td>
<td>2295</td>
<td>1.93</td>
<td>2.01</td>
<td>2405.8</td>
<td>1.92</td>
<td>1.97</td>
<td>96</td>
<td>4.8</td>
</tr>
<tr>
<td>S.4.4 (A)</td>
<td>1072.7</td>
<td>2.05</td>
<td>2.24</td>
<td>1077.9</td>
<td>2.04</td>
<td>2.23</td>
<td>47.6</td>
<td>2.38</td>
</tr>
<tr>
<td>S.4.7 (P)</td>
<td>157.7</td>
<td>1.78</td>
<td>1.72</td>
<td>157.8</td>
<td>1.78</td>
<td>1.71</td>
<td>30.6</td>
<td>1.53</td>
</tr>
<tr>
<td>S.5.3 (P)</td>
<td>883.1</td>
<td>1.87</td>
<td>1.86</td>
<td>710.3</td>
<td>1.9</td>
<td>2.02</td>
<td>95.6</td>
<td>4.78</td>
</tr>
<tr>
<td>S.5.7 (P)</td>
<td>1314.7</td>
<td>1.91</td>
<td>1.88</td>
<td>1258</td>
<td>1.91</td>
<td>1.86</td>
<td>48.4</td>
<td>2.42</td>
</tr>
<tr>
<td>S.5.8 (P)</td>
<td>948.2</td>
<td>1.88</td>
<td>1.92</td>
<td>951.7</td>
<td>1.87</td>
<td>1.87</td>
<td>97.6</td>
<td>4.88</td>
</tr>
<tr>
<td>FKP391 (P)</td>
<td>1488.6</td>
<td>1.99</td>
<td>2.2</td>
<td>1487.6</td>
<td>1.97</td>
<td>2.2</td>
<td>57</td>
<td>2.85</td>
</tr>
<tr>
<td>Population 1 (A)</td>
<td>1259.9</td>
<td>1.93</td>
<td>2.25</td>
<td>1085.8</td>
<td>1.92</td>
<td>2.25</td>
<td>40.4</td>
<td>2.02</td>
</tr>
<tr>
<td>Population 2 (A)</td>
<td>753</td>
<td>1.94</td>
<td>2.26</td>
<td>764.6</td>
<td>1.95</td>
<td>2.27</td>
<td>30.8</td>
<td>1.54</td>
</tr>
<tr>
<td>Population 3 (A)</td>
<td>785</td>
<td>1.9</td>
<td>2.18</td>
<td>722.6</td>
<td>1.91</td>
<td>2.15</td>
<td>54.4</td>
<td>2.72</td>
</tr>
<tr>
<td>Population 4 (A)</td>
<td>632.3</td>
<td>1.88</td>
<td>2.12</td>
<td>622.7</td>
<td>1.88</td>
<td>2.11</td>
<td>32.6</td>
<td>1.63</td>
</tr>
<tr>
<td>Population 5 (A)</td>
<td>695.9</td>
<td>1.93</td>
<td>2.05</td>
<td>676.1</td>
<td>1.91</td>
<td>2.03</td>
<td>62.4</td>
<td>3.12</td>
</tr>
</tbody>
</table>

Figure 4.19: 0.7 % agarose gels of extracted genomic DNA run at 80 V for 1 hour and 20 minutes

4.7 Reverse engineering

As no sequencing data could be obtained before the end of this thesis, no targets for reverse engineering were identified. However, the CRISPR/Cas9 system was set up and can be used when genome data arrives.

4.7.1 pCAS2yl-erg3

The first attempt for setting up the CRISPR/Cas9 system was not successful; none of the 8 screened colonies showed evidence of a knock-out event for ERG3 when using the Gao et al. (2016) strategy [38], even after 4 days of outgrowth. According to Schwartz et al. (2016) the polymerase II design with ribozymes results in low HR frequencies, while the polymerase III design with tRNA hybrid promoters yields much higher HR frequencies (>90 % after 4 days of outgrowth) [44]. Therefore, the CRISPR/Cas9 design was adapted to utilize the design of Schwartz et al (2016) for the expression of the sgRNA.
4. Results

4.7.2 pCAS4yl-hph

The modified design with polymerase III promoter for the sgRNA instead of polymerase II (pCAS2yl-erg3) proved to be more efficient in *Y. lipolytica*. The map and sequence of the final constructed plasmid for the hph knock-out (pCAS4yl-hph) can be seen in section A.3.1. The results of the colony PCR of *Y. lipolytica* FKP355 transformed with pCAS4yl-hph are shown in Figure 4.20. Successful knock-outs should yield a PCR product of 2453 bp, while colonies with intact hph cassette should yield a PCR product of 3890 bp. This matches the results quite well. This strain was cured of the plasmid by YPD outgrowth, named JHYl001 and cryopreservered in 25 % glycerol at -80 °C.

![Figure 4.20: Gel electrophoresis results of colony PCR products of colony 1 (transformed with pCAS4yl-hph and isolated after outgrowth) and wild type FKP355. Knock-out event should yield a 2453 bp product, while intact *HPH* should yield a 3890 bp product.](image)

This means that the single plasmid based design for markerless genome editing is set up and ready for later reverse engineering based on future analysis of genome data of the evolved strains.
Adaptive laboratory evolution is a useful technique to understand how cells adapt to different stresses, and to use that information to construct new, more tolerant strains. During the evolution in high salt medium, the cells spend a lot of energy to pump out excess Na\(^+\) to avoid toxic levels [27]. A mutant with more effluent pumps, higher energy production or some other adaptation that lowers the toxicity of excess Na\(^+\) will be able to spend more energy on growing instead of surviving, resulting in faster growth rate and/or higher biomass yield.

After this adaptive evolution experiment, strains from every population showed increased performance in high salt medium, both in microcultivation settings and shake flask cultivation. The growth rate of the evolved strains did not differ from the wild type during the first 24 hours of cultivation, but the evolved strains showed an increase of roughly 50% in final OD\(_{600}\) (Figure 4.11). This could be due to that the evolved strains somehow handle the Na\(^+\) stress more efficiently, resulting in a higher biomass yield. The mechanism of adaption can only be speculated based on the observed phenotype, as the genome data are yet to be obtained.

None of the tested evolved strains showed improved tolerance in LiOAc medium or high sorbitol medium. The concentration of lithium might have been too high in the Li\(^+\) trial, as *S. cerevisiae* strains have been shown to tolerate up to 20 mM LiCl [28]. A screening for Li\(^+\) tolerance should have been made for *Y. lipolytica* FKP391 prior the testing of the evolved strains. The reason for no improvement in sorbitol medium needs further investigations. The high concentration of sorbitol (2.5 M) made the medium very viscous, which might introduce other factors, such as oxygen transfer limits, and thus affect the results. However, it could be possible that some of the other isolated colonies show increased tolerance towards sorbitol and Li\(^+\).

Both biological replicates of the wild type grew very poorly in the second bioscreen round, which is not consistent with the first round and the later shake flasks cultivations. The difference in the setup of the second round of bioscreen was that the preadaptation was done in precultures containing full stress medium (Delft medium containing 1.4 M NaCl), where the strains in the first round was preadapted on YPD plates containing 1.2 M NaCl prior inoculation into the multiwell plate. Even the evolved strains obtained a lower final OD\(_{600}\) in the second round (see Figure 4.5 and Figure 4.7), even if the difference was small compared to the reduction of the wild type. Maybe the difference of the evolved strains is mainly caused by the error between screening runs and since they were precultured in full stress medium. The reason why the wild type grew poorly during the second round of bioscreen needs to be further investigated.
Interestingly, all 3 tested strains showed improved performance at low pH medium, especially S.3.10 (Figure 4.13 and 4.15), compared to wild type. A evolved cross-tolerance towards low pH and not sorbitol could indicate adaptation towards ionic stress rather than osmotic stress. This phenotype could potentially be connected to improved efficiency of the Nha1 antiporter, which is responsible for most of the Na\(^+\) efflux at low pH [27]. If the efficiency of Nha1 has been improved, the cell needs to be able to cope with excess protons that are taken up during Na\(^+\) export through the Nha1 antiporter. However, this needs genome data and additional experiments to be confirmed.

Similar formation of multicellular clusters, which was seen in the low pH growth trials (Figure 4.14 and 4.16), was found in lactic acid evolved strains in the study of Fletcher et al. (2017). However, if Y. lipolytica had acquired the same mutation (defect ACE2), the multicellular phenotype would have occurred at all growth conditions, which it did not. Formation of flocs have previously been shown to increase the tolerance to higher ethanol concentrations [51] and could be a way for cells to cope with some external stresses. The evolved strain S.3.10 have shown a more yeast-like phenotype in medium without NaCl (Figure 4.17), which might aid the formation of multicellular clusters and improve tolerance towards low pH.

In hindsight, it might have been better to use the BioLector (m2p-labs) for the colony screening, even though the throughput is lower (48 wells), as it has a controlled environment inside. This could reduce the problems with evaporation and make sure that no oxygen limit occurs, which is important as Y. lipolytica requires oxygen to grow. Indications of oxygen limitations in the bioscreen have been previously reported [50].

Even though the CRISPR tool is ready for reverse engineering (see section 4.7.2), it could still use some further improvements. One of the improvements could be to add multiplexing to the CRISPR design. A potential problem with the current design is that the plasmid might become too large if several genes are going to be knocked out at the same time, as each knock out requires 2000 bp in repair fragments and 547 bp for the sgRNA cassette. This could be avoided by integrating Cas9 into the pBR docking platform [52] in the genome of Y. lipolytica, using hygromycin B for selection. This integration would remove the need to transform Cas9 every time genetic changes are going to be made and thus reducing the size of the plasmid by almost 5000 bp. Furthermore, the design can be improved by expressing multiple guides from one transcript and process them using the tRNA machinery, shown to be able to process at least eight sgRNAs from one transcript [48].

Future work involves analysis of genome data and further characterization of the evolved strains in high saline medium in controlled settings using bioreactors. The further characterization would include sampling for substrate uptake rate, compatible solute formation and excretion of metabolites. It would be interesting to obtain and analyze the transcription profiles of the evolved strains during high saline cultivation and compare them to the wild type. The information collected from this evolution could then hopefully be transferred to an industrial strain with the aim for improved tolerance and performance in lignocellulose-derived medium for sustainable production of biodiesel.
Conclusion

After 220 generations of the adaptive evolution, an improved phenotype could be observed for all isolated strains of \textit{Y. lipolytica} in Delft medium containing 1.4 M NaCl. Furthermore, the strains showed increased performance at low pH, indicating evolved cross protection. Samples are sent for genomic sequencing, which is expected to be finished after the summer. A single plasmid design has been optimized for later reverse engineering in \textit{Y. lipolytica} when genome data arrives.
Bibliography


Bibliography


## Appendix 1

### A.1 Primers

Table A.1: Primers used in this study. Uppercase characters hybridize to the template and lowercase characters contain the overlapping overhang.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS1</td>
<td>TCCGTAGGTAACCTGCGG</td>
</tr>
<tr>
<td>ITS4</td>
<td>TCCCTGGCTTATTGATATGC</td>
</tr>
<tr>
<td>erg3-up FW</td>
<td>ttcagctgaagatGAGCCGTACGTGTTGACGTCTCCCGTG</td>
</tr>
<tr>
<td>erg3-up RV</td>
<td>ccacccggccgctctgtGTCTTGTATTTGCAATTAATTG</td>
</tr>
<tr>
<td>erg3-dw FW</td>
<td>tgacccatgtaacggaagctgttgtttTGTAGTGTACGTGCTGTTG</td>
</tr>
<tr>
<td>erg3-dw RV</td>
<td>aaccaagttaacgacATCTTTTGACCATGAAGTGGA</td>
</tr>
<tr>
<td>gRNA-erg3-up FW</td>
<td>gactctaconacgacACAGAGACCGGGTTGGC</td>
</tr>
<tr>
<td>gRNA-erg3-up RV</td>
<td>gctctaaaactgcctgtcgttcgacGACGAGCTTACTCGTTTCG</td>
</tr>
<tr>
<td>gRNA-erg3-dw FW</td>
<td>gctgacagtacgcttcacgtGTAGTTAGCTAGCTAGAATAGCAAGTACGTTAATAGG</td>
</tr>
<tr>
<td>gRNA-erg3-dw RV</td>
<td>gacacaaataacgacccgaacccggctctgcgGACGAGCTTACTCGTTTCG</td>
</tr>
<tr>
<td>cPCR-erg3 FW</td>
<td>ATCCCAAGGTTGTCGCTG</td>
</tr>
<tr>
<td>cPCR-erg3 RV</td>
<td>GATTCACGCGGTCTCGCC</td>
</tr>
<tr>
<td>Pol3 promoter FW</td>
<td>atggattcagccagctgttgcgtttaaacCCCGGAGCTTGCAAGAAATGGAC</td>
</tr>
<tr>
<td>Pol3 promoter RV</td>
<td>aataacgcctacccggtctctctgagcacAAAAGCCACCGACTCG</td>
</tr>
<tr>
<td>hph-up FW</td>
<td>tgacccatgtaacggaagctgttgtttTGTAGTGTACGTGCTGTTG</td>
</tr>
<tr>
<td>hph-up RV</td>
<td>aaccaagttaacgacACAGAGACCGGGTTGGC</td>
</tr>
<tr>
<td>hph-dw FW</td>
<td>gatctggaagctacGACCGGGATCCACTTAACG</td>
</tr>
</tbody>
</table>
| hph-dw RV       | ttcgcaactggtggtggtgtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtg
A.3 Plasmids

A.3.1 pCAS4yl-hph

Figure A.1: Plasmid map pCAS4yl-hph

Sequence:

<table>
<thead>
<tr>
<th>Locus</th>
<th>Exported 13153 bp ds DNA circular SYN 22 MAY 2017</th>
</tr>
</thead>
<tbody>
<tr>
<td>Definition</td>
<td>synthetic circular DNA</td>
</tr>
<tr>
<td>Accession</td>
<td></td>
</tr>
<tr>
<td>Version</td>
<td></td>
</tr>
<tr>
<td>Keywords</td>
<td>pCAS4yl hph</td>
</tr>
<tr>
<td>Source</td>
<td>synthetic DNA construct</td>
</tr>
<tr>
<td>Organism</td>
<td>synthetic DNA construct</td>
</tr>
<tr>
<td>Reference</td>
<td>1 (bases 1 to 13153)</td>
</tr>
<tr>
<td>Authors</td>
<td></td>
</tr>
<tr>
<td>Title</td>
<td>Direct Submission</td>
</tr>
<tr>
<td>Journal</td>
<td>Exported Monday, May 22, 2017 from SnapGene Viewer 3.3.4</td>
</tr>
<tr>
<td>Features</td>
<td>Location/Qualifiers</td>
</tr>
<tr>
<td>Source</td>
<td></td>
</tr>
</tbody>
</table>

http://www.snapgene.com

Key: Created with SnapGene®
ORIGIN

```
1  ccgccggtc  cgcctacacg  acacagttg  aacgctcgg  cgcgtcattg  cgtgcattg  gcgcgcattc  aacatcctg  ggcgcgtgct  aatccgccg
2  ttttcacctg  cagcctcagt  ttcacacag  ccccttgctc  cttgtgtgcg  ggtggtggtc  atctttttgg  gccgtggttc  gccgtggttc  ggcgtggttc
3  61  ttttcaccgt  catcaccgaa  acgcgcgaga  cgaaagggcc  agatctgttc  ggaaatcaac
4  121  ggtatgctcaa  ccgatttcga  cagtaataat  ttgaatcgaa  tcggagccta  aaatgaaccc
5  181  gagatatatct  cataaaattc  tcggtgagag  gtctgtgact  gtcagtacaa  ggtgccttca
6  241  ttatgccctc  aaccttacca  tacctcactg  aatgtagtgt  acctctaaaa  atgaaataca
7  301  gtgccaaaag  ccatggcact  gagctcgtct  aacggacttg  atatacaacc  aattaaaaca
8  361  aatgaaaaga  aatacagttc  tttgtatcat  ttgtaacaat  taccctgtac  aaactaaggt
9  421  attgaaatcc  cacaatattc  ccaaagtcca  cccctttcca  aattgtcatg  cctacaactc
10  481  atataccaag  cactaaccta  ccaaacacca  ctaaaacccc  acaaaatata  tcttaccgaa
11  541  tatacagtaa  caagctacca  ccacactcgt  tgggtgcagt  cgccagctta  aagatatcta
12  601  tccacatcag  ccacaactcc  cttcctttaa  taaaccgact  acacccttgg  ctattgaggt
13  661  tatgagtgaa  tatactgtag  acaagacact  ttcaagaaga  ctgtttccaa  aacgtaccac
14  721  tgtcctccac  tacaaacaca  cccaatctgc  ttcttctagt  caaggttgct  acaccggtaa
15  781  attataaatc  atcatttcat  tagcagggca  gggccctttt  tatagagtct  tatacactag
16  841  cggaccctgc  cggtagacca  acccgcaggc  gcgtcagttt  gctccttcca  tcaatgcgtc
17  901  gtagaaacga  cttactcctt  cttgagcagc  tccttgacct  tgttggcaac  aagtctccga
18  961  cctcggaggt  ggaggaagag  cctccgatat  cggcggtagt  gataccagcc  tcgacggact
19  1021  ccttgacggc  agcctcaaca  gcgtcaccgg  cgggcttcat  gttaagagag  aacttgagca
20  1081  tcatggcggc  agacagaatg  gtggcaatgg  ggttgacctt  ctgcttgccg  agatcggggg
21  1141  cagatccgtg  acagggctcg  tacagaccga  acgcctcgtt  ggtgtcgggc  agagaagcca
22  1201  gagaggcgga  gggcagcaga  cccagagaac  cggggatgac  ggaggcctcg  tcggagatga
23  1261  tatcgccaaa  catgttggtg  gtgatgatga  taccattcat  cttggagggc  tgcttgatga
24  1321  ggatcatggc  ggccgagtcg  atcagctggt  ggttgagctc  gagctggggg  aattcgtcct
25  1381  tgaggactcg  agtgacagtc  tttcgccaaa  gtcgagagga  ggccagcacg  ttggccttgt
26  1441  caagagacca  cacgggaaga  ggggggttgt  gctgaagggc  caggaaggcg  gccattcggg
27  1501  caattcgctc  aacctcagga  acggagtagg  tctcggtgtc  ggaagcgacg  ccagatccgt
28  1561  catcctcctt  tcgctctcca  aagtagatac  ctccgacgag  ctctcggaca  atgatgaagt
29  1621  cggtgccctc  aacgtttcgg  atgggggaga  gatcggcgag  cttgggcgac  agcagctggc
30  1681  agggtcgcag  gttggcgtac  aggttcaggt  cctttcgcag  cttgaggaga  ccctgctcgg
31  1741  gtcgcacgtc  ggttcgtccg  tcgggagtgg  tccatacggt  gttggcagcg  cctccgacag
32  1801  caccgagcat  aatagagtca  gcctttcggc  agatgtcgag  agtagcgtcg  gtgatgggct
33  1861  cgccctcctt  ctcaatggca  gctcctccaa  tgagtcggtc  ctcaaacaca  aactcggtgc
34  1921  cggaggcctc  agcaacagac  ttgagcacct  tgacggcctc  ggcaatcacc  tcggggccac
35  1981  agaagtcgcc  gccgagaaga  acaatcttct  tggagtcagt  cttggtcttc  ttagtttcgg
36  2041  gtccctgttg  ggtggtgtgt  ggttgtatgt  gtgatgtggt  gtgtggagtg  aaaatctgtg
37  2101  gctggcaaac  gctcttgtat  atatacgcac  ttttgcccgt  gctatgtgga  agactaaacc
38  2161  tccgaagatt  gtgactcagg  tagtgcggta  tcggctaggg  acccaaacct  tgtcgatgcc
39  2221  gatagcgcta  tcgaacgtac  cccagccggc  cgggagtatg  tcggagggga  catacgagat
40  2281  cgtcaagggt  ttgtggccaa  ctggtaaata  aatgatgact  caggcgacga  cggaattcga
41  2341  cagcaactac  tcctttcacc  aaccatgtgc  attttagctc  gaataacatt  cacaggcttg
42  2401  gtgatctaca  tccatggtgt  ctggccgattc  accgtggtgt  tttggcagta  acgagaatat
43  2461  tgagtgaact  cttcccatca  cgtcagaaa  aagctggatc  tcaacagcgg  taagagatca
44  2521  caacctatgc  gtggtgcaca  caacactct  cagatctggg  cttgcagttg  gagcgcgaat
45  2581  gcacttttcg  gggaaatgtg  cgcggaaccc  ctatttgttt  atttttctaa  atacattcaa
46  2641  atatgtatcc  gctcatgaga  caataccct  gataaatgct  tcaataatat  tgaaaaagga
47  2701  agagtatgag  tattcaacat  ttccgtgtcg  cccttattcc  cttttttgcg  gcattttgcc
48  2761  ttctggtttt  tgctcaccca  gaaacgctgg  tgaaagtaaa  agatgctgaa  gatcagttgg
49  2821  gtgcacgagt  gggttacatc  gaactggatc  tcaacagcgg  taagagatca
50  2881  aattatgcag  tgctgccata  accatgagtg  ataacactgc  ggccaactta  cttctgacaa
51  2941  acttggttga  gtactcacca  gtcacagaaa  agcatcttac  ggatggcatg  acagtaagag
52  3001  gccccgaaga  acgttttcca  atgatgagca  cttttaaagt  tctgctatgt  ggcgcggtat
53  3061  tatcccgtat  tgacgccggg  caagagcaac  tcggtcgccg  catacactat  tctcagaatg
54  3121  acttggttga  gtactcacca  gtcacagaaa  agcatcttac  ggatggcatg  acagtaagag
55  3181  ctttcggtc  cgtggttaag  cttttttgcc  gcattttgcc  gattttttcg  gtcggttgtg
56  3241  gcagtgggt  tggctgagtt  gtcggttgtg  gtcggttgtg  gtcggttgtg  gtcggttgtg
```
A.4 Bioscreen sample outline

Table A.3: Sample outline of well 1-100 in bioscreen round 1

<table>
<thead>
<tr>
<th></th>
<th>1 - 10</th>
<th>11 - 20</th>
<th>21 - 30</th>
<th>31 - 40</th>
<th>41 - 50</th>
<th>51 - 60</th>
<th>61 - 70</th>
<th>71 - 80</th>
<th>81 - 90</th>
<th>91 - 100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S.1.1</td>
<td>S.2.1</td>
<td>S.3.1</td>
<td>S.4.1</td>
<td>S.5.1</td>
<td>S.1.1</td>
<td>S.2.1</td>
<td>S.3.1</td>
<td>S.4.1</td>
<td>S.5.1</td>
<td></td>
</tr>
<tr>
<td>S.1.2</td>
<td>S.2.2</td>
<td>S.3.2</td>
<td>S.4.2</td>
<td>S.5.2</td>
<td>S.1.2</td>
<td>S.2.2</td>
<td>S.3.2</td>
<td>S.4.2</td>
<td>S.5.2</td>
<td></td>
</tr>
<tr>
<td>S.1.3</td>
<td>S.2.3</td>
<td>S.3.3</td>
<td>S.4.3</td>
<td>S.5.3</td>
<td>S.1.3</td>
<td>S.2.3</td>
<td>S.3.3</td>
<td>S.4.3</td>
<td>S.5.3</td>
<td></td>
</tr>
<tr>
<td>S.1.4</td>
<td>S.2.4</td>
<td>S.3.4</td>
<td>S.4.4</td>
<td>S.5.4</td>
<td>S.1.4</td>
<td>S.2.4</td>
<td>S.3.4</td>
<td>S.4.4</td>
<td>S.5.4</td>
<td></td>
</tr>
<tr>
<td>S.1.5</td>
<td>S.2.5</td>
<td>S.3.5</td>
<td>S.4.5</td>
<td>S.5.5</td>
<td>S.1.5</td>
<td>S.2.5</td>
<td>S.3.5</td>
<td>S.4.5</td>
<td>S.5.5</td>
<td></td>
</tr>
<tr>
<td>S.1.6</td>
<td>S.2.6</td>
<td>S.3.6</td>
<td>S.4.6</td>
<td>S.5.6</td>
<td>S.1.6</td>
<td>S.2.6</td>
<td>S.3.6</td>
<td>S.4.6</td>
<td>S.5.6</td>
<td></td>
</tr>
<tr>
<td>S.1.7</td>
<td>S.2.7</td>
<td>S.3.7</td>
<td>S.4.7</td>
<td>S.5.7</td>
<td>S.1.7</td>
<td>S.2.7</td>
<td>S.3.7</td>
<td>S.4.7</td>
<td>S.5.7</td>
<td></td>
</tr>
<tr>
<td>S.1.8</td>
<td>S.2.8</td>
<td>S.3.8</td>
<td>S.4.8</td>
<td>S.5.8</td>
<td>S.1.8</td>
<td>S.2.8</td>
<td>S.3.8</td>
<td>S.4.8</td>
<td>S.5.8</td>
<td></td>
</tr>
<tr>
<td>S.1.9</td>
<td>S.2.9</td>
<td>S.3.9</td>
<td>S.4.9</td>
<td>S.5.9</td>
<td>S.1.9</td>
<td>S.2.9</td>
<td>S.3.9</td>
<td>S.4.9</td>
<td>S.5.9</td>
<td></td>
</tr>
<tr>
<td>S.1.10</td>
<td>S.2.10</td>
<td>S.3.10</td>
<td>S.4.10</td>
<td>S.5.10</td>
<td>S.1.10</td>
<td>S.2.10</td>
<td>S.3.10</td>
<td>S.4.10</td>
<td>S.5.10</td>
<td></td>
</tr>
</tbody>
</table>

Table A.4: Sample outline of well 181-185 and 191-195 in bioscreen round 1. The rest of the wells were used by Xiaojun Ji

<table>
<thead>
<tr>
<th></th>
<th>181 - 185</th>
<th>191 - 195</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Blank</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>Blank</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>Blank</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>Blank</td>
<td></td>
</tr>
</tbody>
</table>

Table A.5: Sample outline of well 1-100 in bioscreen round 2

<table>
<thead>
<tr>
<th></th>
<th>1 - 10</th>
<th>11 - 20</th>
<th>21 - 30</th>
<th>31 - 40</th>
<th>41 - 50</th>
<th>51 - 60</th>
<th>61 - 70</th>
<th>71 - 80</th>
<th>81 - 90</th>
<th>91 - 100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S.1.3</td>
<td>S.1.6</td>
<td>S.1.8</td>
<td>S.2.2</td>
<td>S.2.5</td>
<td>S.3.1</td>
<td>S.3.3</td>
<td>S.3.10</td>
<td>S.4.4</td>
<td>S.4.8</td>
<td></td>
</tr>
<tr>
<td>S.1.3</td>
<td>S.1.6</td>
<td>S.1.8</td>
<td>S.2.2</td>
<td>S.2.5</td>
<td>S.3.1</td>
<td>S.3.3</td>
<td>S.3.10</td>
<td>S.4.4</td>
<td>S.4.8</td>
<td></td>
</tr>
<tr>
<td>S.1.3</td>
<td>S.1.6</td>
<td>S.1.8</td>
<td>S.2.2</td>
<td>S.2.5</td>
<td>S.3.1</td>
<td>S.3.3</td>
<td>S.3.10</td>
<td>S.4.4</td>
<td>S.4.8</td>
<td></td>
</tr>
<tr>
<td>S.1.3</td>
<td>S.1.6</td>
<td>S.1.8</td>
<td>S.2.2</td>
<td>S.2.5</td>
<td>S.3.1</td>
<td>S.3.3</td>
<td>S.3.10</td>
<td>S.4.4</td>
<td>S.4.8</td>
<td></td>
</tr>
<tr>
<td>S.1.4</td>
<td>S.1.7</td>
<td>S.2.1</td>
<td>S.2.3</td>
<td>S.2.8</td>
<td>S.3.2</td>
<td>S.3.7</td>
<td>S.4.3</td>
<td>S.4.7</td>
<td>S.4.10</td>
<td></td>
</tr>
<tr>
<td>S.1.4</td>
<td>S.1.7</td>
<td>S.2.1</td>
<td>S.2.3</td>
<td>S.2.8</td>
<td>S.3.2</td>
<td>S.3.7</td>
<td>S.4.3</td>
<td>S.4.7</td>
<td>S.4.10</td>
<td></td>
</tr>
<tr>
<td>S.1.4</td>
<td>S.1.7</td>
<td>S.2.1</td>
<td>S.2.3</td>
<td>S.2.8</td>
<td>S.3.2</td>
<td>S.3.7</td>
<td>S.4.3</td>
<td>S.4.7</td>
<td>S.4.10</td>
<td></td>
</tr>
<tr>
<td>S.1.4</td>
<td>S.1.7</td>
<td>S.2.1</td>
<td>S.2.3</td>
<td>S.2.8</td>
<td>S.3.2</td>
<td>S.3.7</td>
<td>S.4.3</td>
<td>S.4.7</td>
<td>S.4.10</td>
<td></td>
</tr>
</tbody>
</table>
Table A.6: Sample outline of well 101-200 in bioscreen round 2

<table>
<thead>
<tr>
<th>101 - 110</th>
<th>111 - 120</th>
<th>121 - 130</th>
<th>171 - 180</th>
<th>181 - 190</th>
<th>191 - 200</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.5.3</td>
<td>S.5.7</td>
<td>S.5.9</td>
<td>WT.1</td>
<td>WT.2</td>
<td>Blank</td>
</tr>
<tr>
<td>S.5.3</td>
<td>S.5.7</td>
<td>S.5.9</td>
<td>WT.1</td>
<td>WT.2</td>
<td>Blank</td>
</tr>
<tr>
<td>S.5.3</td>
<td>S.5.7</td>
<td>S.5.9</td>
<td>WT.1</td>
<td>WT.2</td>
<td>Blank</td>
</tr>
<tr>
<td>S.5.3</td>
<td>S.5.7</td>
<td>S.5.9</td>
<td>WT.1</td>
<td>WT.2</td>
<td>Blank</td>
</tr>
<tr>
<td>S.5.3</td>
<td>S.5.7</td>
<td>S.5.9</td>
<td>WT.1</td>
<td>WT.2</td>
<td>Blank</td>
</tr>
<tr>
<td>S.5.6</td>
<td>S.5.8</td>
<td></td>
<td></td>
<td></td>
<td>Blank</td>
</tr>
<tr>
<td>S.5.6</td>
<td>S.5.8</td>
<td></td>
<td></td>
<td></td>
<td>Blank</td>
</tr>
<tr>
<td>S.5.6</td>
<td>S.5.8</td>
<td></td>
<td></td>
<td></td>
<td>Blank</td>
</tr>
<tr>
<td>S.5.6</td>
<td>S.5.8</td>
<td></td>
<td></td>
<td></td>
<td>Blank</td>
</tr>
<tr>
<td>S.5.6</td>
<td>S.5.8</td>
<td></td>
<td></td>
<td></td>
<td>Blank</td>
</tr>
</tbody>
</table>
Appendix 2

Protocols used in this study

B.1 Genomic extraction for colony PCR

- Prepare eppendorf tubes with 25 µL of 20 mM NaOH for each colony you want to test.
- Add few glass beads (diameter
- With a sterile pipetting tip transfer a 5 µL of culture to the tube and resuspend the yeast cells in the solution.
- Boil the sample for 15 min (heating block at 95-100 ºC)
- Vortex 15 sec
- Spin for 1 min a max. speed in a tabletop centrifuge.
- Transfer 0.5 µL of the solution to a 25 µL PCR reaction.

B.2 Phenol:chloroform genomic extraction

1. Inoculate 1 colony in 5 mL YPD. Grow overnight
2. Spin down cells 4000 g, 5 min, +4 ºC
3. Discard supernatant and remove remaining traces with pipette.
4. Resuspend pellet in 2 mL TE buffer and transfer to 2 mL Eppendorf tube
5. Spin down cells 4000 g, 5 min, +4 ºC
6. Discard supernatant and remove remaining traces with pipette.
7. Resuspend pellet in 1 mL buffer Y1 (1 M sorbitol, 100 mM EDTA, 14 mM 2-Mercaptoethanol (toxic))
8. Add 100 units of Zymolyase or lyticase
9. Incubate 30 ºC with gentle shaking (ex post-stain shaker or normal shaker with 70 rpm) for 1-2 hours
10. Check spheroplasting by measuring OD of in 1 M sorbitol and in 5 % SDS (ex 50 µL sample + 950 µL sorbitol or SDS)
    (a) SDS sample should lyse, yielding high OD reduction
    (b) Handle spheroplasts gently
11. Pellet spheroplasts at 500 g, 10 min, +4 ºC
12. Remove supernatant and dissolve pellet in 600 µL lysis buffer (1 % SDS, 100 mM NaCl, 2 % TritonX-100, 1x TE (10 mM Tris-Cl, 1 mM EDTA, pH 8))
13. Add 12 µL RNase A
14. Incubate 50 ºC for 1 hour with occasional inversions
15. Add 1 volume of phenol:chloroform:isoamyl and vortex 10-20 sec
16. Centrifuge 5 min 16 000 g room temperature and transfer upper phase to new tube.
    (a) Protein will form a visible white precipitate at the interface
17. Repeat step 15-16 until no protein is visible at the interface
18. Add 1 volume chloroform
19. Vortex 10-20 sec
20. Centrifuge 5 min 16 000g RT
21. Transfer upper phase to new tube
22. Add 2.5 volumes of 100 % ethanol.
23. Mix and spin down sample.
24. Place at -80 °C for 60 min (or -20 °C overnight).
25. Centrifuge 16 000 g, 20 min, +4 °C with hinges outward
26. Carefully remove supernatant without disturbing the pellet.
27. Wash pellet with 70 % ethanol (cold).
28. Centrifuge 16 000 g, 5 min, +4 °C with hinges outward
29. Wash again with 70 % ethanol (cold).
30. Pull off all ethanol with pipet tip (use table top centrifuge to collect all ethanol at the bottom of the tube).
31. Air dry pellet under flame for 5 min
32. Resuspend pellet in 50 µl of 1/10 TE (10 mM Tris, pH 7.5-8.0, 0.1 mM EDTA) at 55 °C. 1-2 hours.
33. Measure purity with nanodrop, concentration with Qubit and run 30-60 ng on 0.7 % agarose gel 80 V 1h 20 min
34. Store DNA @ -80 °C or -20 °C.

B.3 Yeast electroporation

10× TE buffer (pH 7.5): 100 mM Tris-HCl, 10 mM EDTA. Filter-sterilized
10× LiAc: 1 M LiAc, pH 7.5 (adjusted using HAc). Filter-sterilized
1 M DTT: stored at -20 °C. Filter-sterilized

Day 1
Pick up single colonies on plates to 5 ml YPD medium. Culture at 30 °C for 12-16 h.

Day 2

1. Inoculate into 50 ml YPD medium in flask. Culture at 30 °C for 6-9 h.
   ««Put sterilized water on ice »»
2. When OD is 0.5-1.2, transfer cell culture into 50 ml cap tube (sterilized).
   «« From here cells should be always on ice »»
3. Collect cells by centrifugation (1100 g, 4 °C, 5 min). Decant supernatant.
5. Treat cells with 20 ml of 0.1 M LiAc (16 ml 1 M sorbitol plus 2 ml 10×TE buffer plus 2 ml 1 M LiAc) at 30 °C for 30 min. Add 0.2 ml 1 M DTT and keep cells at 30 °C for 15 min. Centrifuge and decant supernatant.
   ««Put 1 M sorbitol on ice »»
6. Wash cells twice with 20 ml of 1 M sorbitol (ice-cold). Centrifuge and decant supernatant.
7. Re-suspend cells with 100-200 µl of sterilized ice-cold 1 M sorbitol (final OD=100-200).
8. Take 50 µl of suspended cells into a new 1.5 ml tube on ice.
9. Add 5 µl fragment DNA (> 200 ng/µl). Mix with pipetman and keep on ice for 15 min. Transfer all to a sterilized cuvette (green cap). Add 1 ml of cold 1 M sorbitol to new labeled tubes (used later).

10. Set the cuvette in the holder of Micro Pulser Electroporator. Chose “Manual”, and set voltage at 1.5 kV. Push the pulse button. Read “Time / ms”, if it is between 4.0-6.0, this process is successful.

11. Add 1 ml of cold 1 M sorbitol, immediately after the pulse. Mix well by pipetting up and down. (After this, it is OK to be at RT.) Transfer all to the sorbitol tube ASAP.

12. Incubate at 30 °C for 1-3 h. Centrifuge (3000 g, 1 min) to 150 µl and then spread cells on selection plates. Make a negative control plate. Place the plates at 30 °C air incubator.