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Study of Cooperation in Synthetic Microbial Communities by Division of Labour

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For the convenience of the reader a glossary and acronym list is available in appendix [A](#)

Abstract

Through the power of synthetic biology, cells can be manipulated to produce compounds of interest. Our work focuses on the fungus *Yarrowia lipolytica* due to its abilities as a natural protein cell factory. On one hand our work aims at improving starch digestion in *Y.lipolytica* by tuning the expression of starch degrading enzymes, amylases. On the other hand, we aim to optimise starch digestion by setting up synthetic microbial communities where production of different amylases is split across several cells. We first characterise the metabolic burden caused by the expression of amylases on the host cell. Secondly we study the ability of several strains to use starch as the main carbon source for energy. Our results show that one of the two amylases causes much more burden to the cell than the other. Additionally our study concludes that starch digestion by division of labour is unsuccessful at this stage. Finally, we draw an outline of what the next steps are to improve starch digestion by division of labour and by fine-tuning of production of the amylases.

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Chapter 1

Introduction

1.1 Overview

Synthetic Biology is a young discipline which combines the knowledge of biology, chemistry and engineering to modify the behaviour of cellular organisms. Synthetic biology aims at making biology more engineerable through deepening our understanding of bio-molecular processes. Compounds of pharmaceutical interest have been successfully produced and marketed by genetically engineering organisms such as *Saccharomyces cerevisiae* yeast and *Escherichia Coli* bacteria [1]. Human insulin, human serum albumin or hepatitis surface antigen [2] are just a few of the compounds produced synthetically in single cell organisms that we already use to treat disease. Synthetic biology applications are valuable for industry and medicine as high-value compounds can be engineered by cells on demand and at low cost.

Our work is centred on *Yarrowia lipolytica*. *Y.lipolytica* is a yeast species excellent at degrading protein and lipids and remarkably able to secrete high-levels of heterologous proteins [3]. Compounds of interest such as alpha-linolenic acid [4], beta-carotene [5] or bio-diesel-like [6] compounds among others have been expressed in this yeast.

However, high-level protein expression comes with a trade-off: expressing heterologous proteins utilises resources that the cell would naturally use for vital, more beneficial functions. This effect on the host cell is defined as burden or protein cost[7]. Burden decreases cell fitness, typically causing growth to slow down and resulting in lower protein yield (i.e. of the compound we wish to produce) [8]. To counteract the effect of burden, the task of expressing a set of enzymes or a metabolic pathway can be split and distributed across different cells. This is called metabolic

division of labour (DOL). Figure 1.1 illustrates the difference between a DOL system and a single cell (SC) system as well as the concepts to be introduced next.

In order to compare a SC system to a DOL system we will work on the problem of starch digestion. Starch is a complex carbohydrate molecule which requires two amylases to be broken down into glucose units, to be used as fuel. Moreover, starch is a food industry waste byproduct, thus it could be used as cheap energy source in biotechnology applications.

In this study, we wish to analyse how co-culturing inter-dependent *Y.lipolytica* strains may reduce burden and improve growth with respect to its analogous SC system and the effect this has on heterologous protein yield.

Y.lipolytica does not naturally produce the amylases necessary to utilise starch as a carbon source but strains that are able to digest starch have been engineered. We will study *Y.lipolytica* strains that heterologously express the glucoamylase from the fungus *Thermomyces lanuginosu* (TIGAMY) and the alpha-amylase from rice (*Oryza sativa*) (rice-AA). The expression of TIGAMY is regulated at the secretion stage by a series of characterised signal peptides [9] while the expression of rice-AA was modulated at the transcription level using a characterised promoter library [10].

The burden imposed by expression of each amylase individually was measured and then compared against the burden caused by the co-expression of both amylases in a single cell. The growth of DOL systems and SC systems in starch containing media was then assessed.

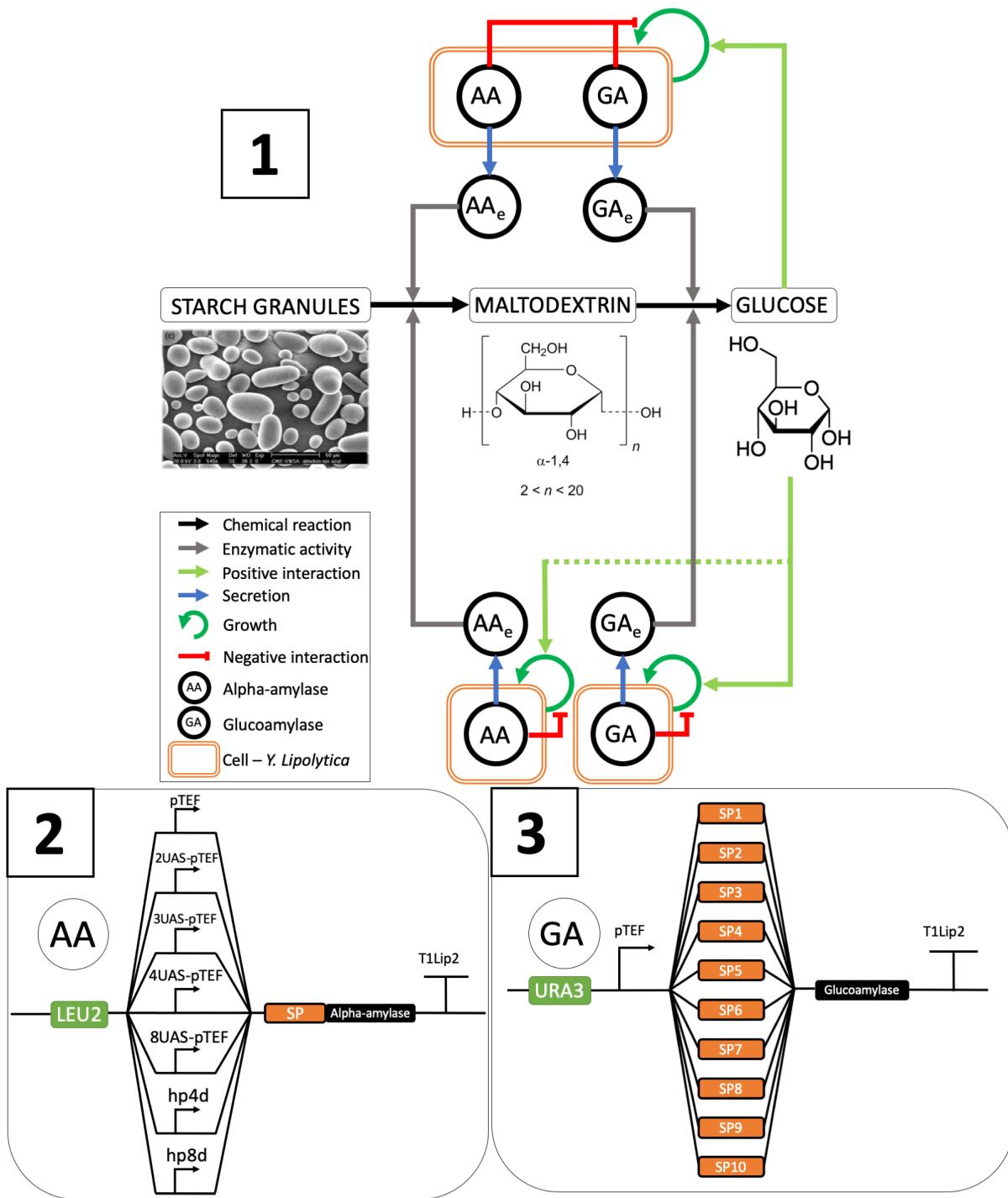


Figure 1.1: Project overview: (1) description of the two system we are trying to study: single cell expression vs. division of labour. (2) Alpha-amylase expression is regulated by a set of 7 different promoters. (3) Glucoamylase secretion is regulated by a set of 10 different signal-peptides.

(1) In this study we wish to compare an SC system (where both amylases (AA and GA) are expressed in a single cell) against a DOL system where the labour of expressing each enzyme is split across two different cells. The diagram illustrates the function of alpha-amylase and glucoamylase in the breakdown of starch as well as the burden caused by expression of these enzymes. (2) Pictures the multiple DNA cassettes in which the alpha-amylase expression is modulated by 7 different promoters. The alpha-amylase insert is paired to the LEU marker. (3) Pictures the cassettes in which glucoamylase secretion is regulated by 10 different signal-peptides. The glucoamylase is paired to the URA3 marker. Note: the e subscript in the AA and GA symbols refers to those enzymes being in the extra-cellular medium.

1.2 Background Theory

1.2.1 Starch digestion: a simple system for the study of cooperation

Starch digestion is a simple system for the study of co-cultures as growth should only occur if both enzymes necessary to break down starch are sufficiently expressed. Only two enzymes are involved, as opposed to systems where a whole metabolic pathway with multiple steps are split across cell types (e.g. [11]).

What is starch? - How can cells make use of it?

What we commonly call starch is, at the microscopic level, composed of starch granules formed of amylose and amylopectin. Amylose is a polymer of glucose units. Amylopectin are shorter polymer chains that branch from the amylose. α - and glucoamylase are essential to break down starch [12]. α - amylase hydrolyses the $\alpha-(1 \rightarrow 4)$ bonds at random sites in the starch polymers, releasing maltodextrin chains[6]. Then, glucoamylase releases β -D-Glucose units by hydrolysis of the released maltodextrins. This process is illustrated in 1.1. At this stage, glucose can be used by the cell as an energy input for metabolic processes. Successful breakdown of starch into glucose requires α -amylase and glucoamylase to be secreted to the extra-cellular space in their active form.

1.2.2 *Yarrowia lipolytica*: a competent protein expressor and se- cretor

Y.lipolytica is a non-conventional yeast, meaning its not a close relative to the most widely used *Saccharomyces Cerevisiae*[13], also known as "Brewer's yeast". *Y.lipolytica* main highlights are its abilities to: hydrolyse lipids, degrade extra-cellular protein and secrete natural and heterologous proteins [3]. This fungus can be found in dairy products and sausages [14] and is classified as a 'Generally Regarded As Safe' (GRAS) organism by the American Food and

Drug Administration (FDA). Therefore, compounds such as citric acid or enzymes for enzyme replacement therapy (ERP) produced in *Y.lipolytica* have been successfully marketed. [14][15].

The following two reasons make *Y.lipolytica* an excellent candidate as a heterologous protein cell factory for biotechnology applications. First *Y.lipolytica* is able to secrete active protein at levels 40-fold greater than the most widely used *S. cerevisiae*[16]. Secondly, a wide genetic engineering toolbox exist for *Y.lipolytica*. Expression levels can be modified with the use of well characterised parts such as the strong constitutive promoter pTEF[17] or the widely used terminator T1Lip2. Additionally, a golden gate assembly method was published in 2017 [18] and its parts have been made available on AddGene recently [19], this facilitates fast and easy assembly of multigene expression cassettes for later integration into the *Y.lipolytica* genome.

1.2.3 Succesful expression of α -amylase and glucoamylase in *Y.lipolytica* achieves survival on starch

Ledesma et. al were succesful in the engineering of a *Y.lipolytica* strain (named Y5017) that expresses α -amylase from *Oryza Sativa*(rice-AA) and glucoamylase from *Aspergillus Niger*(ANGAMY) in 2015, as well as in the engineering of a strain that expresses two copies of each amylase (Y5196)[6]. Additionally, this proved successful in the digestion of soluble starch and in industrial raw starch(figure 1.2). However, the burden caused by expression of the amylases was not studied. We theorise that starch digestion can be optimised either by division of labour or by altering the expression and secretion levels of the amylases with an available library of promoters and signal-peptides respectively.

1.2.4 Signal-peptide and promoter library could improve starch digestion in single cell host

Signal-peptide library shows great improvement of extra-cellular TIGAMY activity

Celinska and colleagues characterised the secretion of TIGAMY and α -amylase (from *Sitophilus oryzae* - SoAMY) under the control of 10 different signal-peptides(SPs)[9]. SPs are recognised by enzymes called signal-peptidases which then secrete the protein through the Endoplasmatic Reticulum (ER) and out to the extra-cellular medium. The use of different SPs associated to a protein alter the rate at which that protein is secreted.

Y5017 and Y5196, the strains co-expressing copies of both rice-AA and ANGAMY, use a modified version of the SP of the extra-cellular lipase Lip2 (SPLip2) with three added X-Ala motifs [6]. As seen in figure 1.2.2, SP3, SP8 and SP4 give the highest TIGAMY activity. It is important to note that SP3 gives a glucoamylase activity almost twice as great as the activity linked to the native SP, SP9 and almost 6-times greater than the activity associated to SPLip2 (labelled SP6 by Celinska and colleagues). From these observations, we hypothesise that if starch digestion in *Y.lipolytica* fails because of insufficient glucoamylase activity, it could be greatly improved by changing the signal peptide associated to glucoamylase.

Promoter library could increase α -amylase activity

Promoters govern how fast DNA is transcribed into RNA by facilitating or inhibiting the binding of RNA polymerase or transcription factors to the DNA. A greater promoter strength implies a greater transcription rate hence a faster protein turnover as long as ribosomes are available. In 2017, Dulermo et al. [10] characterised ANGAMY expression and activity under 7 different promoters in *Y.lipolytica*, which is summarised in table 1.1. In this study Dulermo et al. created new hybrid-promoter by varying the number of upstream activating sequences (UAS) units prior to the promoters pTEF and hp4d. UAS units increase promoter strength by binding to the transcription factors but, they are different parts from promoters. In regards to

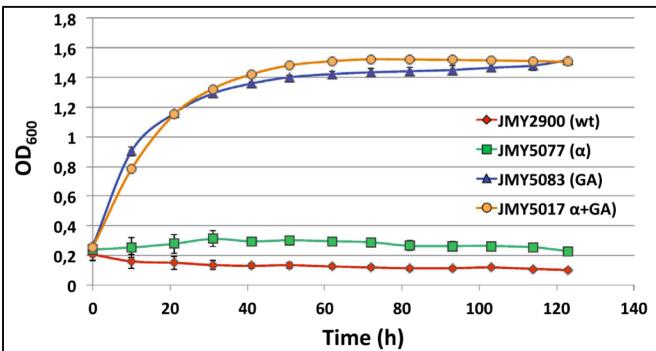
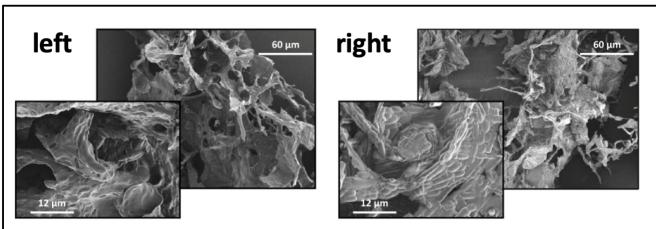
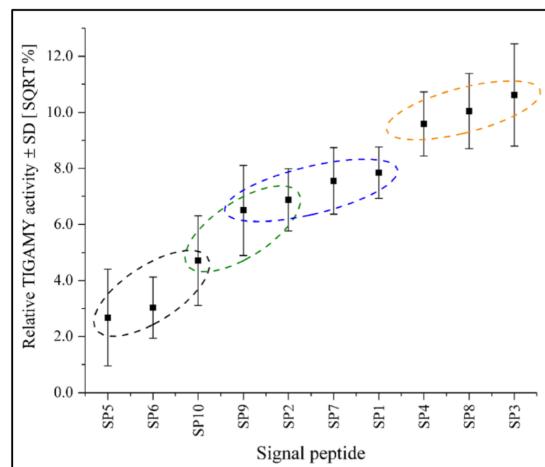
1a**1b****2**

Figure 1.2: (1) Starch digestion in *Y.lipolytica*. (2) SP library characterisation with glucoamylase gene

(1a) Starch digestion occurred in strain expressing ANGAMY only and strain expressing rice-AA and ANGAMY while no growth was observed in the strain expressing rice-AA only. (1b) Starch breakdown was observed under the microscope (left: undigested starch, no cells; right starch broken down by cells) (2) TIGAMY activity was characterised under 10 different signal peptides, SP3, SP8 and SP4 giving the highest activities. (1) from [6] (2) from [9]

ANGAMY activity, the promoters hp8d and 8UAS-pTEF give the highest production and activity of the enzyme. In this case expression and activity both scale up with the number of UAS units. Though, the relationship between activity and promoter strength is not always linear as observed for XylanaseC where a supposedly "weak" promoter (2UAS-pTEF) gives the highest activity. When it comes to transcription, faster or stronger is not necessarily better. Obtaining a functional copy of an enzyme depends on post-transcriptional and post-translational modifications such as folding, mRNA and protein stability [10]. These modifications are partially affected by promoter choice. We aim to characterise expression of rice-AA under the control of 7 different promoters in an effort to optimise the conditions for starch breakdown.

| | Glucoamylase | | XylanaseC | | |
|-------------|--------------|----------|-----------|------------|----------|
| | Production | Activity | RNA | Production | Activity |
| pTEF | + | + | + | + | + |
| 2UAS - pTEF | ++ | ++ | ++ | +++++ | +++++ |
| 4UAS - pTEF | +++ | +++ | ++++ | + | + |
| 8UAS - pTEF | ++++ | ++++ | +++++ | +++ | + |
| hp4d | + | ++ | + | ++ | + |
| hp8d | +++ | ++++ | ++++ | ++++ | +++ |

Table 1.1: Expression and activity levels of different proteins under different promoters. Modified from Dulermo et. al (2017) [10]

Glucoamylase activity is highest when it is under 8UAS-pTEF or hp8d regulation while surprisingly XylanaseC is highest when under the control 2UAS-pTEF regulation.

1.2.5 Metabolic burden, its implications and how it can be surpassed by division of labour

It has been known for a long time that expression of heterologous protein causes metabolic burden in microbes and research on the topic is abundant [7]. The effect known as metabolic burden or protein cost is believed to occur because of the consumption of vital finite cellular resources such as ribosomes, amino acids or polymerases [20]. Consequently, constitutive (always-ON) expression of heterologous enzymes that are unnecessary for survival in a given media typically decrease cell growth and absolute protein yield [8]. When studying expression of several proteins or a metabolic pathway, distributing the task across different cells may help circumvent the problem of burden when diffusivity of the expressed protein in the extra-cellular medium is sufficient [21]. After all, division of labour and cell-cell interactions are ubiquitous in nature where cells tend to form synergistic communities [22][21].

Although *Y.lipolytica* is an excellent protein expressor, we hypothesise greater protein yields could be achieved when distributing the workload of expressing certain metabolic pathways. To our knowledge, studies of co-cultures between cross-feeding *Y.lipolytica* have not been conducted. We hypothesise that support between different *Y.lipolytica* strains is more feasible than between *Y.lipolytica* and a different species because cells from same species will have similar metabolic needs. As an example, when co-culturing *Y.lipolytica* with *Staphylococcus xylosus*,

growth of *Y.lipolytica* is "dramatically reduced"[\[23\]](#).

1.3 Motivation and Objectives

The motivation of my work is to optimise starch digestion in *Yarrowia Lipolytica* to use starch as the sole carbon source in biotechnology applications. The aims of this study are to:

- As shown in table [1.2](#), assemble new strains co-expressing glucoamylase with different signal peptides and alpha-amylase with different promoters(Y100-Y169) as well as strains expressing alpha-amylase only with different promoters(Y11-Y17)(Full details of each strain in table [C](#)).
- Characterise the metabolic burden related to: expressing and secreting rice-AA linked to different promoters in a single *Y.lipolytica* strain, expressing and secreting TIGAMY with different signal peptides in a single *Y.lipolytica* strain and co-expressing rice-AA and TIGAMY in a single *Y.lipolytica* strain.
- Study the ability of the strains co-expressing both amylases to use starch as their main carbon source.
- Study the co-operation between cells expressing alpha-amylase only and cells expressing glucoamylase only in degrading starch.

| Assembly 1 | pTEF | 2UAS pTEF | 3UAS pTEF | 4UAS pTEF | 8UAS pTEF | hp4d | hp8d |
|------------|----------------|------------------------|------------------------|------------------------|------------------------|----------------|----------------|
| RiceAA | Y11 | Y12 | Y13 | Y14 | Y15 | Y16 | Y17 |
| Assembly 2 | pTEF RiceAA | 2UAS pTEF RiceAA | 3UAS pTEF RiceAA | 4UAS pTEF RiceAA | 8UAS pTEF RiceAA | hp4d RiceAA | hp8d RiceAA |
| SP1GA | Y100 | Y101 | Y102 | Y103 | Y104 | Y105 | Y106 |
| SP2GA | Y107 | Y108 | Y109 | Y110 | Y111 | Y112 | Y113 |
| SP3GA | Y114 | Y115 | Y116 | Y117 | Y118 | Y119 | Y120 |
| SP4GA | Y121 | Y122 | Y123 | Y124 | Y125 | Y126 | Y127 |
| SP5GA | Y128 | Y129 | Y130 | Y131 | Y132 | Y133 | Y134 |
| SP6GA | Y135 | Y136 | Y137 | Y138 | Y139 | Y140 | Y141 |
| SP7GA | Y142 | Y143 | Y144 | Y145 | Y146 | Y147 | Y148 |
| SP8GA | Y149 | Y150 | Y151 | Y152 | Y153 | Y154 | Y155 |
| SP9GA | Y156 | Y157 | Y158 | Y159 | Y160 | Y161 | Y162 |
| SP10GA | Y163 | Y164 | Y165 | Y166 | Y167 | Y168 | Y169 |

Table 1.2: Simplified table showing the new strains to be assembled

Assembly 1 consist of the strains expressing rice-AA under regulation of different promoters. Assembly 2 consist of the strains co-expressing rice-AA with different promoters and TIGAMY with different signal peptides. All strains in the tables are prototrophic.

Chapter 2

Methods

2.1 Standard protocols

Standard protocols were used for media and cell culturing ([B.1](#)), DNA extraction ([B.2](#)), restriction enzyme digestion ([B.3](#)), DNA ligation ([B.4](#), polymerase chain reactions (PCR) ([B.5](#)), DNA purification ([B.6](#) & [B.7](#)), golden gate reaction ([B.8](#)), E.Coli transformation ([B.9](#), Y.Lipolytica transformation ([B.10](#) & [B.11](#)) and colony PCR([B.12](#)).

2.2 Extra minimal media: YNB-starch and YNB-1% glucose for the study of starch digestion

To study growth in starch minimal media, a liquid soluble starch solution was created. This recipe is identical to the YNB recipe in [B.1](#) except that the glucose at 2% concentration is switched for soluble starch (Sigma-Aldrich) at 1% concentration. Starch at 2% concentration was not as soluble as at 1% and also was more viscous which complicates experiments in 96-well plates with a volume-per-well of around 200 μL .

To be able to directly compare the growth assays between media containing starch and media containing glucose, YNB-1% glucose was used.

2.2.1 Golden Gate 2.0 - YaTK

Golden gate is a gene assembly method introduced in 2009 by Engler et al. [24] which takes advantage of the off-site nature of the cuts made by type-IIIs restriction enzymes to assemble multiple parts in a one-pot reaction with DNA parts. In 2017, Celinska et. al introduced a golden gate assembly method for the genetic engineering of *Y.lipolytica*[18]. While multiplte transcriptional units can be assembled using this system, the large number of parts in the reaction leads to a lower efficiency. Taking inspiration from the Yeast Tool Kit (YTK)[25], Mauricio Pesantes, a student in RLA lab expanded the golden gate system in *Y.lipolytica* by introducing hierachisation to the toolkit (naming it Yarrowia Toolkit: YaTK - figure 2.1). Hierachisation in this context means the introduction of 3 levels (0,1 and 2) which correspond to: a storage vector of individual parts(promoter, gene or terminator), a storage vector of 1 transcriptional-unit(TU)(promoter, gene and terminator) and the final vector that can store up to 6 TUs. This toolkit includes two yeast markers (LEU2 and URA3), two genome insertion sites (InsUP and InsDOWN) and two backbone vectors (pSB1A and pSB1C) with ampicillin(AMP) and cloramphenicol(CAM) resistance respectively.

2.3 Growth assay study design

Growth of different strains was assessed in volumes of 200 μ L per well in a 96-well plate at 500 RPM, 30°C for 80 hours using the BMG POLARStar plate reader. Dilution from overnight cultures were measured with the 0D600 DiluPhotometer - Thomas Scientific. An experiment in rich and poor media was conducted where the cells were grown overnight and then diluted in a 1:100 ratio and transferred into the 96 well plate. For the growth assay in starch media, cells were grown overnight in rich media (YPD) and then centrifuged and re-suspended in starch media twice before being transferred to the 96-well plate. In the starch growth assay each strain was diluted to initial ODs of 1, 0.5 and 0.25 (measured with the 0D600 DiluPhotometer - Thomas Scientific) and one replicate of each strain at each OD were measured.

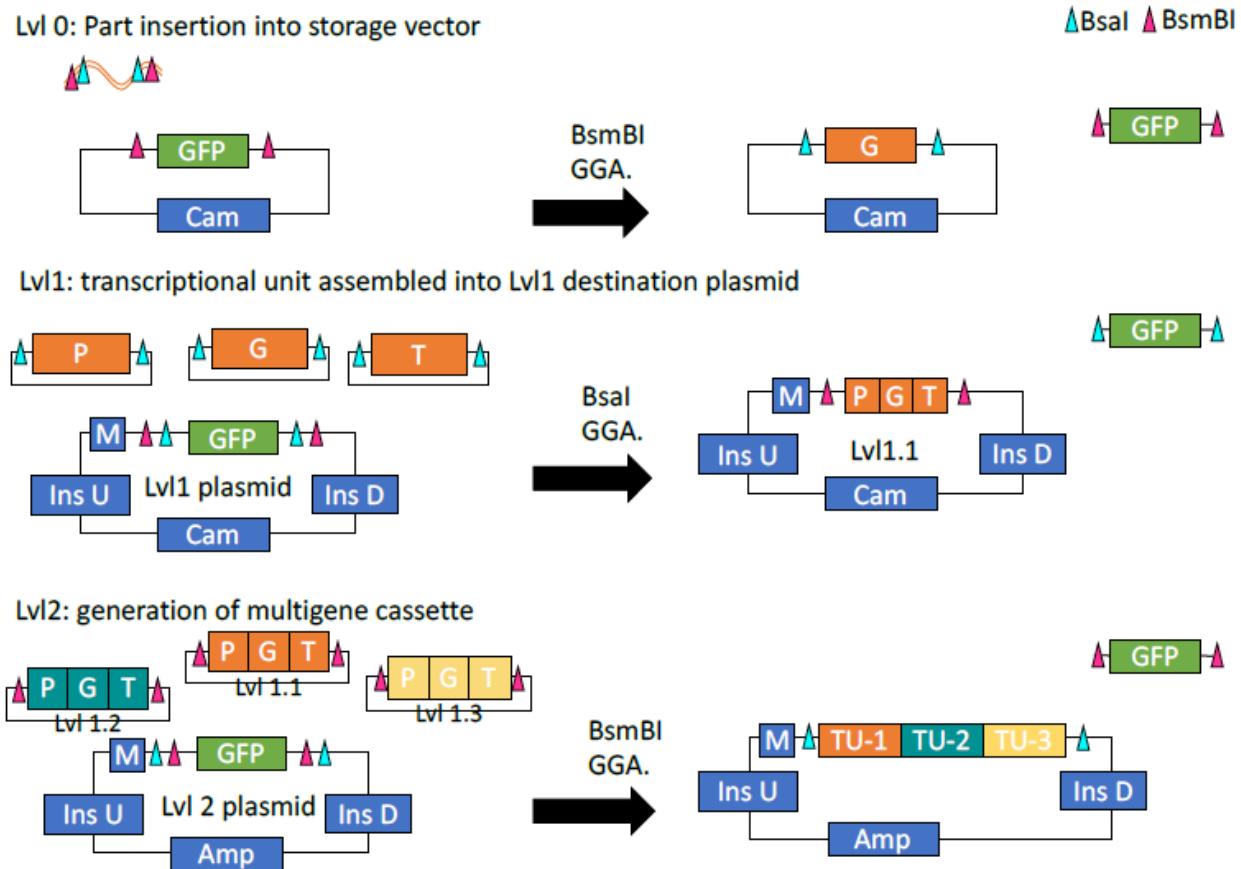


Figure 1 - YaTK overview

Figure 2.1: YaTK overview from YaTK guide (appendix 2.2.1)

Growth in solid media was evaluated using a spot test protocol. Each cell type was cultured at initial ODs of 1, 0.5, 0.25, 0.125 and 0.0625 in three different media: YNB-1% glucose, YNB-soluble starch 1% and YNB-potato starch 1%.

2.4 Curve fitting and parameter retrieval from growth curves

A curve fitting approach was used to extract parameters from growth curves. Growth curves were fitted to a four-parameter Gompertz curve as defined in [26]:

$$OD(t) = OD_0 + OD_{stationary} \cdot \exp(-\exp(-B(t - t_M))) \quad (2.1)$$

where $OD(t)$ is the optical density at time t , OD_0 is the initial OD, $OD_{stationary}$ is the OD when the cells have reached the stationary phase (when growth ceases) relative to the initial OD_0 , B is the relative growth rate (units t^{-1}) and t_M is the time at which the absolute growth rate is maximum. Fitting to a logistic growth curve and to the U-Gompertz curve [27] were tested but more accurate curve fitting results were obtained with the four-parameter Gompertz, as observed in [26][28]. A fit to each curve gave parameters with 95% confidence intervals using the MATLAB function fit from the Curve Fitting Toolbox. A non-linear least square regression method with bisquare weighting of the residuals was used for fitting the curves. A bisquare weighting of the residuals attributes a weight of 0 to extreme outliers and down-weights mild outliers, allowing us to analyse raw data without having to filter the signals. After obtaining growth curve data, the work-flow to use the curve fitting function consists of: fitting the curve to the equation 2.1, obtaining parameters, evaluating the appearance of fit together with the residual plot, evaluating confidence intervals and goodness of fit measures (sum of square error (SSE), root mean squared error (RMSE), R-squared and adjusted R-squared value) and finally discarding anomalous curve fits. Anomalous curves are those which growth curve pattern do not follow a sigmoidal trend or do not reach stationary phase within the time length of the experiment.

2.5 Dilution program for pipetting robot

In an effort to automate labour-intensive and error-prone procedures such as hand dilutions for plate reader experiments, I wrote a MATLAB script which outputs compatible files for the CyBio FeliX pipetting machine. The code for the MatLab script can be found in ??.

Chapter 3

Results

Nomenclature reminder

The following strains are referred to in the following section:

- Y1-Y10, which all express TIGAMY under control of one of 10 signal peptides.
- Strain Y5077 which expresses rice-AA under control of the pTEF promoter
- Strain Y5017 which expresses one copy of ANGAMY and one copy of rice-AA and serves as a positive control for starch digestion.
- Strain Y5196 which expresses two copies of ANGAMY and two copies of rice-AA and serves as a positive control for starch digestion.
- Strain Y2900, which does not express anything extra and serves as negative control for burden and a negative control for growth in starch.
- Strain Y195, which does not express anything extra but cannot make its own uracile and leucine (URA and LEU auxotrophic). It serves as a negative control for growth in minimal media,
- Y11-Y17, the new strains to be assembled, containing the LEU marker, the 7 promoters characterised by Dulermo et. al and alpha-amylase.
- Y100-Y169, the new strains to be assembled, each containing TIGAMY linked to 1 of 10 characterised signal peptides and rice-AA linked to 1 of 7 characterised promoters.

3.1 Assembly of new *Y.lipolytica* strains

In the table 1.2 and in the appendix C can be seen the strains aimed to be constructed by the end of this study as well as the strains that already existed upon start of this project. To transform *Y.lipolytica*, the plasmid containing each gene construct needs to be assembled and cloned into E.Coli. Below is explained how the assembly of the new strains was attempted.

3.1.1 Assembly of new constructs by golden gate assembly

Initially, assembly of the strains Y11 to Y17 was attempted by replacing the gene RedStar by the alpha-amylase gene in plasmids containing the different promoters. The removal of the RedStar gene by digestion with DpnII and AvrII proved unsuccessful (data not shown). Unable to complete the assembly of strains Y11 to Y17 through digestion-ligation reactions, I changed my approach to assembling the plasmids from scratch.

Construction of Level 1 plasmid with Leucine marker

Using the YaTK method (see 2.2.1), we aimed at assembling the different promoters, terminators and the alpha-amylase gene into level 1 expression cassettes (i.e. destination vectors for one transcription unit with the LEU marker).

First we needed to assemble a level 1 destination vector with a Leucine marker(LEU level 1), which was not available. To do so, sfGFP was amplified with the primers p022F and p022R (figure E.1)(see table 8 in appendix D) from the plasmid of pYTK1, adding the BsaI and BsmBI restriction sites to the sfGFP gene. Further on, the InsUP and InsDOWN insertion sites, as well as the LEU2 marker and the pSB1A3 backbone vector were the parts used to assemble the LEU level 1 destination vector. The amplified sfGFP was digested with the BbsI restriction enzyme while the remaining parts (InsUP, InsDOWN, LEU2 and pSB1A3) were digested with BsaI (figure E.3). After PCR-clean up the parts were ligated together with the use of the T7 DNA ligase. Finally, the resulting plasmid was transformed into Escherichia Coli.

The success of the assembly of the LEU level 1 plasmid was evaluated by expression of GFP in the positive transformants (see figure E.4) as well as sequencing of the GFP section and the BsaI and BsmBI recognition and cutting sites where no mutations were observed. BsaI digestion of the LEU level 1 plasmid also showed correct bands (see figure E.5).

Failure of assembly of the alpha-amylase cassettes

Upon successful assembly of the Leu level 1 plasmid, the new plasmids containing the several promoters (pTEF, 2UAS-pTEF, 4UAS-pTEF and 8UAS-pTEF), one alpha-amylase gene and the terminator T1Lip2 were the next goal. To achieve this, the different parts were assembled using a golden gate reaction. Assembly with different alpha-amylases was attempted, either SP1-SoAMY or SP2-SoAMY. Rice-AA was amplified using the primers G1 'D' and 'E' (see table 1 in D) as shown in figure E.2. The backbone used in the golden gate reaction was LEU level 1.

Golden gate assembly of the alpha-amylase cassettes regulated by the four different promoters turned unsuccessful after several rounds of golden gate. The golden reactions worked but gave really poor transformation efficiency. However, a few positive transformants could be selected. When DNA from positive transformants was extracted, digested with NotI and ran in a electrophoresis gel, most inserts seemed shorter (1000 base pairs) than expected (data not shown). Colony PCR revealed the alpha-amylase gene was not fully present in the new strains (data not shown). It was finally confirmed through sequencing (SourceBioScience) that a deletion of 1007 base-pairs had occurred within the rice-AA gene (see table E.1). Due to lack of time, other experimental procedure were prioritised and therefore strains Y11-Y17 & Y100-Y169 were not built and tested.

3.2 Absolute maximum growth rate of *Y.lipolytica* strains in rich and poor media

The parameters for each replicate of the strains Y1-10, Y2900, Y195, Y5017, Y5077 and Y5196 were obtained from fitting each growth curve to equation 2.1. The absolute maximum growth rate is then computed from:

$$\frac{dOD}{dt} \Big|_{max} = \frac{dOD}{dt} \Big|_{t=t_M} = \frac{OD_{stationary} \cdot B}{e} \quad (3.1)$$

The data shown in 3.1 shows the absolute maximum growth rate in YPD and YNB for all the

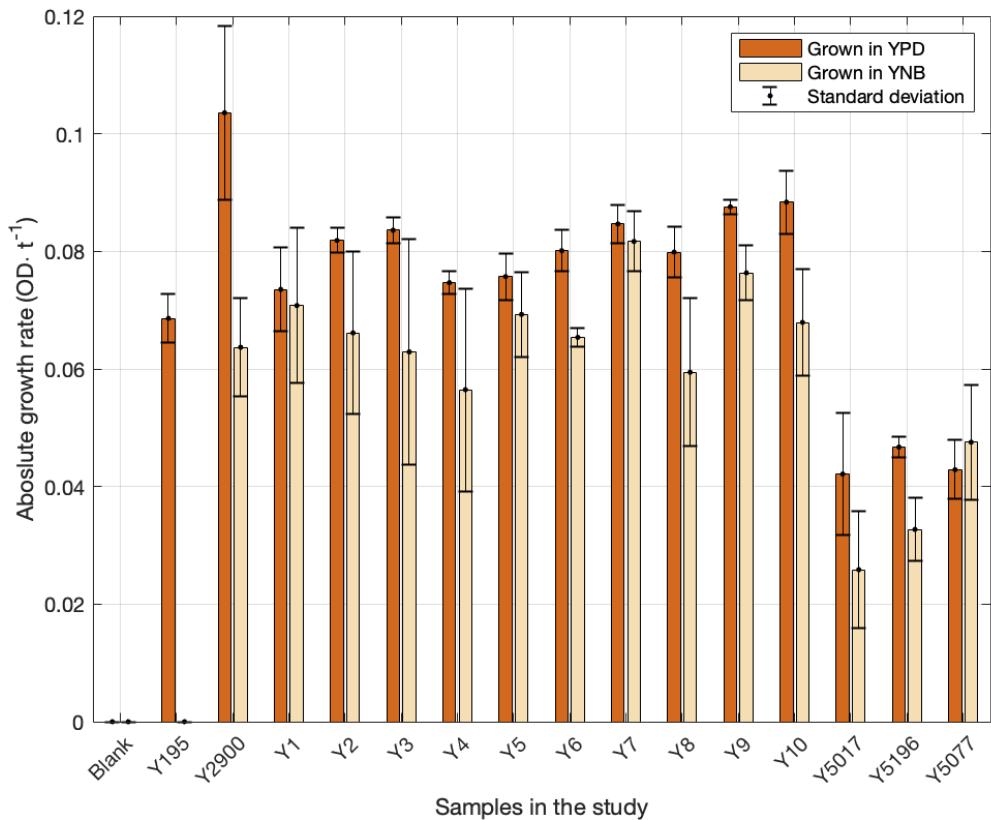


Figure 3.1: Absolute maximum growth rate of strains Y1-Y10, Y5077, Y5017 and 5196 in rich and poor media.

strains studied. Growth rate of strains Y1(n = 3) and Y5 (n=3) which secrete TIGAMY linked to signal peptides SP1 and SP5 is significantly reduced ($p = 0.05$) with respect to the positive

control Y2900(n=3). The growth rate of the other strains expressing glucoamylase (Y2, Y3, Y4, Y6, Y7, Y8 ,Y9 Y10) decreased with respect to the strain Y2900 but is not statistically significant at a p-value of 0.05. The strain expressing alpha-amylase(Y5077, (n=3)) and one or two copies of each of the amylase simultaneously (Y5017(n=2) and Y5196(n=2)) see their growth rate reduced significantly ($p = 0.02$) with respect to the control Y2900 (n=3). As expected no growth was observed in the strain Y195 in minimal media and, the absence of any growth in the blank ensures no cross-contamination occurred between samples.

The average growth curves across repeats for Y3, Y2900, Y195, Y5017, Y5196 and Y5077 are displayed in figure 3.2. As expected growth of all the strains is more limited in poor media than

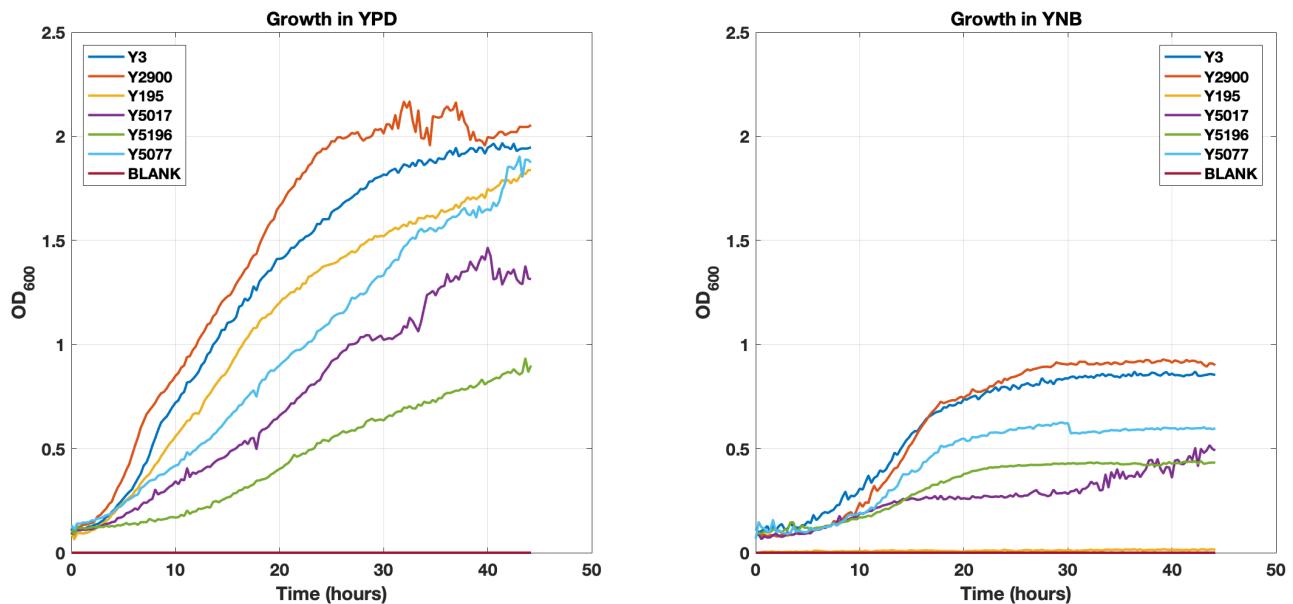


Figure 3.2: Growth of a few *Y.lipolytica* strains in YPD and YNB
Growth in starch of strains Y3, Y2900, Y195, Y5017, Y5196 and Y5077 is depicted.

in rich media. Strains expressing alpha-amylase have even further limited growth irrespective of the media they are present in. The expression of glucoamylase affects growth but not as much as expression of alpha-amylase.

3.3 Growth assay of co cultures in minimal liquid starch media

Growth of the strains Y1-10, Y2900, Y195, Y5017, Y5077 and Y5196 as well of those strains with strain Y5077 added in a 1:1 ratio was measured. The strain Y5017 (used here as positive control of growth in starch) was able to reach an OD₆₀₀ value greater than 2 after approximately 30 hours. The strain Y5077 (only expressing rice-AA) by itself was able to grow on soluble starch after 40 hours while strain expressing under different signal peptides grew initially and then started dying as shown by the decreasing OD over time. The growth pattern of strain Y1-10 is similar in form to the one from Y2900 (the negative control). The co-culture systems where each strain was mixed in equal amount with the strain Y5077 were able to grow to a similar or sometimes higher OD (see Y4 and Y6 in figure 3.3) than Y5077 by itself. A growth lag can be observed between the cells growing in co-cultures (orange line) with respect to the rice-AA expressing cells growing by themselves (red line). Unsurprisingly, cells start to die after a long period of incubation.

In figure E.7, we can see how strain Y5017 (blue line) reaches the highest OD of around 2.3 while the same strain grown im coculture with Y5077 barely reaching an OD of 2(red line), though it grows more than strain Y5077 by itself (purple line) which reaches an OD of 1.4 at best.

3.4 Growth in solid starch media

Figure E.8 shows growth in YNB glucose and YNB starch media of the strains Y1-10, Y2900, Y195, Y5017, Y5077 and Y5196 as well of those strains with strain Y5077 added in a 1:1 ratio after two days of incubation at 30°C. First we note in E.8a that growth was much slower in soluble starch media than in media containing glucose. Additionally strain Y195 could not grow in YNB glucose as expected and halos can be seen around strains Y5017, Y5196 and

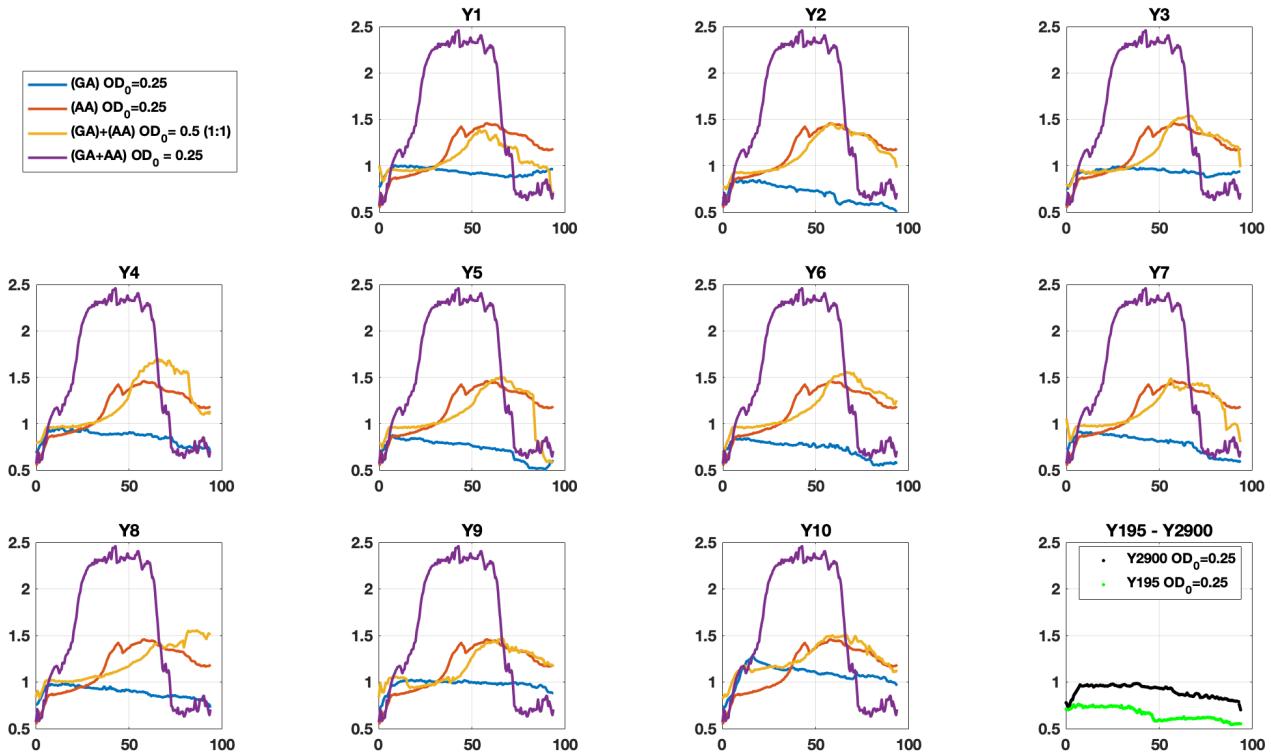


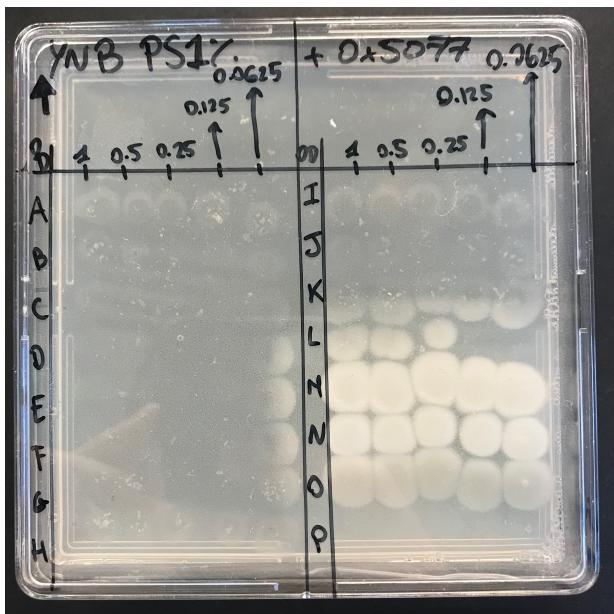
Figure 3.3: Glucoamylase expressing strains Y1-Y10 growing with and without supplementary culturing of alpha-amylase expressing Y5077

Axis: Y-axis is OD_{600} and x-axis is time in hours. In the legend: (GA) stands for cells expressing TIGAMY with different signal peptides (Y1-Y10) as shown on the upper title of each plot at starting OD of 0.25; (AA) stands for the cell expressing rice-AA (Y5077) at starting OD of 0.25; (GA)+(AA) stands for cocultures where cells expressing TIGAMY (Y1-Y10) were initially cultured with strain Y5077 in a 1:1 ratio at an overall OD of 0.5; (GA+AA) stands for the cell where ANGAMY and RiceAA are expressed in a single cell(Y5O17) at starting OD of 0.25.

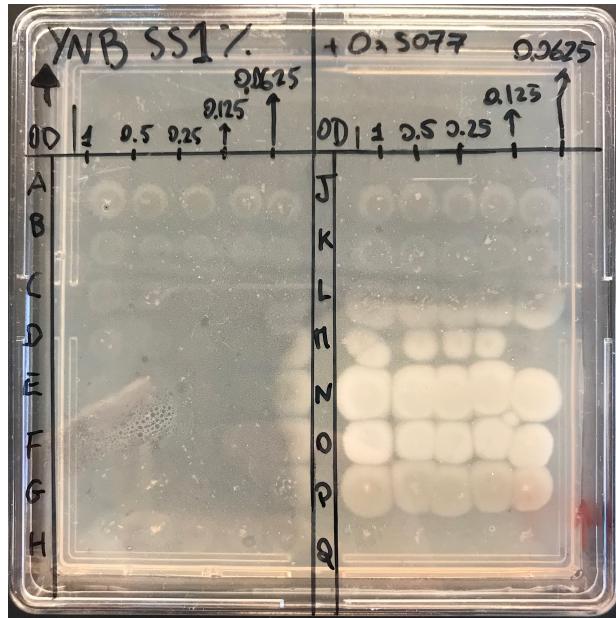
Y5077 proving extracellular activity of alpha-amylase. In figure E.8b, we can see how growth of strains that cannot grow by themselves in potato starch media, occurs when supplemented with the strain that produces alpha-amylase and small halos are seen around all colonies in the co-culture plate. It is hard to discern if growth occurs because of the effect of co-culturing both cell types together or merely because of the presence of the alpha-amylase expressing strain itself.

In figure 3.4, we can observe the state of strains Y1-10, Y2900, Y195, Y5017, Y5077 and Y5196 after 7 days of incubation at 30°C. After such a long time of incubation, the halos of alpha-amylase expressing strains have overlapped and expanded onto the space of cells that do not express alpha-amylase nor glucoamylase, causing some growth in those wells. Strain Y195, has

surprisingly grown in minimal media, though to a lesser extent than the other strains.



(a) Growth of strains in YNB potato starch (1%) media



(b) Growth of strains in YNB soluble starch (1%) media

Figure 3.4: Single cell growth of strains in solid starch media

Shown is the growth of strains Y1-10 (rows A-J), Y2900, Y195, Y5017, Y5077 and Y5196 (row K-O) and blank (no cells, only media - row P) at different starting ODs of 1, 0.5, 0.25, 0.125 and 0.0625 in YNB potato starch (1%) [a] and in YNB soluble starch [b].

Chapter 4

Discussion

4.1 Failure of assembly

Although assembly of LEU level 1 was successful as verified by sequencing and BsaI digestion, the golden gate assembly of plasmids containing the variants of the LEU marker, the promoter pTEF and any alpha-amylase and the terminator T1Lip2 could not be obtained. Initially, positive transformants of E.Coli were obtained meaning the placeholder sfGFP from LEU level 1 was successfully removed and switched from another insert. Through sequencing it was confirmed that the promoters were correctly assembled for the plasmid containing 2-UAS-pTEF and 4UAS-pTEF, meaning that those promoters were correct too. Additionally, positive transformants of *Y.lipolytica* were obtained implying that the yeast marker (LEU2) was correctly integrated into the plasmid and into the genome of the fungus. The latter results also imply that the insertion sites (zeta-InsUP and zeta-InsDown) were correct. Indeed *Y.lipolytica* cells transformed with the new plasmids could grow in minimal media but not in starch. As later colony PCR revealed the alpha-amylase gene was not fully present.

The deletion seen in the rice-AA gene went from within the rice-AA gene to within the zeta-NotInsDown site. All obtained results point at some sort of problem with the rice-AA amplification and potentially with the zeta-NotInsDOWN site. rice-AA was amplified by PCR with Phusion HF polymerase and all PCR can introduce potential mutations or deletions[29]. A mutation in the zeta-InsDOWN could explain the introduction of a BsaI site. In order to reattempt the assembly of strains Y11-Y17 and Y100-Y169 all the parts used in the golden gate reaction should be sequenced. Finally, the golden gate product to verify the rice-AA is

amplified correctly.

4.2 Glucoamylase and alpha-amylase impact on growth

As seen in figure 3.1, the growth rate of strains expressing TIGAMY at different secretion levels is not significantly decreased compared to the positive control Y2900. Producing and secreting TIGAMY decreases cell fitness to a minor extent 3.2 irrespectively of the SP linked to TIGAMY. On the other hand, expression of rice-AA (in Y5077) under the regulation of the pTEF promoter causes a significant reduction of the growth rate and a similar growth rate reduction is observed in the strain Y5017 which expresses ANGAMY and rice-AA and in the strain Y5196 which expresses two copies of ANGAMY, two copies of rice-AA and the CRE recombinase and a set of enzymes that makes it accumulate lipids. The similar reduction in growth rate between strains Y5017, Y5077 and Y5196 suggests the expression of alpha-amylase could be the main cause of burden wherever it is expressed.

Protein cost or burden is conceptually defined as "the fractional reduction in growth rate" [7], thus, the results in figure 3.1 gives a measure of how much burden is caused to each strain by the expression of the heterologous compounds it is expressing. The smaller the maximum growth rate the greater the burden and viceversaa.

4.3 Alpha-amylase expressing strain grows in soluble cooked starch

E.7 shows the ability of the rice-AA expressing strain Y5077 to grow on raw cooked soluble starch. This contradicts previous results shown in 1.2(1a) and previous research on starch hydrolysis. It could be thought that the cooking of soluble starch breaks down the starch to a certain extent, thus liberating maltodextrin chains and glucose units. However, this hypothesis cannot be confirmed because in such scenario we would see growth of the TIGAMY expressing

strains and the negative control (Y2900). On another note, the initial minimal amount of growth that can be seen in strains Y1-Y10 and Y2900 could be due to the use of: cellular energy storage or resources they carry over from the previous media they were in before being transferred to starch. Another explanation for such initial growth could be altruistic suicide, where most cells in the colony die to feed of the remaining. Such event of programmed cell death has been observed in unicellular organisms[30]

4.4 Co-cultures: cooperation or competition?

Coculture of rice-AA and TIGAMY secreting strains does not yield as much growth in soluble starch than SC expression observed in strain Y5017 (figure 3.3). On top of this, growth of the co-culture system appears to only be causing lag in the growth of the cells, which could be caused by the competition of resources between the two different strains or the simple fact that one of the two strains is not doing any useful work on breaking down the starch and simply consuming resources. The most important result from figure 3.3 is that the co-culture systems reach a growth 40% lower than the positive control Y5017. Thus, at this stage using co-cultures to improve the efficiency of starch digestion is not useful. We will now seek to explain why this could be happening.

4.5 Alpha-amylase causing burden could be caused by codon non-optimality

Expressing alpha-amylase under the control of the pTEF promoter causes a significant amount of burden to the host cell. The level of burden caused by the expression of rice-AA is very similar to the burden in strains Y5017 and Y5196, which leads us to think the expression of the rice-AA is the main cause of burden in the aforementioned strains. Even though the rice-AA gene was previously codon-optimized in [6] using the GenScript optimisation tool, when submitting the rice-AA sequence to the ([IDT codon optimisation tool](#)) and choosing to

optimise for *Y.lipolytica*, large discrepancies arise between the two sequences. 248 nucleotides and 246 codons were modified between the two sequences(figure E.6). This discrepancies could be explained by the fact that when a codon optimisation tool is used for several iterations, different results will be obtained each time. However, as it is shown in 4.2, the codon bias is less balanced in the original rice-AA used ([6]) than in the version optimised by IDT. The discrepancies between the sequence used in the work of Ledesma et. al and one of the iterations obtained from the IDT tool is illustrated in figure 4.1 where the difference in codon usage frequency between the original and the optimised gene sequences is illustrated. The research that links codon-optimality and costly protein production is abundant[31][32]. Additionally this study [33], found up to a 2.6-fold increase in alpha-amylase expression when optimising alpha-amylase of *Bacillus subtilis* expressed in *Pichia pastoris*. Re-optimising the rice-AA sequence should be considered as well as the use of other alpha-amylases from other organisms.

One thing that is clear is that expressing rice-AA does not help the cell hence it does not help us solve the problem of starch digestion. We are now going to explore several possible reasons for this could.

4.6 Limitations of our study

The initial aim of the study was to characterise the metabolic burden on cells imposed by: expressing rice-AA under control of different promoters, expressing TIGAMY under control of different SPs and by co-expressing alpha-amylase and TIGAMY under control of different promoters and signal peptides respectively. This could not be accomplished because of failure of the rice-AA assembly, thus SC systems could not be compared effectively to their equivalent DOL systems.

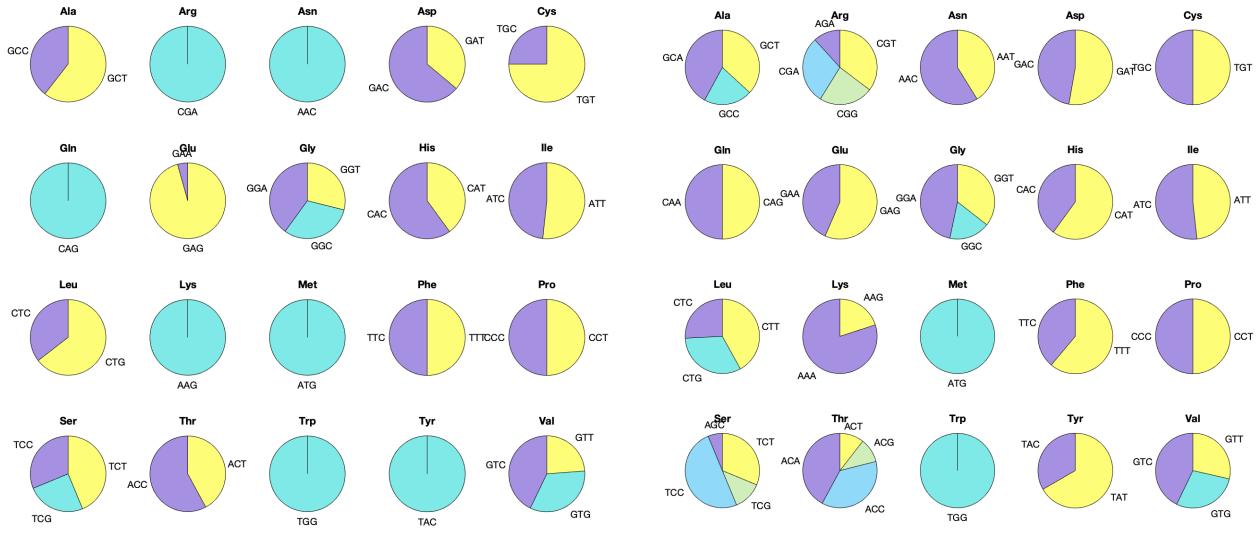
Instead, the assessment of burden was performed on: cells expressing TIGAMY with different SPs, one cell variant expressing rice-AA and cells expressing one or two copies of rice-AA and ANGAMY. The discrepancy between different glucoamylase being used weakens the power to compare the behaviour of the different strains. However, our results show a particular problem

| | | | | | | | |
|---------------|-------------------------------|---------------|--|---------------|--------------------------|---------------|-----------------------|
| TTT -0.005 | (Phe/F) Phenylalanine | TCT 0.005 | | TAT -0.024 | (Tyr/Y) Tyrosine | TGT 0.002 | (Cys/C) Cysteine |
| TTC 0.005 | | TCC -0.007 | | TAC 0.024 | | TGC -0.002 | |
| TTA 0 | | TCA 0 | | TAA 0 | STOP | TGA 0 | STOP |
| TTG 0 | | TCG 0.005 | | TAG 0 | STOP | TGG 0 | (Trp/W) Tryptophan |
| CTT -0.031 | (Leu/L) Leucine | CCT 0 | | CAT -0.007 | (His/H) Histidine | CGT -0.014 | |
| CTC 0.007 | | CCC 0 | | CAC 0.007 | | CGC 0 | |
| CTA 0 | | CCA 0 | | CAA -0.014 | (Gln/Q) Glutamine | CGA 0.028 | (Arg/R) Arginine |
| CTG 0.024 | | CCG 0 | | CAG 0.014 | | CGG -0.009 | |
| ATT 0.002 | | ACT 0.014 | | AAT -0.017 | (Asn/N) Asparagine | AGT 0 | (Ser/S) Serine |
| ATC -0.002 | (Ile/I) Isoleucine | ACC 0.009 | | AAC 0.017 | | AGC -0.002 | |
| ATA 0 | | ACA -0.019 | | AAA -0.038 | (Lys/K) Lysine | AGA -0.005 | (Arg/R) Arginine |
| ATG 0 | (Met/M) Methionine - START | ACG -0.005 | | AAG 0.038 | | AGG 0 | |
| GTT -0.002 | | GCT 0.021 | | GAT -0.014 | (Asp/D) Aspartic acid | GGT -0.007 | |
| GTC 0 | (Val/V) Valine | GCC 0.017 | | GAC 0.014 | | GGC 0.014 | (Gly/G) Glycine |
| GTA 0 | | GCA -0.038 | | GAA -0.021 | (Glu/E) Glutamic acid | GGA -0.007 | |
| G TG 0.002 | | GCG 0 | | GAG 0.021 | | GGG 0 | |

Figure 4.1: Change in codon usage between original and optimised rice-AA sequence. Displayed is the difference in codon frequency between the original and the optimised rice-AA sequence. Red values indicate a negative difference (deficit in the original sequence) while blue values indicate a positive difference (superhabit in original sequence)

related to the expression of rice-AA which if addressed would improve starch digestion efficiency. Also, none of the TIGAMY linked to the different signal-peptides cause any significant burden on the cells. This validates the use of the TIGAMY with the SP3, SP8 or SP4 which yields the greatest extra-cellular TIGAMY activity.

Additionally, when studying co-culture only a simple 1:1 ratio between cells expressing glucoamylase and cells expressing alpha-amylase was evaluated and any knowledge on the kinetics of the enzymes was unknown. Synthetic co-cultures are difficult to engineer and reality often comes short of expectation [34]. Potentially, cell co-cultures can be more robust than single cells [35], though a major challenge remains the fine tuning of the performance of the different cell populations [35].



(a) Codon bias of the original rice-AA sequence (b) Codon bias of optimised rice-AA sequence

Figure 4.2: Codon bias for original and optimised rice-AA sequence
Codon bias is more balanced in the optimised rice-AA sequence than in the original one used in [6]

4.7 Future work

The different golden gate parts need to be sequenced and high throughput methods should be used for the assembly of the new inserts and transformation of the new strains [36].

Growth assays should equally be performed in high throughput set-ups with automated pipetting stations and high number of biological replicates for each strain or co-culture to be tested, if reliable results for industrial purposes wish to be obtained.

For a co-culture to be successful several cell-cell interactions and also cell-media interactions need to be very finely regulated. To better understand how cooperation between cells could yield starch breakdown we would need to know:

1. The kinetics of both enzymes activities
2. The stoichiometry of the starch and of the maltodextrin hydrolysis reactions
3. The diffusivity of each amylase in the extra-cellular medium
4. The optimal pH and temperature conditions of the reactions

5. the expression and secretion rate of each amylase from each cell type
6. The burden (or absence) of it caused by expressing each amylase

Items 1,2,3 and 4 from the list above could be obtained by performing large in-vitro analysis of enzymes produced by cells. This would allow for a more rational design of starch digestion in *Y.lipolytica*. In parallel, obtaining the results from items 5 and 6 from the list would allow for modelling of the system to be performed, in a similar way to the modelling presented by Tsoi et. al[21]. Several experiments could be performed to track the concentration of each enzyme in the intracellular and extracellular medium. The rice AA could be tagged with a fluorescent protein as it was done in [37]. Otherwise, other fluorescent tags could be considered [38] To perform high throughput studies of co-cultures systems microfluidic devices such as the one presented in [22]could be used.

Chapter 5

Conclusion

Our study concludes that the rice-AA is the main cause of burden in the strains expressing it. Additionally we find TIGAMY to be a valid candidate as the glucoamylase enzyme even when secreted at the highest levels. Third, our results show that starch digestion by division of labour is unsuccessful at this stage. Finally, we draw an outline of what the next steps in improving starch digestion by division of labour and by fine-tuning of production of the amylases should be.

Appendices

Appendix A

Glossary & acronyms

A.1 Glossary

DNA and nucleotides: Deoxyribonucleic acid (DNA) is a common molecule across all living beings which encodes genetic information. Structurally it is composed of two complimentary strands which hold together because of hydrogen bonds between nucleotide pairs, also called base pairs. There are four DNA nucleotides: adenine(A) is complementary to thymine(T) and cytosine(C) is complementary to guanine (G).

Central dogma of molecular biology: central dogma describes the information transfer between DNA (where genetic information is stored) and proteins where information turns into action. DNA is first transcribed to ribonucleic acid (RNA) by RNA polymerase. RNA is a single strand of nucleic acids where the base thymine (T) is now replaced by uracile (U). Thereafter, RNA is translated to a protein. In this process each triplet of nucleotides, also called a codon, codes for an amino-acid. Sequentially, a protein is built from the concatenation of amino-acids.

Restriction enzymes (REs): Restriction enzymes constitute a family of proteins that make cuts in the DNA. There are five types of REs but only type II REs will be defined here. Type IIS REs recognise a short DNA sequence called the recognition site (4-7 nucleotides) and either cut DNA on the recognition site, leaving what is known as a blunt-end (e.g. NotI RE) or can cut the DNA at a defined distance from the recognition site leaving so-called 'sticky-ends' or overhangs of single stranded DNA (type IIS REs e.g. BsaI, BsmBI). The latter gained popularity at the beginning of this century as they are used in the gene assembly method known as Golden Gate Assembly. **DNA digestion:** The reaction between DNA molecules and restriction enzymes is

commonly referred to as "DNA digestion".

Genetic parts or parts: Genetic parts are very well-known sequences of DNA that have a very well understood function. The most common ones are: promoters, which initiate transcription; ribosome binding sites (RBS) which initiate translation; terminator, which terminate transcription, coding sequences (CDS) or open-reading frame (ORF) which encodes for a specific gene.

Golden gate assembly method: The golden gate assembly method takes advantage of type IIS recognition sites that leave overhangs (also referred to as sticky ends) which allow for orientation specific annealing of DNA fragments with complementary parts. Post digestion by the Type II restriction enzyme, the sticky ends anneal to their appropriate partners by Watson-Crick basepairing, this is followed by a ligation reaction catalysed by an enzyme, T4 ligase, which covalently links the backbones of the fragments, making the entire DNA fragment one molecule. More information can be attained from the original paper: Engler, C., Kandzia, R. and Marillonnet, S. (2008). A One Pot, One Step, Precision Cloning Method with High Throughput Capability. PLoS ONE, 3(11), p.e3647.

Transcriptional unit or expression cassette: TUs, also called expression cassette generally consist of a promoter, a coding sequence and a terminator. Several TUs make a multigene expression cassette.

Plasmid: Plasmids are short sequences of DNA which form circular structures, they do not integrate into the genome of the cell in which they are present. They are sometimes also referred to as vectors as they often carry foreign DNA in the form of a synthetic genetic insert that is aimed to be carried into a cell. Genetic insert often refers to one or multiple transcriptional units (TUs). Synthetic plasmids are often equipped with: an origin of replication, which will dictate how many copies of that plasmid need to be made and an antibiotic selection marker, to track the cells that hold the vector in selective media (in presence of antibiotic).

Destination vector: A destination vector is a plasmid DNA molecule into which we wish to insert one or several transcriptional units or DNA parts.

Transformation: Transformation of a cell refers to the act of introducing a foreign DNA vector to a given cell type. Transformation protocols include a phase where the cell membrane is disrupted so that the foreign DNA vector can make its way into the cell. Once inside the cell the DNA may be integrated in the genome or stay as a plasmid.

Auxotrophy: An auxotrophic cell or organism is that which lacks the ability of making one or more vital nutrients by itself compared to its parental wildtype. Auxotrophic organisms require supply of that nutrient from its environment. As an example a uracil auxotrophic *Yarrowia Lipolytica* strain will not be able to synthesise uracil, therefore it needs to be provided with uracile in the media it is grown in.

Prototrophic: A prototrophic organism has the same nutritional requirement than its equivalent wildtype.

Growth curve: Microbial growth curves have three main characteristic phases: the lag phase, where growth does not occur as cells are adapting to the new medium; an exponential growth phase, where cells replicate at an exponential rate and are at their most active state and a stationary phase where cells cease growing as resources become limited for a large population.

A.2 Acronyms

TIGAMY:Thermophilus glucoamylase

ANGAMY:Aspergillus Niger glucoamylase

Rice-AA:Rice α -amylase from *Oryza Sativa*

DOL:Division of labour

SP:signal peptide

Y1: Y. *Lipolytica* expressing TIGAMY with SP1

Y2: Y. *Lipolytica* expressing TIGAMY with SP2

Y3:Y. *Lipolytica* expressing TIGAMY with SP3

Y4:Y. *Lipolytica* expressing TIGAMY with SP4

Y5:Y. *Lipolytica* expressing TIGAMY with SP5

Y6:Y. Lipolytica expressing TIGAMY with SP6

Y7:Y. Lipolytica expressing TIGAMY with SP7

Y8:Y. Lipolytica expressing TIGAMY with SP8

Y9:Y. Lipolytica expressing TIGAMY with SP9

Y10:Y. Lipolytica expressing TIGAMY with SP10

Appendix B

Protocols

B.1 Growth Media

Preparation of bacterial growth media(LB broth and LB agar) and yeast growth media (YPD and YNB) is explained below. Minimal media(YNB) for yeast was used for selection of positive transformants and supplemented with uracile or leucine when appropriate.

B.1.1 Bacterial growth media

LB media was used for Escherichia Coli (E.Coli) growth in both solid media (LB agar - Sigma Aldrich) and liquid media (LB broth - Sigma Aldrich). The latter were made accordingly to the instructions provided in their respective containers. Antibiotics such as Ampicillin, Kanamyacin and Cloramphenicol where used in antibiotic-media in appropriate ratios.

B.1.2 *Yarrowia Lipolytica* growth media

To prepare liquid media with any of the following recipes addition of agar was avoided.

Rich Media - YPD

Yeast extract Peptone Dextrose media was used to grow *Yarrowia lipolytica* in accordance with the recipe in the book [39]. The YPD recipe can be found in table B.1. Note that due to

| Ingredient | For 1 L |
|----------------------|---------|
| Yeast extract | 10g |
| Peptone | 20g |
| Glucose | 20g* |
| Bacteriological agar | 20g |
| Water | to 1L** |

Table B.1: Ingredients and amounts to prepare YPD.

caramelisation of glucose in the autoclave, YPD obtains a darker colour. When using YPD for spectrophotometry or plate reader experiments, glucose can be added after sterilisation in the autoclave (for 1L add 40mL of glucose at 50% and **only add water to 960 mL before autoclaving the solution).

Minimal Media - YNB

Yeast Nitrogen Base media was used to select positive transformants of *Yarrowia Lipolytica* after insertion of a certain selection marker (URA3 or LEU2 gene) together with an expression cassette of interest. YNB can be made following the recipe on the table B.2.

| Ingredient | For 100 ml | For 400 ml |
|------------------------------------------|------------|------------|
| YNB without AA with ammonium sulphate | 0.68g | 2.72g |
| Bacteriological agar | 2g | 8g |
| Water | to 86ml | to 344ml |
| AUTOCLAVE | | |
| Glucose 50% | 4ml | 16 ml |
| Phosphate buffer pH 6.8 | 10ml | 40ml |

Table B.2: Ingredient & amounts to prepare YNB agar.

B.2 DNA extraction

Isolated plasmid DNA from E.Coli was obtained in the following way:

1. Growing E.Coli cells overnight in 5mL of liquid LB media with the adequate antibiotic

2. Centrifuge tubes at 4000 RPM for 10 minutes.
3. Remove media supernatant leaving a pellet at the bottom of the culture tube.
4. Resuspend cell in 250 μ L of P1 buffer
5. Add 250 μ L of P2 buffer and let sit for no longer than 5 minutes.
6. Add 350 μ L of N3 buffer to the solution.
7. Transfer content to an eppendorf tube and centrifuge for 10 minutes at 14800 RPM.
8. Remove supernatant and add to a DNA collection column (provided by QIAGEN: QIAprep Spin Miniprep Columns).
9. Centrifuge for 1 minute at 14800 RPM.
10. Discard supernatant
11. Add 750 μ L of PE buffer and centrifuge for 1 minute at 14800 RPM.
12. Discard supernatant
13. Add 750 μ L of PE buffer and centrifuge for 1 minute at 14800 RPM.
14. Discard supernatant
15. Centrifuge for 1 minute at 14800 RPM.
16. Transfer column into new eppendorf tube and discard collection tube.
17. Add 30-50 μ L of PCR-water or elution buffer to the column, making sure the liquid is in contact with the column filter.
18. Let sit for 10 minutes, centrifuge for 1 minute at 14800 RPM.
19. Measure DNA concentration with NanoDrop machine (ThermoFisher Scientific)

B.3 Digestion protocols

Restriction enzyme digestions were performed as detailed below. Multiple digestion enzymes were used for different purposes such as: AvrII, DpnII, BsaI, BsmBI, BbsI and NotI. All digestions were performed with NEB enzymes following their protocols at [NEBcloner](#)

B.4 Ligation of DNA

Ligation reactions were in some cases performed with T4 ligase in T7 buffer(New England Biolabs, NEB). Detailed protocols can be found below.

The following ligation protocol (from NEB ligation protocol [NEB T4 ligation protocol](#)) was used:

1. Add following component into a PCR tube after allowing T4 buffer to thaw and after resuspending it:

| Component | 20 μ l reaction |
|-----------------------------|----------------------|
| T4 DNA Ligase Buffer (10X)* | 2 μ l |
| Vector DNA (4 kb) | 50 ng (0.020 pmol) |
| Insert DNA (1 kb) | 37.5 ng (0.060 pmol) |
| Nuclease-free water | to 20 μ l |
| T4 DNA Ligase | 1 μ l |

2. Gently mix the reaction by pipetting up and down and microfuge briefly.
3. For cohesive (sticky) ends, incubate at 16°C overnight or room temperature for 10 minutes.
4. For blunt ends or single base overhangs, incubate at 16°C overnight or room temperature for 2 hours (alternatively, high concentration T4 DNA Ligase can be used in a 10 minute ligation).
5. Heat inactivate at 65°C for 10 minutes.
6. Chill on ice and transform 1-5 μ l of the reaction into 50 μ l competent cells.

B.5 Polymerase Chain Reactions(PCR)

Polymerase chain reaction for DNA amplification were performed according to the protocols below. Depending on the need, different polymerases and PCR mixes were used, when more precision was needed Phusion high fidelity polymerase or Q5 polymerase were used and when faster reactions were preferred GoTaq Green or Phire polymerase were used. PCR were performed with Phusion High-Fidelity DNA Polymerase (M0530 - NEB), GoTaq Green Master Mix (Promega) and Phire Hot Start II DNA Polymerase (ThermoFisher Scientific). Phusion was used when higher copying efficiency was required, when amplifying a sequence for cloning for example while the other two were used for colony PCR experiments. PCR annealing temperature calculators can be found at the following sites: [NEB Tm calculator](#), [ThermoFisher Tm calculator](#) and [Promega biomath calculator](#).

- Phusion PCR protocol:

| Component | 20 μ l Reaction | 50 μ l Reaction | Final Concentration |
|----------------------------|---------------------|---------------------|--------------------------|
| Nuclease-free water | to 20 μ l | to 50 μ l | |
| 5X Phusion HF or GC Buffer | 4 μ l | 10 μ l | 1X |
| 10 mM dNTPs | 0.4 μ l | 1 μ l | 200 μ M |
| 10 μ M Forward Primer | 1 μ l | 2.5 μ l | 0.5 μ M |
| 10 μ M Reverse Primer | 1 μ l | 2.5 μ l | 0.5 μ M |
| Template DNA | variable | variable | <250 ng |
| Betaine 5M | (4 μ l) | (10 μ l) | 1M |
| Phusion DNA Polymerase | 0.2 μ l | 0.5 μ l | 1.0 units/50 μ l PCR |

Table B.3: Phusion PCR components and quantities

| Step | Temperature | Time |
|--------------------------------------|-------------------------|-------------------------------------------------------|
| Initial Denaturation: 1 cycle | 98°C | 30 seconds |
| Amplification: 25-35 Cycles | 98°C 45-72°C 72°C | 5-10 seconds 10-30 seconds 15-30 seconds per kb |
| Final Extension: 1 cycle | 72°C | 5-10 minutes |
| Hold | 4-10°C | ∞ |

Table B.4: Phusion PCR Thermocycler protocol

- GoTaq Green Protocol:

| Component | Volume | Final Concentration |
|-------------------------------|--------------------|---------------------|
| GoTaq Green Master Mix, 2X | 12.5 μ l | 1X |
| upstream primer, 10 μ M | 0.25 - 2.5 μ l | 0.1 - 1.0 μ M |
| downstream primer, 10 μ M | 0.25 - 2.5 μ l | 0.1 - 1.0 μ M |
| DNA template | 1 - 5 μ l | <250ng |
| Nuclease-Free Water | to 25 μ l | N.A. |

Table B.5: GoTaq Green reaction mix for a 25 μ l reaction

| Step | Temperature | Time |
|--------------------------------------|-------------------------|--------------------------------------------------------|
| Initial Denaturation: 1 cycle | 98°C | 2 minutes |
| Amplification: 25-35 Cycles | 98°C 45-72°C 72°C | 30-60 seconds 10-30 seconds 15-30 seconds per kb |
| Final Extension: 1 cycle | 72°C | 5-10 minutes |
| Hold | 4-10°C | ∞ |

Table B.6: GoTaq Green Thermocycler protocol

- Phire PCR protocol:

| Component | Volume | Final Concentration |
|-------------------------------|-------------|---------------------|
| Phire buffer, 5X | 4 μ l | 1X |
| Betaine, 5M | 4 μ l | 1M |
| dNTPs | 0.4 μ l | ?? μ M |
| upstream primer, 10 μ M | 1 μ l | 0.5 μ M |
| downstream primer, 10 μ M | 1 μ l | 0.5 μ M |
| DNA template | 2 μ l | <250ng |
| Phire Hot Start II | 0.4 μ l | 1X |
| Nuclease-Free Water | 7.2 | N.A. |

Table B.7: Phire PCR reaction mix for a 20 μ l reaction

| Step | Temperature | Time |
|--------------------------------------|---------------------|------------------------------------------------|
| Initial Denaturation: 1 cycle | 98°C | 30 seconds |
| Amplification: 25-35 Cycles | 98°C X°C 72°C | 5 seconds 5 seconds 10-15 seconds per kb |
| Final Extension: 1 cycle | 72°C | 5 minutes |
| Hold | 4-10°C | ∞ |

Table B.8: Phire PCR Thermocycler protocol

B.6 DNA purification

DNA purification from a PCR reaction or from a RE digestion mix was purified with the use of the QIAquick PCR Purification Kit from QIAGEN.

B.7 DNA gel extraction

DNA purification from an agarose gel was extracted and purified with the use of the QIAquick Gel Extraction Kit from QIAGEN.

B.8 Golden Gate reaction

Golden gate one-pot reaction were performed using the protocol below and with specific parts as specified in each situation. After completion of this protocol the resultant solution was transformed into E.Coli competent cells. Competent cells are cells "ready" to take in foreign DNA. To optimise the results of a Golden gate reaction, DNA needs to be pure and all different DNA vectors need to be equimolar (at 50 fmol/ μ L). The following protocol can be used for a golden gate reaction:

1. Combine the following components in a PCR tube as shown in table B.9.

| Component | Volume (μ L) |
|-----------------------------|-------------------|
| T4 DNA ligase buffer | 1 |
| Type IIS REs | 0.50 |
| Destination vector backbone | 0.25 |
| Each insert/DNA part/GGF | 0.50 |
| PCR water | to 10 |

Table B.9: Golden gate components to be added together to perform a golden gate reaction

2. Insert all used PCR tubes in a thermocycler and run the program found in 2

| Step | Temperature($^{\circ}$ C) | Time (minutes) |
|--------------------------------------------------|----------------------------|----------------|
| RE digestion & ligation 25 cycles | 42 | 2 |
| | 16 | 5 |
| RE digestion (1 cycle) | 60 | 10 |
| Heat inactivation (1 cycle) | 80 | 10 |
| Hold | 4 | ∞ |

Table B.10: Golden gate components thermocycler protocol

B.9 E.Coli Transformation

E.Coli transformation was performed following a heat-shock protocol as specified below.

1. Transfer a set of competent cells from the -80C freezer into a bucket with ice. Let thaw for 5-10 minutes.
2. Add 50 μ L of KCM into the competent cell tube and resuspend. KCM (5x): 500 mM KCL, 150mM CaCl₂ and 250 mM MgCl₂ (from Cold Spring Harbor protocols)
3. Mix 1-10 μ L of DNA with 50 μ L of the cells+KCM mix.
4. In a thermocycler, program 10 minutes at 4 $^{\circ}$ C, followed by 1 minutes at 42 $^{\circ}$ C and terminated by another minute at 4 $^{\circ}$ C.
5. Take cells from thermocycler and incubate at 37 $^{\circ}$ in 1 mL of LB media without antibiotics, to allow for cells to recover. Incubate for 45-60 minutes.
6. Plate 100 μ L of cells in an LB agar plate with the appropriate antibiotic.

B.9.1 Making E. Coli competent cells

Protocol can be found in [40]

B.10 Lithium Acetate (LiAc) Y. Lipolytica Transformation Protocol

Initially the Lithium Acetate protocol was used to transform Yarrowia Lipolytica strains. Later on the DTT protocol was used as it is a faster protocol with good transformation efficiency.

Materials

- Acetic acid 10%: dissolve 10 g into 100 mL of distilled water and mix well.
- Lithium Acetate(LiAc) 0.1M pH 6.0: for 500 mL add 5.1g of lithium acetate (Molar mass: 102 g/mol) to 400mL of distilled water. Correct pH to 6.0 with a 10% acetic acid solution and add water to 500 mL. Sterilise in autoclave.
- PEG 40% in LiAc: dissolve 40g of PEG 4000 in 50 mL LiAc 0.1M pH 6.0. Adjust pH to with 10% acetic acid solution. Add LiAc 0.1M pH 6.0 to 100 mL. Filter sterilise under sterile conditions.
- Carrier DNA (Salmon sperm DNA): from a stock of salmon sperm DNA (see ThermoFisher) aliquot 50 μ L of solution to several PCR tubes and store at -20°C.
- TE buffer: Tris(10mM)-EDTA(1mM) pH 6.0: (for 100 mL) combine 1mL of Tris (1M) with 0.2 mL of EDTA (0.5M), add water to 80 mL; adjust pH with HCl and add water to 100 mL.
- Digested DNA: (for digestion with InsUP-InsDOWN sites) grow E.Coli strain with desired insert overnight in selective media. Perform miniprep (DNA extraction) and set 500 ng DNA digestion with NotI enzyme in Cutsmart buffer for at least 4 hours at 37°C. For higher transformation efficiency perform PCR clean up, as shown in section [B.6](#).

Day 1

1. Spread the strain you wish to transform in a YPD plate, incubate at 28-30°C for 16 to 24 hours.

Day 2

1. With a big loop, pick a loop-full of cells and suspend them in 1mL of TE buffer in a sterile eppendorf tube.
2. Centrifuge mix for 1 min at 10000 RPM and discard supernatant.
3. Resuspend cell pellet in 600 μ L of the LiAc solution.
4. Incubate for 1 hour at 28-30°C in a water bath or heat block.
5. Centrigue for 2 minutes at 3000 RPM and discard the supernatant.
6. Resuspend pellet in 60 μ L of LiAc.
7. In a sterile eppendorf tube mix combine: 3 μ L of carrier DNA (after boiling it for 8 minutes), 2-5 μ L of the digested DNA insert and 40 μ L of competent cells. Mix gently with a pipette by slow resuspension or stirring.
8. incubate for 15 minutes at 28-30°C.
9. Add 350 μ L of the PEG solution.
10. Incubate for 1 hour at 28°C.
11. Heatshock for 10 minutes at 39°C.
12. Add 600 μ L of LiAc solution, centrifuge for 2 minutes at 3000 RPM.
13. Discard enough supernatant to leave approximately 100 μ L in the tube.
14. Resuspend the remaining 100 μ L and spread in a culture plate with appropriate selection conditions.

15. Allow to grow for 48 hours and perform colony PCR.

B.11 DTT Y.Lipolytica Transformation protocol

Materials

- 50% PEG - 50 g of PEG 4000 water up to 100ml - sterilize at 121 °C
- 2M LiAc pH 6.0 - 6.6 g of LiAC - water up to 40mL of water - Adjust pH and add water to 50 mL - sterilize in autoclave
- 2M DTT - To make aliquots, add 0.616 g (0.62g) of DTT into a 2mL tube and then filter it from there into 1 mL aliquots - sterilize using 0.22 μ m membrane - store in small aliquots (1 mL) and keep in fridge
- Digested DNA - Digest approximately 200 - 500 ng of insert DNA before transformation
- DNA carrier: Salmon sperm DNA - useful to have it in 50 μ L aliquots in PCR tubes - boil for 8 minutes before use

Day 1

1. Spread cells to be transformed on a YPD agar plate (one plate-one strain), incubate at 28 °C, overnight

Day 2

1. Mix (for 4 transformations): 450 μ L 50% PEG, 25 μ L 2M LiAc pH 6.0 and 25 μ L 2M DTT
2. Put 100 μ L of transformation solution into sterile 2 mL Eppendorf tubes
3. Add a whole loop of biomass

4. Add 5 μ L carrier DNA and 10 μ L expression cassette
5. Vortex
6. incubate at 28°C for 30 minutes
7. Vortex
8. 42°C for 10 minutes or 30 minutess at 39°C for improved efficiency.
9. Plate onto selection media, incubate 28-30°C for 24-48h
10. Verify the transformants

B.12 Colony PCR

Colony PCRs are performed to check if a certain DNA insert has been successfully inserted after transformation.

In E.Coli

Colony PCRs for E.Coli were performed in the same way that normal PCRs were performed with the modification of switching the template DNA by an inoculate of the E.Coli colony to be verified.

In Y.Lipolytica

For fast verification of transformation success in Y. Lipolytica, the protocol in section below was performed. Inserts of up to 1.2 thousand base pairs were successfully amplified using this protocol. The protocol was not tested with longer fragments. Usage of the Phire or the Phusion HF protocol was preferred over GoTaq green because of the improved precision of the first two polymerases.

1. For each colony to be tested, aliquot 20 μ L of 20 mM of NaOH into a PCR tube and inoculate each tube with a fraction of a colony with the help of a toothpick or a small pipette tip.
2. Insert PCR tubes in a thermocycler machine and boil cells (at 98-100 °C) for 15 minutes.
3. Let cool to room temperature.
4. Set up a PCR using any of the protocols shown in [B.5](#), ideally the Phire or the Phusion one.

Appendix C

Strains

Table of used *Y. Lipolytica* strains found in next page.

| STRAINS WE ALREADY HAD | CHARACTERISTICS | AUXOTROPHY | MAIN FEATURE |
|---------------------------|--------------------------------------------------------------------------------|------------|-------------------------------------------------------------------------------------------------------------------------------------------|
| format | ParentalStrain[insert] | | |
| W29 | wildtype | URA+ LEU+ | wildtype |
| Y195 or po1d | W29 [ura3-leu2-AEP+SUC2] | URA- LEU- | Leucine and uracile auxotrophy and deletion ofAlkaline Extracellular Protease(AEP) plus added invertase for sucrose breakdown |
| JMY330 | po1d[ura3] | URA+ LEU- | po1d with ura3 marker |
| JMY2101 | po1d[leu2] | URA- LEU+ | po1d with leu2 marker |
| Y2900 | po1d [ura3-leu2] | URA+ LEU+ | po1d made prototroph |
| Y3820 | po1d[(MATa ura3-302 leu2-270 xpr2-322 pox1-6 tgl4 + pTEF-DGA2 + pTEF-GPD1)] | URA+ LEU+ | obese strain unable to breakdown fats |
| Y-EVA1 | JMY2101[pSB1A-zeta/NotUP-Mura3- pTEF-SSP1-TIGAMY-tLip2-zeta/NotDOWN] | URA+ LEU+ | Thermomyces lanuginosus glucoamylase (TIGAMY) with signal peptide number 1 (SP1) and under pTEF expression |

| | | | |
|--------|-----------------------------------------------------------------------|-----------|---------------------------------------------------------------------------------------------------------------------|
| Y-EVA2 | JMY2101[pSB1A-zeta/NotUP-Mura3-pTEF-SSP2-TIGAMY-tLip2-zeta/NotDOWN] | URA+ LEU+ | Thermomyces lanuginosus glucoamylase (TIGAMY) with signal peptide number 2 (SP2) and under pTEF expression |
| Y-EVA3 | JMY2101[pSB1A-zeta/NotUP-Mura3-pTEF-SSP3-TIGAMY-tLip2-zeta/NotDOWN] | URA+ LEU+ | Thermomyces lanuginosus glucoamylase (TIGAMY) with signal peptide number 3 (SP3) and under pTEF expression |
| Y-EVA4 | JMY2101[pSB1A-zeta/NotUP-Mura3-pTEF-SSP4-TIGAMY-tLip2-zeta/NotDOWN] | URA+ LEU+ | Thermomyces lanuginosus glucoamylase (TIGAMY) with signal peptide number 4 (SP4) and under pTEF expression |
| Y-EVA5 | JMY2101[pSB1A-zeta/NotUP-Mura3-pTEF-SSP5 - TIGAMY-tLip2-zeta/NotDOWN] | URA+ LEU+ | Thermomyces lanuginosus glucoamylase (TIGAMY) with signal peptide number 5 (SP5) and under pTEF expression |

| | | | |
|--------|---------------------------------------------------------------------|-----------|---------------------------------------------------------------------------------------------------------------------|
| Y-EVA6 | JMY2101[pSB1A-zeta/NotUP-Mura3-pTEF-SSP6-TIGAMY-tLip2-zeta/NotDOWN] | URA+ LEU+ | Thermomyces lanuginosus glucoamylase (TIGAMY) with signal peptide number 6 (SP6) and under pTEF expression |
| Y-EVA7 | JMY2101[pSB1A-zeta/NotUP-Mura3-pTEF-SSP7-TIGAMY-tLip2-zeta/NotDOWN] | URA+ LEU+ | Thermomyces lanuginosus glucoamylase (TIGAMY) with signal peptide number 7 (SP7) and under pTEF expression |
| Y-EVA8 | JMY2101[pSB1A-zeta/NotUP-Mura3-pTEF-SSP8-TIGAMY-tLip2-zeta/NotDOWN] | URA+ LEU+ | Thermomyces lanuginosus glucoamylase (TIGAMY) with signal peptide number 8 (SP8) and under pTEF expression |
| Y-EVA9 | JMY2101[pSB1A-zeta/NotUP-Mura3-pTEF-SSP9-TIGAMY-tLip2-zeta/NotDOWN] | URA+ LEU+ | Thermomyces lanuginosus glucoamylase (TIGAMY) with signal peptide number 9 (SP9) and under pTEF expression |

| Y-EVA10 | JMY2101[pSB1A-zeta/NotUP-Mura3-pTEF-SSP10-TIGAMY-tLip2-zeta/NotDOWN][| URA+ LEU+ | Thermomyces lanuginosus glucoamylase (TIGAMY) with signal peptide number 10 (SP10) and under pTEF expression |
|-------------|-----------------------------------------------------------------------|------------|-----------------------------------------------------------------------------------------------------------------------|
| JMY4926 | po1d[pTEF-riceAlphaAmylase-Ura3] | URA+ LEU- | po1d with Rice (<i>Oryza Sativa</i>) alpha-amylase under pTEF expression |
| JMY5077 | Y4926[LEU2] | URA+ LEU+ | Prototroph JMY4926 |
| JMY5017 | Y5077[pTEF-A.NigerGlucoAmylase-LEU2] | URA+ LEU+ | Y5077 with <i>Aspergillus Niger</i> gluco-amylase (Rice alpha amylase) under pTEF expression |
| Y5196 | Y3820[(RiceAlphaAmylase + A.NigerGlucoAmylase)x2 + CRE] | URA+ LEU+ | obese strain with 2 copies of Rice alpha-amylase and 2 copies of A. <i>Niger</i> glucoamylase |
| NEW STRAINS | CHARACTERISTICS | AUXOTROPHY | MAIN FEATURES |
| Y-11 | JMY330[InsUP_zeta/-leu2-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Rice alpha-amylase under pTEF expression. Equivalent to Y5017 |

| | | | |
|------|-------------------------------------------------------------------------|-----------|------------------------------------------------|
| Y-12 | JMY330[InsUP_zeta/-leu2-2UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Rice alpha-amylase under 2UAS-pTEF expression. |
| Y-13 | JMY330[InsUP_zeta/-leu2-3UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Rice alpha-amylase under 3UAS-pTEF expression. |
| Y-14 | JMY330[InsUP_zeta/-leu2-4UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Rice alpha-amylase under 4UAS-pTEF expression. |
| Y-15 | JMY330[InsUP_zeta/-leu2-8UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Rice alpha-amylase under 8UAS-pTEF expression. |
| Y-16 | JMY330[InsUP_zeta/-leu2-hp4d-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Rice alpha-amylase under hp4d-pTEF expression. |
| Y-17 | JMY330[InsUP_zeta/-leu2-hp8d-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Rice alpha-amylase under hp8d-pTEF expression. |
| Y100 | Y-EVA1[InsUP_zeta/-leu2-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA1 combined with Y-11 insert. |
| Y101 | Y-EVA1[InsUP_zeta/-leu2-2UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA1 combined with Y-12 insert. |
| Y102 | Y-EVA1[InsUP_zeta/-leu2-3UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA1 combined with Y-13 insert. |

| | | | |
|------|-------------------------------------------------------------------------|-----------|-----------------------------------|
| Y103 | Y-EVA1[InsUP_zeta/-leu2-4UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA1 combined with Y-14 insert. |
| Y104 | Y-EVA1[InsUP_zeta/-leu2-8UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA1 combined with Y-15 insert. |
| Y105 | Y-EVA1[InsUP_zeta/-leu2-hp4d-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA1 combined with Y-16 insert. |
| Y106 | Y-EVA1[InsUP_zeta/-leu2-hp8d-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA1 combined with Y-17 insert. |
| Y107 | Y-EVA2[InsUP_zeta/-leu2-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA2 combined with Y-11 insert. |
| Y108 | Y-EVA2[InsUP_zeta/-leu2-2UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA2 combined with Y-12 insert. |
| Y109 | Y-EVA2[InsUP_zeta/-leu2-3UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA2 combined with Y-13 insert. |
| Y110 | Y-EVA2[InsUP_zeta/-leu2-4UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA2 combined with Y-14 insert. |
| Y111 | Y-EVA2[InsUP_zeta/-leu2-8UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA2 combined with Y-15 insert. |

| | | | |
|------|-------------------------------------------------------------------------|-----------|-----------------------------------|
| Y112 | Y-EVA2[InsUP_zeta/-leu2-hp4d-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA2 combined with Y-16 insert. |
| Y113 | Y-EVA2[InsUP_zeta/-leu2-hp8d-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA2 combined with Y-17 insert. |
| Y114 | Y-EVA3[InsUP_zeta/-leu2-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA3 combined with Y-11 insert. |
| Y115 | Y-EVA3[InsUP_zeta/-leu2-2UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA3 combined with Y-12 insert. |
| Y116 | Y-EVA3[InsUP_zeta/-leu2-3UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA3 combined with Y-13 insert. |
| Y117 | Y-EVA3[InsUP_zeta/-leu2-4UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA3 combined with Y-14 insert. |
| Y118 | Y-EVA3[InsUP_zeta/-leu2-8UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA3 combined with Y-15 insert. |
| Y119 | Y-EVA3[InsUP_zeta/-leu2-hp4d-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA3 combined with Y-16 insert. |
| Y120 | Y-EVA3[InsUP_zeta/-leu2-hp8d-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA3 combined with Y-17 insert. |

| | | | |
|------|-----------------------------------------------------------------------------|-----------|-----------------------------------|
| Y121 | Y-EVA4[InsUP_zeta/-leu2-pTEF- RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA4 combined with Y-11 insert. |
| Y122 | Y-EVA4[InsUP_zeta/-leu2-2UAS-pTEF- RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA4 combined with Y-12 insert. |
| Y123 | Y-EVA4[InsUP_zeta/-leu2-3UAS-pTEF- RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA4 combined with Y-13 insert. |
| Y124 | Y-EVA4[InsUP_zeta/-leu2-4UAS-pTEF- RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA4 combined with Y-14 insert. |
| Y125 | Y-EVA4[InsUP_zeta/-leu2-8UAS-pTEF- RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA4 combined with Y-15 insert. |
| Y126 | Y-EVA4[InsUP_zeta/-leu2-hp4d- RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA4 combined with Y-16 insert. |
| Y127 | Y-EVA4[InsUP_zeta/-leu2-hp8d- RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA4 combined with Y-17 insert. |
| Y128 | Y-EVA5[InsUP_zeta/-leu2-pTEF- RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA5 combined with Y-11 insert. |
| Y129 | Y-EVA5[InsUP_zeta/-leu2-2UAS-pTEF- RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA5 combined with Y-12 insert. |

| | | | |
|------|-------------------------------------------------------------------------|-----------|-----------------------------------|
| Y130 | Y-EVA5[InsUP_zeta/-leu2-3UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA5 combined with Y-13 insert. |
| Y131 | Y-EVA5[InsUP_zeta/-leu2-4UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA5 combined with Y-14 insert. |
| Y132 | Y-EVA5[InsUP_zeta/-leu2-8UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA5 combined with Y-15 insert. |
| Y133 | Y-EVA5[InsUP_zeta/-leu2-hp4d-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA5 combined with Y-16 insert. |
| Y134 | Y-EVA5[InsUP_zeta/-leu2-hp8d-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA5 combined with Y-17 insert. |
| Y135 | Y-EVA6[InsUP_zeta/-leu2-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA6 combined with Y-11 insert. |
| Y136 | Y-EVA6[InsUP_zeta/-leu2-2UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA6 combined with Y-12 insert. |
| Y137 | Y-EVA6[InsUP_zeta/-leu2-3UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA6 combined with Y-13 insert. |
| Y138 | Y-EVA6[InsUP_zeta/-leu2-4UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA6 combined with Y-14 insert. |

| | | | |
|------|-------------------------------------------------------------------------|-----------|-----------------------------------|
| Y139 | Y-EVA6[InsUP_zeta/-leu2-8UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA6 combined with Y-15 insert. |
| Y140 | Y-EVA6[InsUP_zeta/-leu2-hp4d-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA6 combined with Y-16 insert. |
| Y141 | Y-EVA6[InsUP_zeta/-leu2-hp8d-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA6 combined with Y-17 insert. |
| Y142 | Y-EVA7[InsUP_zeta/-leu2-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA7 combined with Y-11 insert. |
| Y143 | Y-EVA7[InsUP_zeta/-leu2-2UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA7 combined with Y-12 insert. |
| Y144 | Y-EVA7[InsUP_zeta/-leu2-3UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA7 combined with Y-13 insert. |
| Y145 | Y-EVA7[InsUP_zeta/-leu2-4UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA7 combined with Y-14 insert. |
| Y146 | Y-EVA7[InsUP_zeta/-leu2-8UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA7 combined with Y-15 insert. |
| Y147 | Y-EVA7[InsUP_zeta/-leu2-hp4d-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA7 combined with Y-16 insert. |

| | | | |
|------|-------------------------------------------------------------------------|-----------|-----------------------------------|
| Y148 | Y-EVA7[InsUP_zeta/-leu2-hp8d-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA7 combined with Y-17 insert. |
| Y149 | Y-EVA8[InsUP_zeta/-leu2-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA8 combined with Y-11 insert. |
| Y150 | Y-EVA8[InsUP_zeta/-leu2-2UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA8 combined with Y-12 insert. |
| Y151 | Y-EVA8[InsUP_zeta/-leu2-3UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA8 combined with Y-13 insert. |
| Y152 | Y-EVA8[InsUP_zeta/-leu2-4UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA8 combined with Y-14 insert. |
| Y153 | Y-EVA8[InsUP_zeta/-leu2-8UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA8 combined with Y-15 insert. |
| Y154 | Y-EVA8[InsUP_zeta/-leu2-hp4d-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA8 combined with Y-16 insert. |
| Y155 | Y-EVA8[InsUP_zeta/-leu2-hp8d-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA8 combined with Y-17 insert. |
| Y156 | Y-EVA9[InsUP_zeta/-leu2-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA9 combined with Y-11 insert. |

| | | | |
|------|--------------------------------------------------------------------------|-----------|------------------------------------|
| Y157 | Y-EVA9[InsUP_zeta/-leu2-2UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA9 combined with Y-12 insert. |
| Y158 | Y-EVA9[InsUP_zeta/-leu2-3UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA9 combined with Y-13 insert. |
| Y159 | Y-EVA9[InsUP_zeta/-leu2-4UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA9 combined with Y-14 insert. |
| Y160 | Y-EVA9[InsUP_zeta/-leu2-8UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA9 combined with Y-15 insert. |
| Y161 | Y-EVA9[InsUP_zeta/-leu2-hp4d-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA9 combined with Y-16 insert. |
| Y162 | Y-EVA9[InsUP_zeta/-leu2-hp8d-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA9 combined with Y-17 insert. |
| Y163 | Y-EVA10[InsUP_zeta/-leu2-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA10 combined with Y-11 insert. |
| Y164 | Y-EVA10[InsUP_zeta/-leu2-2UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA10 combined with Y-12 insert. |
| Y165 | Y-EVA10[InsUP_zeta/-leu2-3UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA10 combined with Y-13 insert. |

| | | | |
|------|--------------------------------------------------------------------------|-----------|------------------------------------|
| Y166 | Y-EVA10[InsUP_zeta/-leu2-4UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA10 combined with Y-14 insert. |
| Y167 | Y-EVA10[InsUP_zeta/-leu2-8UAS-pTEF-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA10 combined with Y-15 insert. |
| Y168 | Y-EVA10[InsUP_zeta/-leu2-hp4d-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA10 combined with Y-16 insert. |
| Y169 | Y-EVA10[InsUP_zeta/-leu2-hp8d-RiceAlphaAmylase-T1Lip2/InsDOWN_zeta] | URA+ LEU+ | Y-EVA10 combined with Y-17 insert. |

Appendix D

YaTK guide by Mauricio Pesantes

YaTK How to Use Guide

Mauricio Pesantes

Important

*changes have been made to nomenclature: what previously was considered Lvl1, Lvl2, and Lvl3 have now been renamed Lvl0, Lvl1 and Lvl2 respectively.

Vector List:

<https://docs.google.com/spreadsheets/d/10NjM65ljG8Z8LwlcDTBqYJOHDZFiiZ8w9xXX2cn1LI/edit?usp=sharing>

Benchling:

https://benchling.com/rilalab/f_nHW3YmLo-gq-v2/?sort=name§ion=inventory&filter=archivePurposes%3ANOT_ARCHIVED

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Introduction

Based on the Yeast Toolkit (YTK) described by Michael E. Lee (Lee et al., 2015), we developed a new toolkit compatible with the existing Yarrowia Golden Gate modular system (Celińska et al., 2017) to address efficiency issues associated the single step assembly of all multiple parts of a multi-gene cassette.

This toolkit seeks to generate multigene cassettes in 3 stages (or levels) see Figure 1. First biopart (Golden Gate fragments, GGF) are cloned into storage vectors using a BsmBI type-II restriction enzyme (RE) Golden Gate reaccion (GGR). Second, through *Bsal* type-II RE GGR, single transcriptional units (TU), consisting of a promoter (P) a gene (G) and a Terminator (T), are assembled into one of the different premade Lvl1 destination vectors which determine the TU's position in the final construct. Finally through a BsmBI type-II RE GGR, the different TUs are liberated from the Lvl1 plasmids and assembled into one of the different Lvl2 vectors, varing in the number of TUs the can lodge (2 to 6).

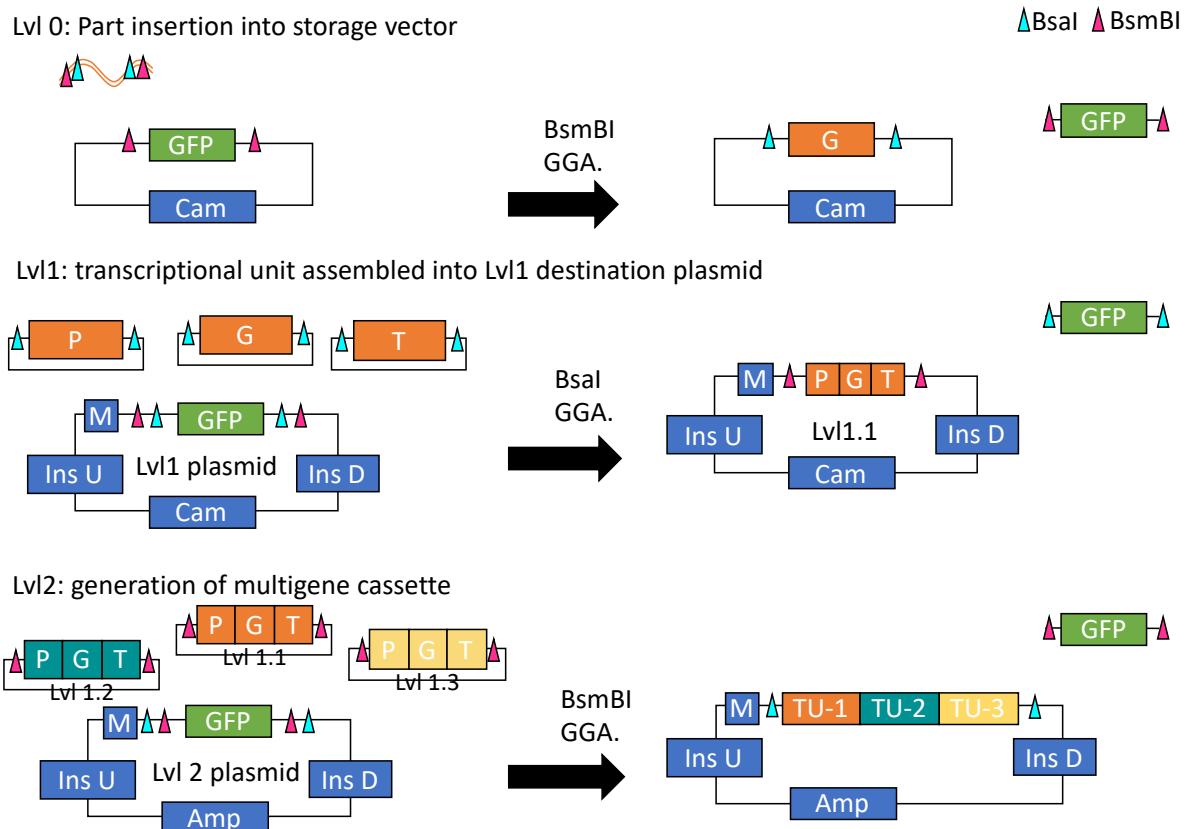


Figure 1 - YaTK overview

This toolkit seeks to optimize efficiency by reducing the number of parts required for each GGR. To this end, upstream and downstream insertion sequences (InsUP and InsDown) as well as Yeast marker (M) are preassembled into the Lvl 1 and 2 destination plasmids containing bacterial marker and origin. Therefore, the assembly of a multigene cassette requires 3 different type of GGF: promoters P, genes G, and terminators T; and an array Lvl1 vectors, and Lvl2 vectors.

For greater flexibility and to reduce the amount of predesign needed for a final construct, Lvl1 destination vectors are preassembled with BsmBI restriction sites required for Lvl2 assembly.

Parts Design

New promoters, genes and terminators can be designed as standard parts and used interchangeably by addition of 5' and 3' sequences, respective to each part type, see Table 1. These sequences contain BsmBI restriction sites used for a Lvl0 reaction and Bsal restriction sites used for a Lvl1 GG reaction enabling assembly of single TUs into Lvl1 plasmids according to a predesigned scaffold, see Table 4.

Backward compatibility

This toolkit was designed to be fully compatible with the *Yarrowia* Golden Gate modular system (Celińska et al., 2017), and so GGFs and destination vectors have the same Lvl1 Bsal restriction enzyme 4nt overhang than their counter part in the *Yarrowia* Golden Gate modular system scaffold. See Table 3 and Table 4. Standard parts use the respective overhangs of P1, G1 and T1/3. New compatible parts for P2, G2, T2/3, P3, G3 and T3 can be made by addition of 5' and 3' sequences, respective to each part type, shown in Table 2.

These parts require different Lvl1 destination plasmids made specifically for “G2” and “G3” TUs with the appropriate 4nt Bsal overhangs, further explained below.

- In these pseudoprimer, some common notation will be used. “gcat” will be standard junk DNA to allow the type IIs sites to cut, BsmBI sites and overhangs are highlighted in grey, and Bsal sites and overhangs are colored in red (note overlaps).
- To use these, copy-paste the left and right overhangs upstream and downstream respectively of your part sequence. Take note that you need to reverse complement the sequence when ordering the reverse primer.
- You will need to remove these cut sites from your part: BsmBI, Bsal, BbsI, NotI. For more information on how to do this, consult the “YTK Protocols.docx” guide.

| Type | Description/Notes | 5' Overhang | 3' Overhang |
|------|--------------------------------------------------------------------|----------------------------------|--------------------------------|
| P | Standard promoter (utilizes GG – P1 ‘C’ and ‘D’ overhangs) | gcatCGTCTCATC GGTCTCAACGG | AATGTGAGACC TGAGACGgcat |
| G | Standard gene (utilizes YGG – G1 ‘D’ and ‘E’ overhangs) | gcatCGTCTCATC GGTCTCAATG | TCTATGAGACC TGAGACGgcat |
| T | Standard Terminator (utilizes YGG – T1/3 ‘E’ and ‘L’ overhangs) | gcatCGTCTCATC GGTCTCATCTA | GAGTTGAGACC TGAGACGgcat |

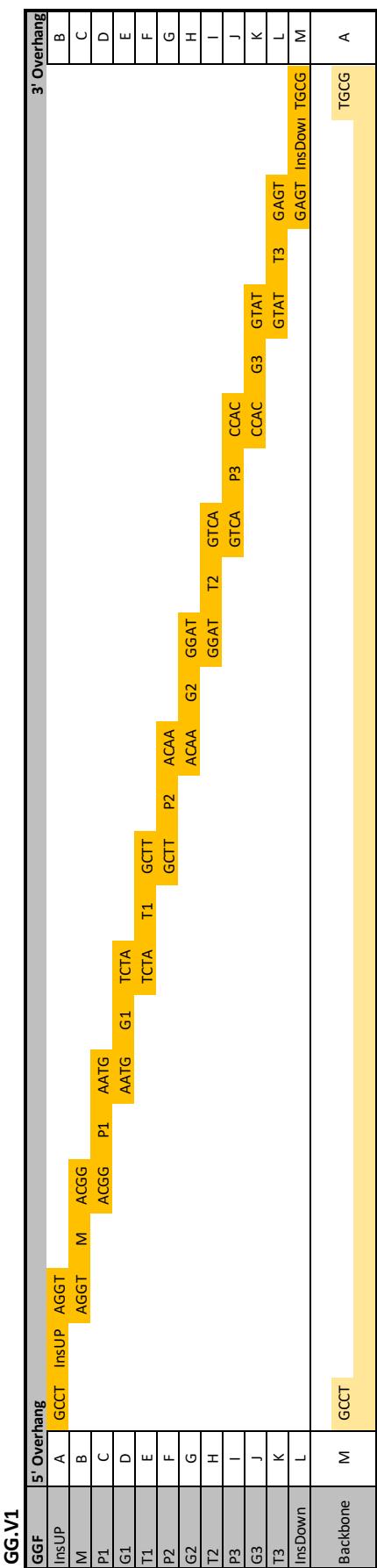
Table 1 - Standard part overhangs for part design

| Type | Description/Notes | Left Overhang | Right Overhang |
|------|----------------------------------------------------------------|-----------------------------------|--------------------------------|
| P2 | P2 Promoter (utilizes YGG – P2 'F' and 'G' overhangs) | gcatCGTCTCATC GGTCTCAGCTT | ACAATGAGACC TGAGACGgcat |
| G2 | G2 Gene (utilizes YGG – G2 'G' and 'H' overhangs) | gcatCGTCTCATC GGTCTCAACAA | GGATTGAGACC TGAGACGgcat |
| T2 | T2/3 Terminator (utilizes YGG – T2/3 'H' and 'L' overhangs) | gcatCGTCTCATC GGTCTCAGGAT | GAGTTGAGACC TGAGACGgcat |
| P3 | P3 Promoter (utilizes YGG – P3 'I' and 'J' overhangs) | gcatCGTCTCATC GGTCTCAGTCA | CCACTGAGACC TGAGACGgcat |
| G3 | G3 Gene (utilizes YGG – G2 'J' and 'K' overhangs) | gcatCGTCTCATC GGTCTCACCCAC | GTATTGAGACC TGAGACGgcat |
| T3 | T3 Terminator (utilizes YGG – T3 'K' and 'L' overhangs) | gcatCGTCTCATC GGTCTCAGTAT | GAGTTGAGACC TGAGACGgcat |

Table 2 - Backward compatible overhangs for part design

Available in benchling are these sequences elongated with type I RE BamHI and AvrII for major flexibility.

https://benchling.com/rllab/f_JxA5D8wV-yarrowia-gg-v2-overhangs-for-new-parts/?sort=name§ion=inventory&filter=archivePurposes%3ANOT_ARCHIVED



| GGA | Parts | Total | | | | | | |
|-------|--------|--------|----|----|------|----------|----|------|
| 1 TU | Ins UP | Marker | P1 | G1 | T1/3 | Backbone | | |
| 2 TUs | Ins UP | Marker | P1 | G1 | T1 | P2 | G2 | T2/3 |
| 3 TUs | Ins UP | Marker | P1 | G1 | T1 | P2 | G2 | T2 |

Table 3 - Golden Gate V.1

Top: Four nucleotides overhang scaffold for the single tier modular GG assembly of multigene cassettes for *Yarrowia lipolytica*.

Bottom: GG.V1 single tier assembly of expression cassette of 1, 2 or 3 TUs.

GG.V2

Lvl1 Bsal - Standard

| GGF | 5' Overhang | | | | | | 3' Overhang | |
|--------------------|-------------|----------------|--|--|--|--|-------------|--------|
| P | C | ACGG P1 AATG | | | | | | D |
| G | D | AATG G1 TCTA | | | | | | E |
| T | E | TCTA T1/3 GAGT | | | | | | L |
| Lvl1.n.1 Vector | L | ACGG | | | | | | C GAGT |

Backward compatibility

| GGF | 5' Overhang | | | | | | 3' Overhang | |
|--------------------|-------------|----------------|--|--|--|--|-------------|--------|
| P2 | C | GCTT P2 ACAA | | | | | | D |
| G2 | D | ACAA G2 GGAT | | | | | | E |
| T2/3 | E | GGAT T2/3 GAGT | | | | | | L |
| Lvl1.n.2 Vector | L | GCTT | | | | | | C GAGT |
| P3 | C | GTCA P3 CCAC | | | | | | D |
| G3 | D | CCAC G3 GTAT | | | | | | E |
| T3 | E | GTAT T1/3 GAGT | | | | | | L |
| Lvl1.n.3 Vector | L | GTCA | | | | | | C GAGT |

Lvl2 BsmBI

| GGF | 5' Overhang | | | | | | | | | | 3' Overhang | | | |
|----------------------|-------------|------|------|------|------|---------|------|----------|------|---------|-------------|--------|------|---------|
| TU-1 | S | CTGA | TU-1 | CCAA | | | | | | | | 1 | | |
| TU-2 | 1 | | | CCAA | TU-2 | GATG | | | | | | 2 | | |
| TU-3 | 2 | | | | GATG | TU-3 | GTTC | | | | | 3 | | |
| TU-4 | 3 | | | | | GTTC | TU-4 | GGTA | | | | 4 | | |
| TU-5 | 4 | | | | | | GGTA | TU-5 | AAGT | | | 5 | | |
| TU-6 | 5 | | | | | | | AAGT | TU-6 | AGCA | | E | | |
| Desination Vector | S | CTGA | | | GATG | Lvl2-II | GTTC | Lvl2-III | GGTA | Lvl2-IV | AAGT | Lvl2-V | AGCA | Lvl2-VI |

Table 4 - Golden Gate V.2 scaffolds

Four nucleotides overhang scaffolds for multi-tier GG.v2. (Top) Bsal digestion scaffold for standard assembly of Lvl1 plasmids containing transcriptional units containing a single promoter, gene and terminator. (Middle) Bsal digestion scaffold for assembly of non-standard parts, using promoters, genes and terminators designed for GG.v1 TUs positions 2 and 3. (Bottom) BsmBI digestion scaffold for assembly of multiple TUs into a multigene Lvl2 cassette.

Lvl0 : Assemble Parts

You can insert PCRs (made with the above overhangs), gBlocks or genestrings, annealed oligos, or any other double-stranded DNA into the pYTK001 vector, running a BsmBI

GoldenGate reaction. Once produced, you will need to sequence the resulting part to check for possible point mutations.

Lvl1 : TU assembly

The desired promoter, gene and terminator are assembled into a transcriptional unit into a Lvl1 destination vector, through a Bsal GGR. The destination vector is selected according to the desired position of the TU in the final construct; as well as the desired yeast marker M and insertion sites InsUP and InsDown.

Preassembled Lvl1 plasmids carry the InsUP/ InsDown and M modules, and therefore an assembled Lvl1 vector can be directly used to transform *Y. lipolytica* with a single TU.

Adjacent to the Bsal sites used for Lvl1 GGR, are BsmBI type-II RE sites which liberate the TU for subsequent Lvl2 assembly. Accordingly to a Lvl2 scaffold, see **Error! Reference source not found.**, BsmBI sites in the preassembled Lvl1 plasmid encode the position in the final construct of the assembled TU.

A set of multiple Lvl1 destination plasmids carrying different BsmBI restriction sites encoding upto 6 different positions for the host TU (Lvl1-1, Lvl1-2, Lvl1-3, Lvl1-4, Lvl1-5 and Lvl1-6) were designed, see Table 5. And different sets can be made using different yeast selection markers (e.g. Ura3 or Leu2) as well as different inseretion sites (e.g. NotI zeta, or SfiI zeta sequences).

TUs that are to be assembled into a multigene cassette cannot share the same position in the final construct.

| Lvl 1 | | | |
|------------------|----|----|------|
| Plasmid | P | G | T |
| Lvl 1.1.1 | P1 | G1 | T1/3 |
| Lvl 1.2.1 | P1 | G1 | T1/3 |
| Lvl 1.2.2 | P2 | G2 | T2/3 |
| Lvl 1.3.1 | P1 | G1 | T1/3 |
| Lvl 1.3.3 | P3 | G3 | T3 |
| Lvl 1.4.1 | P1 | G1 | T1/3 |
| Lvl 1.5.1 | P1 | G1 | T1/3 |
| Lvl 1.6.1 | P1 | G1 | T1/3 |

Table 5 - Lvl1 Assembly

List of parts needed for assembly of GG.v2 Lvl1 cassettes.

Lvl2 : Multi-gene cassette assembly

The different transcriptional units contained in Lvl1 vectors are assembled into a Lvl2 plasmid through a BsmBI GGR, making a multigene cassette.

The preassembled Lvl2 plasmids carry the InsUP/ InsDown and M modules, enabling *Y. lipolytica* genomic insertion, transformation and selection.

Lvl2 plasmids carry a different bacterial selection marker than Lvl1 plasmids to avoid transformations with of undisgested Lvl1 plasmids. Lvl1 are CamR while Lvl2 are AmpR.

A set of multiple Lvl2 destination plasmids carrying accepting up to 6 different TUs were designed (Lvl2-II, Lvl2-III, Lvl2-IV, Lvl2-V and Lvl2-VI), see Table 6. Different sets can be made using different yeast selection markers (e.g. Ura3 or Leu2) as well as different inseretion sites (e.g. NotI zeta, or SfiI zeta sequences).

See vector list for details of the different Lvl 1 and 2 destination plasmids:

<https://docs.google.com/spreadsheets/d/10NjM65ljG8Z8LwlcDTBqYJOHDZFiiZ8w9xXX2cn1Ll/edit?usp=sharing>

The Lvl2 BsmBI scaffold (see Table 4) requires using TU contained in continues Lvl1 starting with Lvl1-1 (ie: With plasmids Lvl1.1, Lvl1.2, Lvl1.3 and Lvl1.4 containing respectively TU-A, TU-B, TU-C, TU-D, it is possible to assemble constructs AB, ABC and ABCD, respectively into plasmids Lvl2.II, Lvl2.III and Lvl2.IV.) For more flexibility ‘linker’ sequences could be designed, although additional part would reduce assembly efficiency.

Lvl2 vectors contains Bsal sites with resulting 4nt overhangs (left: AACG; right: GCTG) that would allow a future Lvl4.

| Lvl2 | | TU1 | TU2 | TU3 | TU4 | TU5 | TU6 |
|----------|--------|--------|--------|--------|--------|--------|-----|
| Plasmid | | | | | | | |
| Lvl2-II | Lvl1.1 | Lvl1.2 | | | | | |
| Lvl2-III | Lvl1.1 | Lvl1.2 | Lvl1.3 | | | | |
| Lvl2-IV | Lvl1.1 | Lvl1.2 | Lvl1.3 | Lvl1.4 | | | |
| Lvl2-V | Lvl1.1 | Lvl1.2 | Lvl1.3 | Lvl1.4 | Lvl1.5 | | |
| Lvl2-VI | Lvl1.1 | Lvl1.2 | Lvl1.3 | Lvl1.4 | Lvl1.5 | Lvl1.6 | |

Table 6 - Lvl2 Assembly

List of parts needed for assembly of GG.v2 Lvl2 cassettes.

Generation of storage plasmids

The part vector from YTK :pYTK001 can be used as a Lvl0 storage vector.

Lvl1 and Lvl2 destination plasmids can be assembled using mostly parts compatible with the Yarrowia Golden Gate modular system (Celińska et al., 2017).

A set of primers were designed for the each Lvl1 and Lvl2 plasmid, see Table 8. These primers contain the unique Bsal and BsmBI restriction site that differentiate each plasmid, but also contain flanking BbsI restriction sites yielding 4nt overhangs that match the Bsal scaffold’s ‘C’ and ‘L’ Bsal 4nt overhangs (see Table 4). Primers are used to amplify GFP coding sequence

from pYTK001 (Phusion or Q5 DNA polymerase, NEB) which is used as a placeholder for the target insert (i.e: for Lvl1 plasmids: P+G+T; for Lvl2 plasmids: the different TUs).

The PCR products are digested with **BbsI** (NEB) and 1X CutSmart buffer (NEB) for 2 hours at 37°C, thus protecting the desired Bsal and BsmBI sites for the Lvl1/ Lvl2 destination vectors. On the other hand, Backbone vectors ([pSB1C3](#) or [pSB1A3](#)), yeast marker M (Ura or Leu), InsUp and InsDown (zeta notI) are digested with **Bsal** (NEB) for 2 hours at 37°C. All digested parts are purified through DNA gel-purification.

Plasmid is assembled through complementary *Bsal* and *BbsI* 4nt overhangs, see Table 7, and ligated by overnight incubation at 16°C with T7 DNA ligase (NEB), and 1X T7 DNA ligase buffer(NEB).

GG.V2 vectors assembly

| GGF | 5' Overhang | | | | 3' Overhang | |
|----------|-------------|------|-------|------|---------------|---------------------|
| InsUP | A | GCCT | InsUP | AGGT | | B |
| M | B | | | AGGT | M | ACGG |
| GFP | C | | | | ACGG | Vector Specific GFP |
| InsDown | L | | | | GAGT | InsDown |
| Backbone | M | GCCT | | | TGCG | A |
| | | | | | Cut with Bsal | |
| | | | | | Cut with BbsI | |

Table 7 - Destination Vectors assembly scaffold

The GGFs InsUP, M, InsDown and the Backbone are digested with Bsal and gel purified. Separately, the sfGFP PCR product is digested with BbsI, to protect the Bsal and BsmBI restriction sites specific to each destination plasmid, and also gel purified. The Plamid can then be assembled overnight from all required GGFs and T7 DNA ligase.

| | | |
|--------------------|----------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------|
| MP-P022 Lvl2.1.1-F | ttgcatgaagacggACGGcgctcgCTGAAACGGgGAGACCgaaagtgaaaacg | Amplify pYTK001 sfGFP sequence while adding the Bsal, BsmBI and BbsI restriction site for generating YaTK destination vector Lvl2.1.1 |
| MP-P023 Lvl2.1.1-R | tgacttGAAGACggACTCcgcttcTTGGACTCtgagacctataacgcagaaagg | |
| MP-P024 Lvl2.2.1-F | ttgcatgaagacggACGGcgctcgCCAACGGgGAGACCgaaagtgaaaacg | for generating YaTK destination vector Lvl2.2.1 |
| MP-P025 Lvl2.2.1-R | tgacttGAAGACggACTCcgcttcCATCACTCtgagacctataacgcagaaagg | |
| MP-P026 Lvl2.2.2-F | ttgcatgaagacggACGGcgctcgCCAACGGTgGAGACCgaaagtgaaaacg | for generating YaTK destination vector Lvl2.2.2 |
| MP-P027 Lvl2.2.2-R | tgacttGAAGACggACTCcgcttcCATCACTCtgagacctataacgcagaaagg | |
| MP-P028 Lvl2.3.1-F | ttgcatgaagacggACGGcgctcgGATGACGGgGAGACCgaaagtgaaaacg | for generating YaTK destination vector Lvl2.3.1 |
| MP-P029 Lvl2.3.1-R | tgacttGAAGACggACTCcgctccGAACACTCtgagacctataacgcagaaagg | |
| MP-P030 Lvl2.3.3-F | ttgcatgaagacggACGGcgctcgGATGGTCgAGGACCgaaagtgaaaacg | for generating YaTK destination vector Lvl2.3.3 |
| MP-P031 Lvl2.3.3-R | tgacttGAAGACggACTCcgctccGAACACTCtgagacctataacgcagaaagg | |
| MP-P032 Lvl2.4.1-F | ttgcatgaagacggACGGcgctcgGTTACCGGgGAGACCgaaagtgaaaacg | for generating YaTK destination vector Lvl2.4.1 |
| MP-P033 Lvl2.4.1-R | tgacttGAAGACggACTCcgctccTACCACTCtgagacctataacgcagaaagg | |
| MP-P034 Lvl2.5.1-F | ttgcatgaagacggACGGcgctcgGTAAACGGgGAGACCgaaagtgaaaacg | for generating YaTK destination vector Lvl2.5.1 |
| MP-P035 Lvl2.5.1-R | tgacttGAAGACggACTCcgctccACTTACTCtgagacctataacgcagaaagg | |
| MP-P036 Lvl2.6.1-F | ttgcatgaagacggACGGcgctcaAAAGTACGGgGAGACCgaaagtgaaaacg | for generating YaTK destination vector Lvl2.6.1 |
| MP-P037 Lvl2.6.1-R | tgacttGAAGACggACTCcgctccTGCTACTCtgagacctataacgcagaaagg | |
| MP-P038 Lvl3.II-F | ttgcatGAAGACggACGGAACGtGAGACCaCTGAtGAGACGgaaagtgaaaacg | for generating YaTK destination vector Lvl3-II |
| MP-P039 Lvl3.II-R | tgacttGAAGACggACTCCAGCtgagaccCATCtgagacctataacgcagaaagg | |
| MP-P040 Lvl3.III-F | ttgcatGAAGACggACGGAACGtGAGACCaCTGAtGAGACGgaaagtgaaaacg | for generating YaTK destination vector Lvl3-III |
| MP-P041 Lvl3.III-R | tgacttGAAGACggACTCCAGCtgagaccGAACtgagacgtataacgcagaaagg | |
| MP-P042 Lvl3.IV-F | ttgcatGAAGACggACGGAACGtGAGACCaCTGAtGAGACGgaaagtgaaaacg | for generating YaTK destination vector Lvl3-IV |
| MP-P043 Lvl3.IV-R | tgacttGAAGACggACTCCAGCtgagaccTACCTtgagacctataacgcagaaagg | |
| MP-P044 Lvl3.V-F | ttgcatGAAGACggACGGAACGtGAGACCaCTGAtGAGACGgaaagtgaaaacg | for generating YaTK destination vector Lvl3-V |
| MP-P045 Lvl3.V-R | tgacttGAAGACggACTCCAGCtgagaccACTTtgagacgtataacgcagaaagg | |
| MP-P046 Lvl3.VI-F | ttgcatGAAGACggACGGAACGtGAGACCaCTGAtGAGACGgaaagtgaaaacg | for generating YaTK destination vector Lvl3-VI |
| MP-P047 Lvl3.VI-R | tgacttGAAGACggACTCCAGCtgagaccTGCTtgagacgtataacgcagaaagg | |

Table 8 – primers

Note that primers retain previous nomenclature. Lvl1, Lvl2, and Lvl3 have now been renamed Lvl0, Lvl1 and Lvl 2 respectively.

Appendix E

Supplementary files

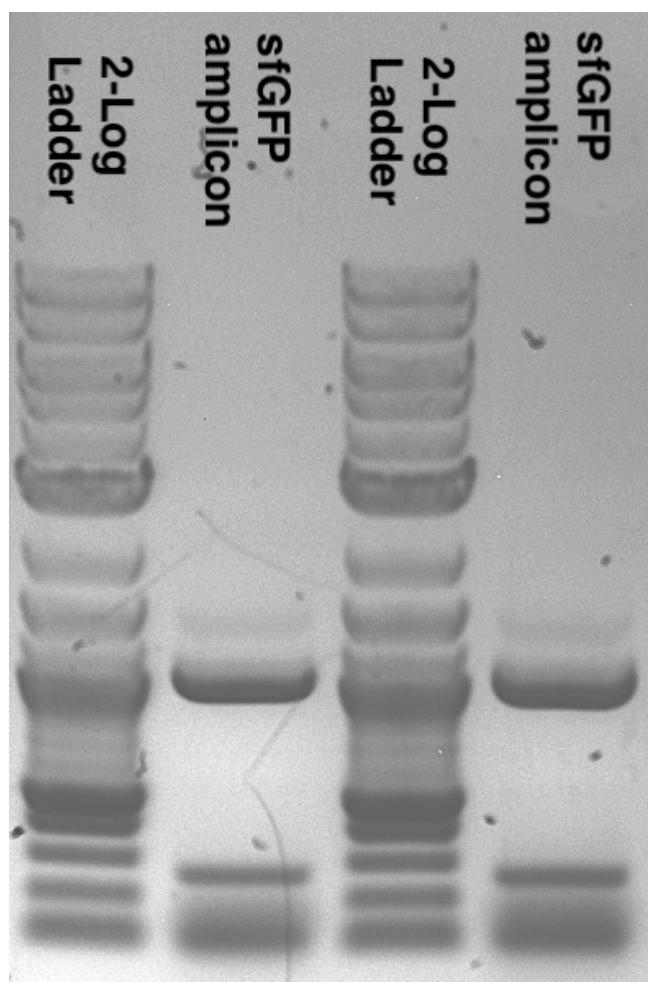


Figure E.1: sfGP amplicon from pYTK1 showing correct band length

[h] [h] [h]

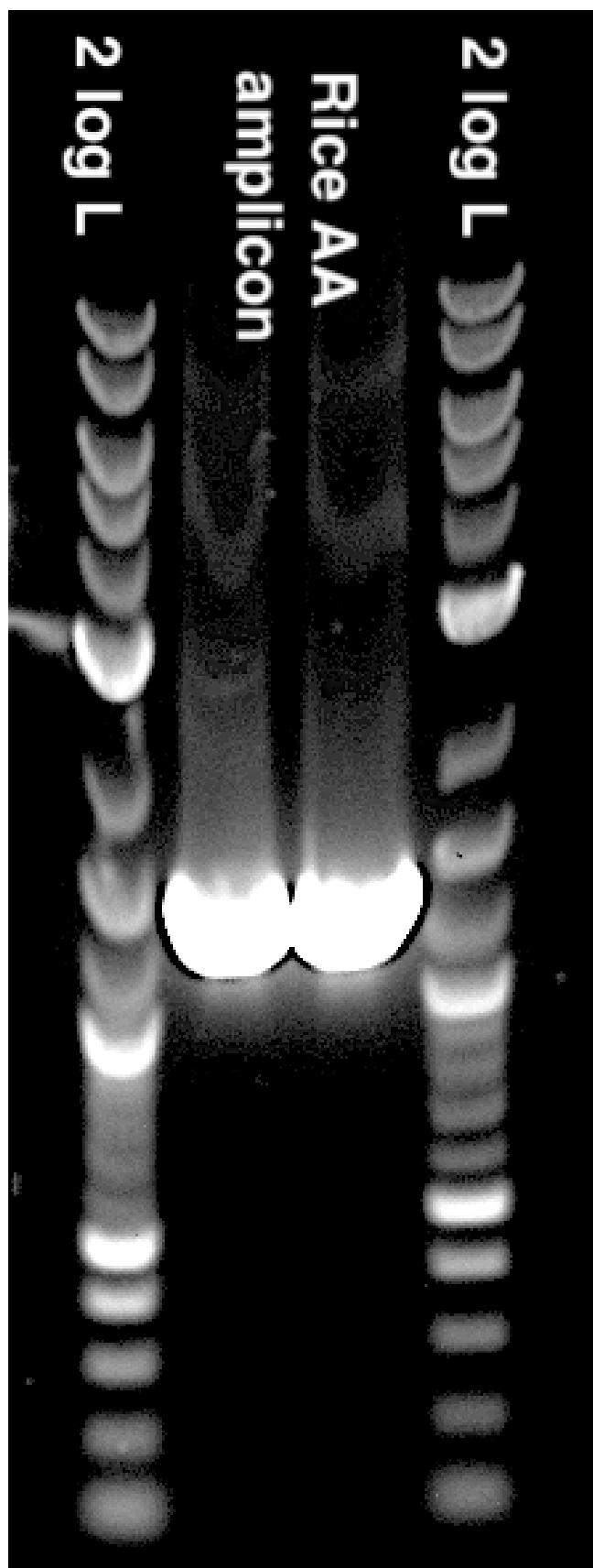


Figure E.2: Rice-AA amplicon showing correct band length

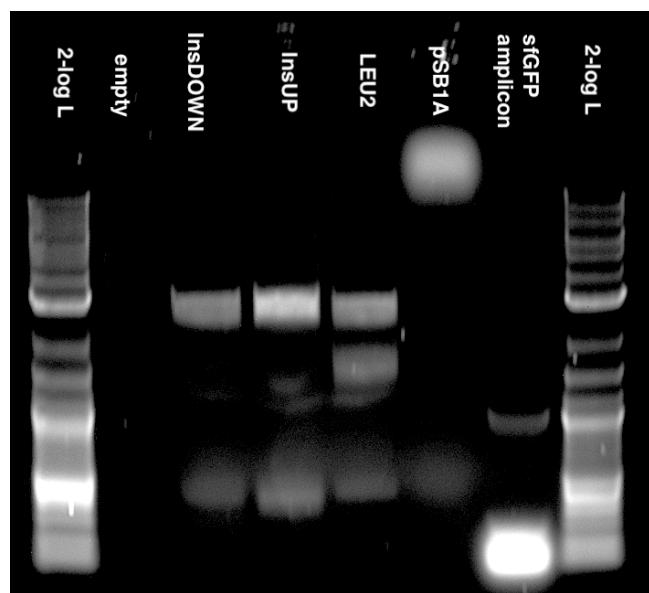


Figure E.3: Different parts to make LEU vector showing correct bands

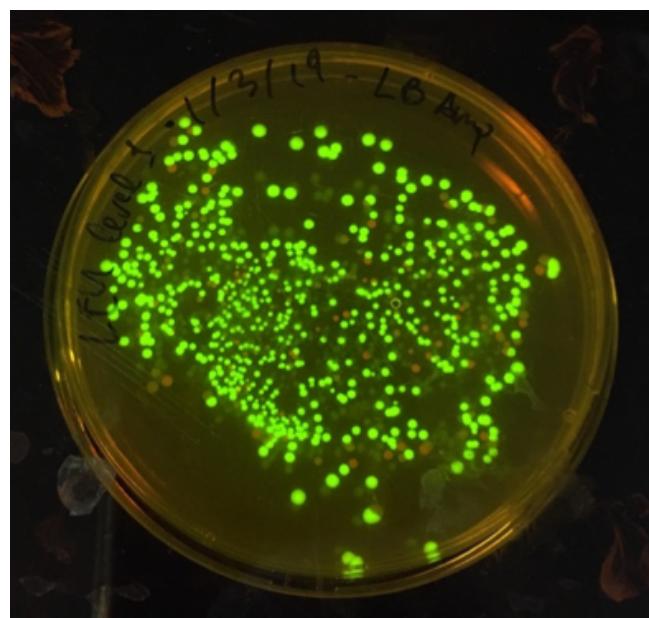


Figure E.4: Positive transformants with LEU level 1 vector glowing from GFP expression

Table E.1: Discrepancy between expected alpha-amylase amplicon and sequenced one

E.1 Growth in solid media of starch degrading strains does not improve in co-culture

E.2 Growth in starch media

E.3 Big deletion in Rice AA gene

E.4 Curve fitting code

E.5 Dilution pipette robot code

```
1 %% Calculate dilutions for Felix Robot
2
3 clear all; clc; close all; %#ok<CLALL>
4
5 %% Open .txt File
6
7 %%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
8 % Open the .txt output file from the plate-reader. Format of this file ...
9 %     should be the following:
10 % A1;0.152
11 % A2;0.313
12 % etc for the appropriate wells
13
14 file = uigetfile('*.txt');
15 fileID = fopen(file); % change to the name of the working file
16 C = textscan(fileID, '%s %f', 'Delimiter', ';');
17 fclose(fileID);
18
19 %% Define M9 index to correct ABS600
20
21 %%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
22 % Need to subtract the media background to obtain the "real ABS600". Be
23 % careful that this is not OD600, it's the value of the absorbance given by
24 % the plate-reader. Pathlength correction can be used to obtain a
25 % conversion to OD600 (or need to do a calibration curve, typically need to
26 % multiply ABS600 by 4 to obtain OD600).
27 %%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
28
29
30 %% Plot how to place boxes inside Felix machine
31 figure('units','normalized','outerposition',[0 1/2 1/2 1/2])
32 subplot(2,3,1)
33 text(0.1,0.5,'Mixing Plate')
34 yticklabels('')
```

```
35 xticklabels('')  
36 subplot(2,3,2)  
37 text(0,0.5,'Overnight Plate')  
38 title('please ensure components are placed as shown below in the Felix ...  
        upper tray')  
39 yticklabels('')  
40 xticklabels('')  
41 subplot(2,3,3)  
42 text(00,0.5,'H_2O Reservoir')  
43 xticklabels('')  
44 yticklabels('')  
45 subplot(2,3,4)  
46 text(0,0.5,'Final Plate')  
47 xticklabels('')  
48 yticklabels('')  
49 subplot(2,3,5)  
50 text(0,0.5,'Ethanol Reservoir')  
51 yticklabels('')  
52 xticklabels('')  
53 subplot(2,3,6)  
54 text(0,0.5,'Media Reservoir')  
55 xticklabels('')  
56 yticklabels('')  
57  
58  
59 %%  
60 idx_m9 = [20 32 44];  
61 C{1,2}(idx_m9)  
62 % C{1,2} = [C{1,2}] - mean(C{1,2}(idx_m9));  
63 % C{1,2} = [C{1,2}];  
64  
65 ODplate = reshape(C{:,2},[12,8]);  
66 ODplate = ODplate';  
67 figure('units','normalized','outerposition',[1/2 1/2 1/2 1/2])  
68 microplateplot(ODplate)
```

```
69
70 title('Normalised ABS600 in diluted O/N plate')
71 % whiteToBlack = [linspace(1,0,256)', linspace(1,0,256)',linspace(1,0,256)'];
72 % colormap(whiteToBlack)
73 colorbar
74
75 %% Calculate volume of fresh media to add in the mixing plate
76
77 %%%%%%
78 % Calculate how much fresh media should be added to the mixing plate after
79 % 50uL (ask the user) of cells have been transferred from the diluted ...
80 % overnight
81 % plate to the mixing plate. At the end, we want to make sure that there ...
82 % is at
83 % least 100uL of cells in each well and that there is less than 300uL
84 % (maximum volume that a plate can contain: to be adapted if using a
85 % different plate).
86 %%%%%%
87
88 %97 rows are needed as the first row needs to include: Well_ID,
89 %Media_Volume
90 Felix = cell(97,2);
91 Felix{1,1} = 'Well_ID';
92 Felix{1,2} = 'Media_Volume';
93 % Felix_plate = zeros(12,8);
94 mask_vector = cell(96,1);
95 % mask = cell(12,8);
96
97 % Ask the user how much cells are being transferred from the diluted ...
98 % overnight plate
99 % to the mixing plate. (Default is 50ul)
100
101 prompt = 'What is the volume of cells that you are transferring from the ...
102 % diluted overnight plate to the mixing plate? ';
103 Vcells = input(prompt);
```

```
100 prompt2 = 'What is your desired OD in the final plate?';
101 OD = input(prompt2);
102 ODmax = OD*300/Vcells; %based on equation OD_o/n*V_o/n = OD_mix*Vmix,max
103 warning(['Given your selected OD and the volume desired to transfer from ...
           the O/N plate to the mixing plate the maximum OD you should have in ...
           your O/N wells must be ',num2str(ODmax),'. Please adjuts the values ...
           accordingly or dilute the wells further if necessary so that the OD in ...
           all your wells is between, ',num2str(3*OD),' and ',num2str(ODmax),'.']);
104 counter1 = 1;
105 counter2 = 1;
106
107 for i = 1:length(C{1,2})
108
109     if C{1,2}(i) < 3*OD
110
111         warning{counter1} = C{1,1}{i}; %#ok<*>SAGROW>
112         mask_vector{i} = 'X';
113         counter1 = counter1 + 1;
114
115     if Vcells ≥ 50
116         C{1,3}(i) = 2*Vcells; % if the user doesn't want to set up a ...
117         new diluted overnight plate, do a 1:2 dilution if enough ...
118         cells to reach colume of 100uL in the well
119     else
120         C{1,3}(i) = 150; % if the user doesn't want to set up a new ...
121         diluted overnight plate, add 100uL in the well to be sure ...
122         the reach the minimum volume required (100uL)
123     end
124 else
125
126     Vtot = (Vcells*C{1,2}(i))/OD; % final volume in the mixing plate ...
127     well (in uL)
128     C{1,3}(i) = Vtot - Vcells; % volume of media to add(in uL)
129
130     if Vtot > 300
```

```
126     warning2{counter2} = C{1,1}{i};  
127     mask_vector{i} = 'O';  
128     counter2 = counter2+1;  
129  
130     end  
131  
132 %well names into 1st column  
133 Felix{i+1,1} = C{1,1}{i};  
134 %volume into 2nd column  
135 Felix{i+1,2} = C{1,3}(i);  
136 end  
137  
138 %% Make felix columns into plate (for visualisation only)  
139 %use this for Felix as matlab doesnt like vector to cell change  
140  
141 for i = 1:96  
142 Felixvector(i) = Felix{i+1,2};  
143 end  
144 Felix_plate = reshape(Felixvector,[12,8]);  
145 Felix_plate = Felix_plate';  
146  
147 mask = reshape(mask_vector,[12,8]);  
148 mask = mask';  
149  
150 %% Plot plate reader  
151 figure('units','normalized','outerposition',[0 0 1/2 1/2])  
152 microplateplot(Felix_plate)  
153 title('Amount of media that will be transferred to the mixing plate in ...  
          order to reach the right ABS/OD')  
154 whiteToRed = [ones(256,1), linspace(1,0,256)',linspace(1,0,256)'];  
155 colormap(whiteToRed)  
156 colorbar  
157  
158 figure('units','normalized','outerposition',[1/2 0 1/2 1/2])  
159 microplateplot(Felix_plate,'TEXTLABEL',mask)
```

```
160 title({'Warning: Wells labelled with an o exceed the maximum volume that ...
161     can be transferred to a well,',' wells labelled with an x wont reach ...
162     the desired OD as the intial value of OD is too low'})
163 whiteToRed = [ones(256,1), linspace(1,0,256)',linspace(1,0,256)'];
164 colormap(whiteToRed)
165 colorbar
166 set(groot,'defaultFigureVisible','on')
167
168 %% Write dilution file for Felix Robot
169
170 prompt3 = 'Please indicate the name of your output file: ';
171 out_file = input(prompt3,'s');
172 fileID = fopen([out_file,'.txt'],'w');
173 formatSpec = '%s, %.0f \r\n';
174 [nrows,ncols] = size(Felix);
175 fprintf(fileID,'%s\r\n','Well_ID, Media_Volume');
176 for row = 2:nrows
177     if isnan(Felix{row,2}) == 0
178         fprintf(fileID,formatSpec,Felix{row,:});
179     end
180 end
181 fclose(fileID);
182
183 % close_input = 'Press q to close all windows';
184 % while input(close_input) ~= 'q'
185 % if input(close_input) == 'q'
186 %     close all
187 % end
188 % type test.txt;
189 %
190 %
191 % % ABS600 is too low, give a warning so that the user can increase ...
192     concentration
```

```
192 % % of these wells in a nex diluted overnight plate.  
193 %  
194 % msgbox(sprintf(['WARNING!! ABS600 of the following wells is below', ...  
    num2str(OD),':\n\n %s \n\nYou might want to increase cell density of ...  
    these wells in your diluted overnight plate.'],...  
195 %           strjoin(warning,'\\n ')), 'Warning');  
196 %  
197 % % Give a warning if final volume in the well is above 300uL, in which case  
198 % % the user should dilute the overnights by a larger dilution factor.  
199 %  
200 % if exist('warning2','var')  
201 %     msgbox(sprintf('WARNING!! Total volume of the following wells in ...  
    the mixing plate will be above 300uL:\n\n %s \n\nYou might want to ...  
    dilute the overnights in the diluted overnight plate by a larger ...  
    dilution factor.',...  
202 %           strjoin(warning2,'\\n ')), 'Warning');  
203 % end  
204 %
```

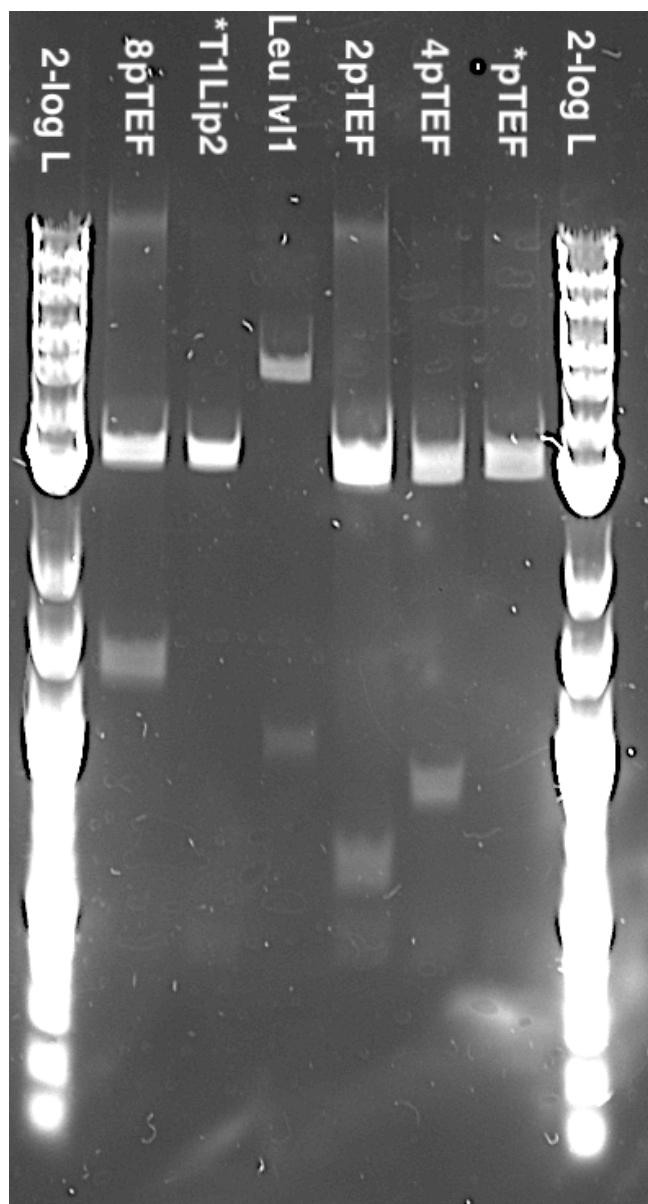


Figure E.5: New alpha-amylase cassettes parts

BsaI digestion of Dulermo promoters and T1lip2, and LEU level 1 and BbsI digestion of sfGFP amplicon showing correct length. T1Lip2 and pTEF are very small parts, hence their bands are not visible, a gel with a greater agarose concentration.

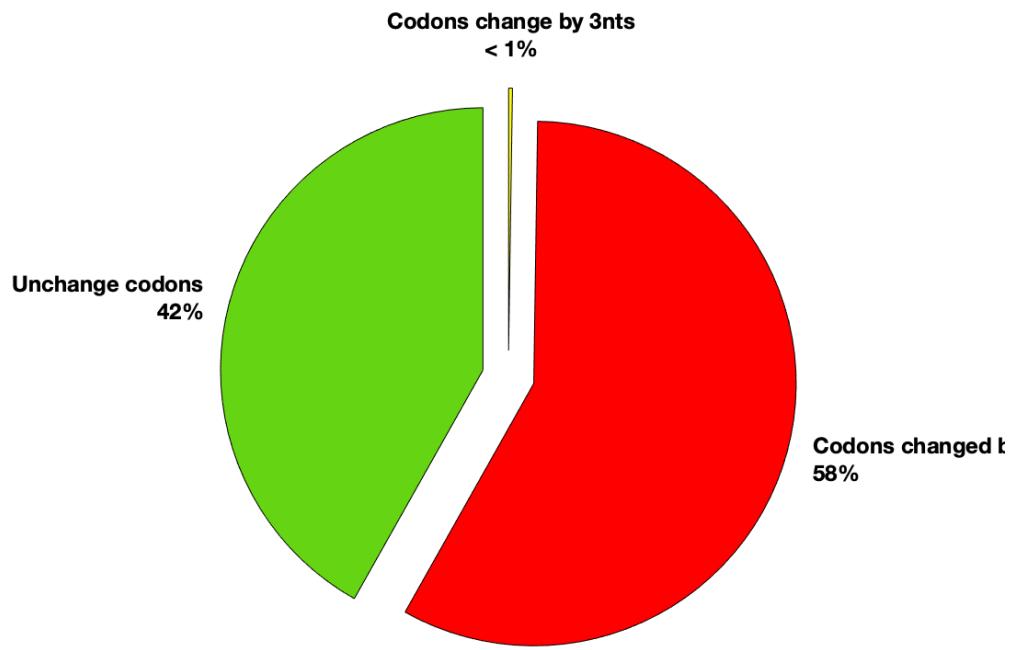


Figure E.6: Percentage of codon modification between the original rice-AA and the optimised sequence by IDT

42% of codons remain unchanged while most codon are changed due to a 1 nucleotide change between the original and the optimised sequence

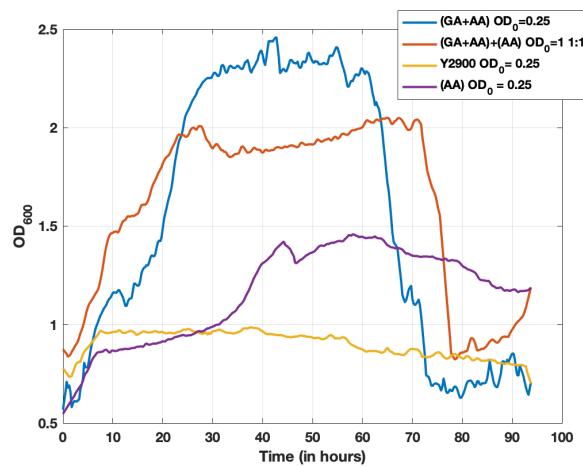
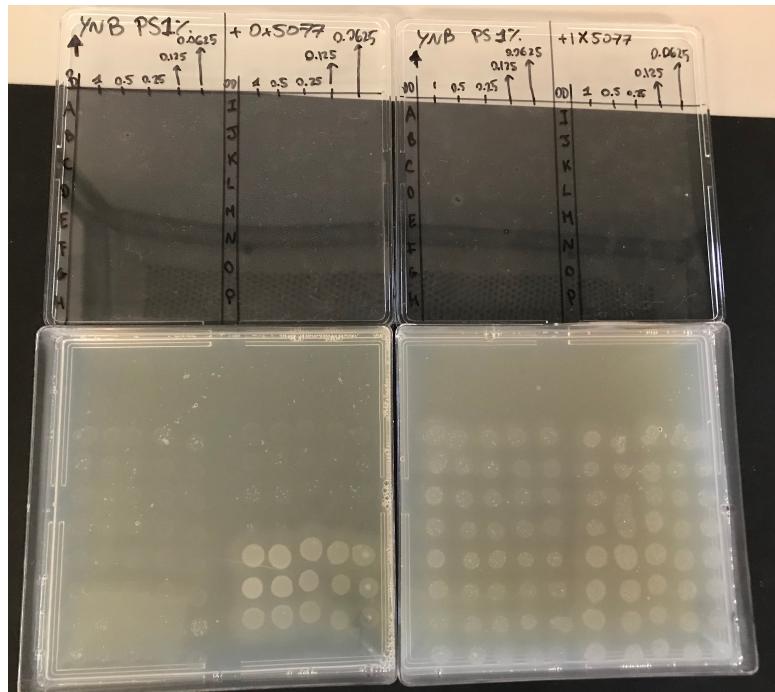


Figure E.7: Growth in starch media not improved when co-culturing

Shown is the growth of cells expressing ANGAMY and Rice AA simultaneously (Y5017 - blue line) at a starting OD of 0.25, the growth of strain Y5017 together with strain Y5077 (red line) at an overall starting OD of 1; growth of strain Y5077 by itself at a starting OD of 0.25 is shown in purple and growth of strain Y2900 (negative control) at a starting OD of 0.25 is shown in orange.



(a)



(b)

Figure E.8

Appendix F

GitHub repository

By following [this link](#), the raw experimental data as well as curve fitting algorithms and sequencing data can be accessed.

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