# Development of a molecular toolbox for the oleaginous yeast *Cutaneotrichosporon oleaginosus*

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Long Master thesis in Bioengineering

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**Abstract:**

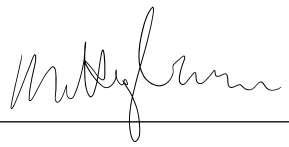
Oleaginous yeasts are highly effective for the production of biofuels and high-value poly-unsaturated fatty acids. Among these yeasts, the basidiomycete *Cutaneotrichosporon oleaginosus* is an excellent candidate due to its ability to accumulate up to 70% of its dry biomass in lipids and tolerate common fermentation inhibitors such as furfurals, ammonium ions and acetate at a much higher concentration compared to other common oleaginous yeasts. However, introducing exogenous DNA into *C. oleaginosus* has been proven to be challenging due to its thick cell wall and natural predisposition towards non-homologous end joining. In this report, an electroporation protocol was developed using a combination of nourseothricin and hygromycin B supplemented with cefoxitin for selection of transformants. It was possible to introduce linear DNA fragments of 9000+ bp, which were randomly integrated into the genome. This protocol was then used to create a *C. oleaginosus* strain carrying a tetracycline-induced TetON system for Cre-Lox recombination, able to excise the nourseothricin selection marker from the genome, allowing for multiple subsequent transformations of genes of interest using nourseothricin for selection. Full genome sequencing was performed in order to prove the effectivity of the transformation protocol and recycling of the selection marker. Results proved inconclusive as the examined samples resulted to be *Y. lipolytica* instances without any proof of integration. Further research is required to investigate if and when an accidental swap of samples might have taken place before estimating the efficacy of both the developed electroporation protocol and selection marker recycling system.

**Preface**

This report was completed by the student Mattia Gamberoni, group BIO9-2-E22, as long master thesis at Aalborg University Esbjerg, section of applied bioprocess design and engineering, fall semester 2022 and spring semester 2023. A special acknowledgement for the help and assistance provided throughout the two semesters goes to my supervisor Jens Laurids Sørensen, as well as Tobias Bruun Pedersen, PhD and BSc student Katharina Røhrt Bech. Furthermore, special thanks are owed to laboratory technician Linda Birkebæk Madsen and Dorte Spangsmark for their help with the equipment and sharing their expertise. Finally, gratitude is owed to Aalborg University Esbjerg for providing access to the laboratories, together with the resources and materials needed to complete this project.

The pictures and vector maps in this report have been created using BioRender and SnapGene Viewer, respectively. Further information about growth mediums, primers and the different experimental trials performed will be provided in the appendix.

Aalborg University, May 31, 2023



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# Contents

# 1 Abbreviations

| Abbreviation | Description |
|---|---|
| PUFA | Polyunsaturated Fatty Acids |
| DCW | Dry cell weight |
| TAG | Triacylglycerol |
| ASTM | American Society for Testing and Materials |
| PDH | Pyruvate dehydrogenase |
| et al. | And others |
| HR | Homologous recombination |
| NHEJ | Non-homologous end joining |
| MIC | Minimum inhibiting concentration |
| PEG | Polyethylene glycol |
| OD600 | Optical density measured at a wavelength of 600 nm |
| ATMT | *Agrobacterium tumefaciens* mediated transformation |
| rpm | Revolutions per minute |
| MCS | Multiple Cloning Site |
| ORF | Open Reading Frame |
| sp. | Species (singular) |
| spp. | Species (plural) |
| v/v | Volume per volume |
| w/v | Weight per volume |

## 2 Introduction

Biofuels play a crucial role in tackling the environmental impact of the transportation sector, but the increased exploitation of soybean oil and palm oil has led to deforestation and negatively impacted biodiversity [1]. Oleaginous yeasts have been studied as a promising alternative for sustainable biofuel production, but are yet to become economically viable. They can nevertheless be grown on various substrates and for various purposes besides biofuels, including food supplements and animal feed [1–3]. Research on these yeasts has increased over the last decade, with approximately 100 papers published annually [1]. The oleaginous yeast known as *Cutaneotrichosporon oleaginosus* is one of such intensively studied oleaginous yeasts in the recent years [1]. This microorganism was originally isolated at the Iowa State University Dairy Farm from factory drains in the late 1970s [3]. The principal distinguishing characteristic of this yeast is its ability to accumulate large amounts of intracellular lipids under specific conditions, as well as being able to metabolise a large variety of biomass hydrolysates, including traditionally inhibitory compounds, such as furfurals and weak organic acids [3]. This microbe has switched nomenclature multiple times, being originally registered as *Candida curvata* D, then published as *Apiotrichum curvatum*, *Cryptococcus curvatus*, *Trichosporon cutaneum* and *Trichosporon oleaginosus* [3, 4].

As mentioned earlier, oleaginous yeasts have seen a recent resurgence in interest as potential workhorses for the production of biodiesel, which can be obtained through transesterification of fatty acids with an alcohol, yielding monoalkyl esters. Biodiesel represents a solid and greener alternative to its fossil counterpart, due to the lower carbon footprint. Furthermore, domestic production of biodiesel would eliminate the need to rely on imports of crude oil and/or natural gas to satisfy a growing energy demand. Although far from taking over the energy market, the biofuel business has been steadily growing for the past 5 yeast and is projected to grow even further in the near future [5, 6].

Despite a great number of mentions in literature of similar oleaginous yeasts, there is a lack of replicable protocols dedicated to the genetic transformation of *C. oleaginosus* specifically, as well as no dedicated molecular toolboxes, plasmids and mutants aimed at simplifying further genetic experimentation with this microorganism.

This report will therefore attempt to develop a quick, reliable and easily replicable transformation protocol for *C. oleaginosus* with the equipment available at Aalborg University Esbjerg. Once this goal has been achieved, it is intended to create a *C. oleaginosus* mutant carrying an inducible Cre-recombinase system, able to cleave selection markers in-vivo when desired. This property will then allow to use the same selection marker for multiple transformations.

The development of a quick and reliable method for transformation of *C. oleaginosus*, together with a system for the recycling of selection markers, may allow for the future use of CRISPR/Cas9 in this yeast to target and disrupt genes of interest, which in turn will open the doors to the "hijacking" of the powerful native enzymatic machinery and switch biosynthesis from lipids to other biomolecules of interest. More specifically, it will be attempted to test out the ability of such a mutant to produce bioquinones, originally synthesised by multiple species of the filamentous fungi genus *Fusarium*, and used as electrolytes in the experimental red-ox flow battery developed at Aalborg University Esbjerg.

## 3 Genetic engineering of oleaginous yeasts

Oleaginous yeasts and yeasts in general have always been one of the main targets of genetic engineering because of their capability to accumulate significant amounts of triacylglycerols (TAGs) in the form of lipid droplets within their cells. The lipid content varies between a minimum of 20% dcw, required to be considered oleaginous, and more than 70% dcw, depending on both the species and culture conditions. Some examples of oleaginous yeast strains that have been investigated include *Yarrowia lipolytica*, *Candida* 107, *Rhodotorula glutinis*, *Rhodosporidium toruloides*, *Cutaneotrichosporon oleaginosus*, *Trichosporon pullulan*, and *Lipomyces lipofer*. Screening studies nevertheless continue to be conducted, leading to the discovery of several new species of oleaginous yeasts [7]. The versatility of fatty acids, which these microorganisms are naturally versed to produce in large quantities, allow for the synthesis of multiple industrially relevant compounds. This spans from low value, high volume products such as unsaturated fatty acids (PUFA) to biofuels and oleochemicals. Countless experiments have been performed though manipulation of metabolic pathways aiming towards boosting the synthesis of fatty acids or the synthesis of fatty acids derivates. In addition, the availability of acetyl-CoA in oleaginous yeasts is very high as this unit is the main building block for the biosynthesis of fatty acids chains. This efficient supply renders oleaginous yeasts ideal candidates for the production of other acetyl-CoA derivative products, such as poly-3-hydroxybutyrate PHB, terpenoids and polyketides. Finally, aside from their vast molecular toolbox, oleaginous yeasts are close to model organisms for fermentation, as they are able to quickly grow to high cell densities, can be scaled up easily and are able to metabolise a wide variety of substrates, as well as naturally being able to thrive at low pH, which is a considerable advantage in preventing the growth of bacterial contamination [8–10].

The success of genetic manipulation in microorganisms is highly dependent on the type and efficiency of the vector used, and oleaginous yeasts are no exception. Autonomously replicating vectors allow for greater diversity of transformants and subsequent manipulation. The use of such systems is already well established in a multitude of model organisms such as *E. coli* and *S. cerevisiae* but their availability is limited in most oleaginous yeasts. *Y. lipolytica* is an exception as it has autonomously replicating sequences (ARS) that can be adapted for use as replication elements. Self replicating plasmids for other yeasts such as *R. toruloides*, *L. starkeyi* and *C. oleaginosus* are still unavailable. Therefore, foreign genes can only be loaded on suicide or integrating plasmids, which limits their genetic manipulating power [11]. Other essential components of a transformation vector and crucial for its success are selection markers and recognisable promoter/terminator sequences. Again, *Y. lipolytica* has a wide variety of validated and widely used auxotrophic selection markers, such as the uracil-deficient Δura3/ura3d4,

leucine-deficient Δleu2, tryptophan-deficient Δtrp1, and histidine-deficient ΔhisG [11–13]. Auxotrophic strains are unable to synthesize certain metabolites that are crucial for their survival. This leads to cell death if these metabolites are not present in the growth medium or if the corresponding genes, which can produce these metabolites when expressed, are not included in the transformation vector as selection markers [14]. Other selection markers available for oleaginous yeasts are antibiotics, but only hygromycin B and bleomycin/phleomycin could successfully be used in only certain *Y. lipolytica* strains [15]. *Rhodosporidium toruloides* on the other hand has been proven to be able to use hygromycin B, bleomycin/phleomycin, neomycin and geneticin for selection, as well as also having viable auxotrophic selection markers and reporter genes in the form of luciferase and green fluorescent protein [11, 16].

Promoter/terminator sequences are more simple to screen for and can be easily identified on a microorganism genome after sequencing. A wide variety of both constituive and inducible promoters with different expression ranges have been extensively studied and characterised. Studies have also found that the strength of the promoter can be controlled by engineering the TATA box sequence and upstream activating sequences. A panel of terminators that regulate gene expression was also developed, resulting in improved fluorescence protein output and transcript level[11].

Overall, basic genetic elements have been developed in different oleaginous yeasts to facilitate metabolic engineering. *Y. lipolytica* has by far the most available genetic elements, including a one-step transformation method using heat shock [17]. Similar transformation methods are also available for the oleaginous yeasts *R. toruloides* and *L. starkeyi* in the form of LiAc/PEG-mediated chemical transformation and PEG-mediated protoplast transformation. Both of these methods require the addition of chemical agents and a heat shock in order to open pores in the cell membrane or degrade the cell wall and allow the insertion of foreign DNA into the microorganism [18, 19]. More specifically, the PEG protoplast transformation process is more laborious to carry out and the efficiency depends largely on the quality of the protoplasts produced [18]. The LiAc/PEG-mediated chemical method on the other hand is simpler and quicker to carry out, but the transformation efficiency is usually low and can also be affected by multiple factors [19].

Electroporation has also been investigated as a transformation method for oleaginous yeasts with moderately positive results. While the transformation process is usually simple and relatively fast to carry out, the efficiency and number of transformants can be drastically affected by multiple factors, such as cell growth phase, pretreatment of the cells, composition of the suspension buffer [20, 21]

Another transformation method which has been shown to be effective on a multitude of different organisms including *R. toruloides*, *L. starkeyi* and *C. oleaginosus* is *Agrobacterium tumefaciens* mediated transformation or ATMT. This is a relatively complicated and slow transformation procedure, but

reliable and efficient if performed correctly. Much like the LiAc/PEG-mediated chemical transformation method however, ATMT is only able to transform linear DNA fragments. Transformation efficiency has also been shown to drop significantly on ΔKu70 mutants [11, 22, 23]. The protein coded by *Ku70* is used for the formation of the "Ku heterodimer" (Ku70/Ku80–protein complex), which in turn together with the DNA ligase IV–Xrcc4 complex is responsible for the NHEJ DNA repair mechanism inside the cell. The elimination of this gene does therefore allow to obtain mutants with enhanced HR efficiency and significantly improved gene-targeting frequency. This modification is in fact a common approach to tackle the strong preference of oleaginous yeasts for non-homologous end-joining (NHEJ) and low homology-directed repair (HR) activity [10, 22, 24].

Overall, there is growing interest in using oleaginous yeasts other than *Y. lipolytica* such as *Rhodosporidium toruloides*, *Lipomyces starkeyi* and *Cutaneotrichosporon oleaginosus* due to their higher lipid content, but their genetic engineering is limited by the lack of genetic elements. Efforts are needed to establish efficient genetic elements in these yeasts [10].

## 4 The oleaginous yeast *Cutaneotrichosporon oleaginosus*

*Cutaneotrichosporon oleaginosus*, formerly also known as *Apiotrichum curvatum*, *Cryptococcus curvatus*, *Trichosporon cutaneum* and *Trichosporon oleaginosus*, is a relatively newly discovered oleaginous yeast which displays promising traits both for fermentation of biomasses and biosynthesis of specific high-value compounds, such as polyunsaturated fatty acids (PUFA) [4, 25].

This oleaginous yeast belongs to the phylum Basidiomycota, one of seven major phyla of fungi and includes mushrooms, puffballs, rusts, smuts, and yeasts. Organisms belonging to this classification are known for their adaptability to different environments, where they generally grow and feed largely on lignocellulosic biomasses [26].

In particular, yeast belonging to the phylum Basidiomycota differ from their counterparts in the phylum Ascomycota in a number of ways. Unlike Ascomycota yeasts, the budding sites in basidiomycete yeasts are confined to either or both poles of the cells and during budding, the inner wall of the mother cell extends to form the wall of the daughter cell. This is in contrast to the budding process in most ascomycete yeasts, where the daughter cell is continuous with the entire wall of the mother cell [27]. Basidiomycete yeasts can also produce asexual propagules called ballistoconidia, which are actively liberated in a manner similar to basidiospores. Sexual reproduction is relatively rare in basidiomycete yeasts, but when it occurs, it involves mating between compatible yeast cells, leading to a limited dikaryotic mycelium that produces basidia [27]. Transmission electron microscopy has shown that

basidiomycete yeasts have multi-layered lamellate walls, which can be differentiated from the two-layered cell walls of ascomycetes through staining techniques. In addition, septa in basidiomycete yeasts can also be examined by transmission electron microscopy when present and their structure can be an important feature in classification [27].

From a phylogenetic point of view, *C. oleaginosus* has been recently relocated to the novel genus *Cutaneotrichosporon* from *Trichosporon*, which was instead revised and placed in the order of *Trichsponales*. The new *Cutaneotrichosporon* genus currently contains only 13 species, around half of which are actually harmful to humans [4].

*C. oleaginosus* has been found to grow as a filamentous fungi in nature, proliferating in soil and on dead leaves [4]. One of the main distinguishing characteristics of this microbe, i.e. the ability to metabolise large amount of intracellular lipids, is triggered only as a response to environmental stimuli and variation of the supply of nutrients. Nevertheless, under these specific conditions *C. oleaginosus* has been shown to be able to produce enough TAGs to account for up to 70% of its DCW, which puts it on pair with other oleaginous yeasts, such as *Yarrowia lipolytica*, *Rhodotorula glutinis* and *Rhodosporidium toruloides* [4].

Genome analysis of *C. oleaginosus* has also predicted cellulase and chinase enzymes. However, the organism has been shown to not be able to grow on substrates of polysaccharide nature, such as chitin and lignocellulose. This suggests that these enzymes are responsible for intracellular activities, such as remodelling of the cell wall [4].

*C. oleaginosus* is able to metabolise a vast variety of carbon sources, such as glucose, glycerol, xylose, arabinose, lactose, volatile fatty acids, cellobiose and complex waste materials, while also being able to tolerate relatively high concentrations of well-known fermentation inhibitors like acetate, ammonium ions and furfurals.

Interestingly, the composition of the fatty acids produced by this yeast is predominantly made up of C16 and C18 units (16-33 and 43-57 %DCW, respectively), which are also the most common in oils of plant-based origin. Moreover, the B20 blend with biodiesel derived from *C. oleaginosus* lipids does meet the D6751 ASTM specification for biodiesel blends in diesel fuel oil [3]. Nevertheless, the production of yeast-based biofuel remains roughly twice as expensive than the one of first generation biofuels from conventional sources, such as sunflower seed oil, soybeans and rapeseed [3].

Genetic manipulation of *C. oleaginosus* in order to further boost the powerful fatty acid metabolism has been attempted, but so far only a handful of successful transformation experiments could be found in literature, which will be reported below.

### 4.1 Random mutagenesis

Auxotrophic mutants commonly used in genetic engineering lack the ability of autonomously producing one or more essential organic nutrient, which if not provided in the growth medium will cause the organism to die. This allows for the use of genes responsible for the synthesis of these essential nutrients as efficient selection markers during transformations [28]. One of the most simple and direct ways of creating auxotrophic mutants of a microorganism is the introduction of random mutations by a mutagenic agent [29].

In the case of *C. oleaginosus*, creation of auxotrophic mutants was attempted by Ykema et al. on a WT strain (ATCC 20509) using different known mutagenic agents and subsequently screening the treated cells on growth media with varying concentrations of essential amino acids and UFA. [30] . Overall, unsaturated fatty acids (UFA) and amino acids auxotrophs could be produced using N-Methyl-N'-nitro-N'-nitrosoguanidine (MNNG) and acridine mustard (ICR-170), while ethyl methanesulfonate (EMS) and UV light proved to be unsuccessful at generating any mutants [30]. Two strains, a methionine auxotroph and an UFA auxotroph from this study, were combined through the fusion of spheroplasts, in order to create a new mutant with a modified fatty acid composition in the produced lipids. The spheroplasts were created by dissolving the cell wall using the enzyme Novozym 234 [31]. In addition, revertants of the auxotrophs produced via random mutagenesis still displayed an altered fatty acid composition [32]. In a different study, the use of EMS did prove successful in creating more fatty acid mutants [33].

### 4.2 *Agrobacterium* mediated transformation (ATMT)

*Agrobacterium tumefaciens* mediated transformation (ATMT) is a widely used method for plant and fungal transformation. The method is based on the discovery of a link between crown gall disease and *A. tumefaciens* in 1907 [34]. *A. tumefaciens* can perform a horizontal gene transfer from a large Ti-plasmid into the chromosomal DNA of eukaryotes, which has been harnessed for DNA modification of plants and fungi. The transfer is facilitated by a series of virulent genes on the Ti-plasmid and in the *A. tumefaciens* chromosomal DNA, which induce tumours in the host [34, 35]. However, mutant strains of *A. tumefaciens* with disarmed Ti-plasmids and deleted oncogenic genes are used to insert heterologous DNA fragments in a target host. ATMT is best suited for smaller modifications, such as heterologous expression, introduction of smaller genes, and promoter swapping, while larger modifications may require other methods such as protoplast transformation. ATMT has been evaluated for different technical parameters and multiple binary vector systems containing T-DNA have been

created, resulting in different protocols depending on the species of the host [35].

Only a single research paper by Görner et al. could be found in literature that successfully managed to create *C. oleaginosus* mutants though the development of an *A. tumefaciens* mediated transformation protocol [36]. For this purpose, a cassette was created containing a codon-optimised version of yellow fluorescent protein (*yfp*), regulated by the constituve GDH (glyceraldehyde-3-phosphate dehydrogenase) promoter from *C. oleaginosus* and the respective terminator. To these three elements, truncated versions of the same promoter and terminator were added to the cassette in order to regulate the expression of a hygromycin b phosphotransferase from *E. coli*, to be used for the selection of successful mutants upon growth on the antibiotic hygromycin B. After the successful integration of this cassette, the same protocol was used for the heterologous expression of various bacterial enzymes acting on fatty acid modification. The resulting mutants were able to produce significantly more more α-linoleic acid (from 2.8% to 21% of the total lipid fraction). In addition, purposefully engineered mutants were able to synthesise the non-native PUFA acid chains eicosatrienoic, eicosadienoic acid and 10(E),12(Z)-conjugated linoleic acid, overall amounting to 16%, 9% and 2.6% of total produced fatty acids.

### 4.3 Electroporation

Similary to *Agrobacterium tumefaciens* mediated transformation, only a single research paper could be found in literature reporting the successful transformation of *C. oleaginosus* though electroporation [2]. The article by Koivuranta et al. describes the assembly of a series of cassettes using cerulenin, hygromycin B and G418 as selection markers, which were used to introduce the genes acetaldehyde dehydrogenase, pyruvate decarboxylase and acetyl-CoA synthetase with the aim of improving the production of TAGs through PDH bypass. Various mutants expressing different combinations of the aforementioned genes were produced, which showed an overall improvement on the total lipid yield, especially at high C/N ratios [2]. The electroporation protocol is described in great detail and makes use of a considerable 6µg of DNA for each transformation [2]. Nevertheless, no further information on the results and efficiency of the protocol is actually provided.

## 5 Cre-Lox systems

Cre-Lox systems are a site specific genetic engineering technology, which allow to perform in-vivo targeted genetic modifications. When expressed, this system is able to excise a specific sequence of DNA, invert it or perform translocation by combining fragments from two separate DNA molecules. The performed action depends on the way the system has been design [37, 38]. Currently, one of the most common applications of such systems is the excision and recycling of selection markers from the genome of a transformant [39, 40].

Recombination though Cre-lox systems makes use of the Cre-recombinase enzyme and LoxP DNA sites (locus of x-over, P1) on a target DNA fragment [37, 38]. The former is a tyrosine site-specific recombinase (T-SSRs), a category of enzymes which also includes flippase and D specific recombinase, while the latter is a 34 bp sequence consisting of two 13 bp inverted and palindromic fragments and a central 8 bp core sequence [38]. Cre-recombinase, which originates from a gene of bacteriophage P1, is able to recognise two identical LoxP sites and affect the DNA sequence between them. More specifically, depending on the orientation of the cores of the LoxP sites, the sequence between the two LoxP sites will be excised if the cores have the same orientation, while if the cores have opposite orientation the sequence will be inverted [37, 38, 41]. Additionally, if the LoxP sites are located on different DNA molecules, Cre-recombinase is able to perform a translocation, recombining the different fragments on either side of the sites from different molecules with each other [37].

Table 1: Sequence of a LoxP site

| 13 bp | 8 bp "core" | 13 bp |
|---|---|---|
| ATAACTTCGTATA | - NNNTANNN - | TATACGAAGTTAT |

The inversion of a sequence surrounded by two LoxP sites facing opposite directions does not result in the modification of either of the LoxP sites, thereby allowing the inversion process to proceed an indefinite amount of times over. This continuous flipping of a single sequence does not serve any practical use in molecular engineering [37]. However, as depicted in Figure 1, the addition of an extra pair of LoxP sites allows for a permanent inversion of the sequence, followed by the excision of one of the LoxP sites, leaving behind only a pair of incompatible LoxP sites. This strategy can be used as a permanent *in-vivo* on/off switch for any gene by changing the orientation with respect to the promoter located outside the region surrounded by LoxP sites [37, 38, 41].

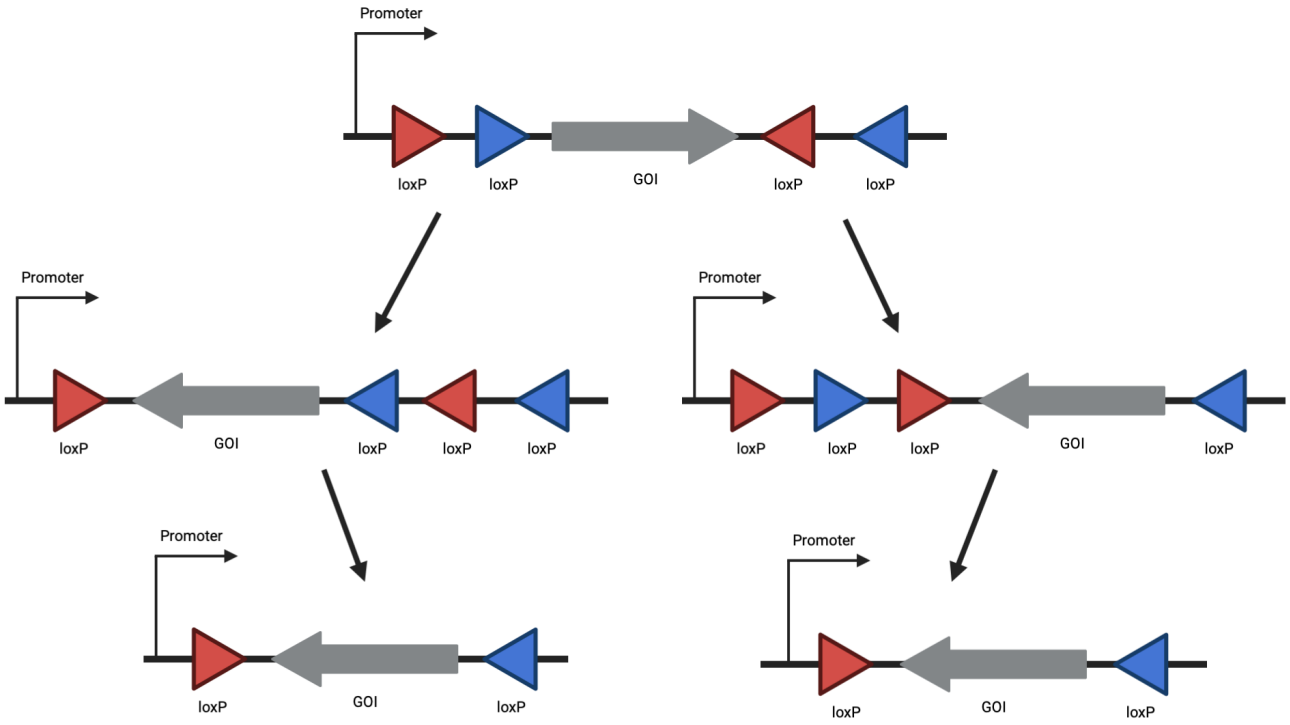


Figure 1: Schematic representation of permanent deactivation of a gene through inversion with LoxP and Cre-recombinase. The same process can also be used for permanent activation of the gene

Oftentimes the gene for Cre-recombinase is positioned after an inducible promoter, which allows for the controlled activation, deactivation or excision of one or multiple genes only at the desired moment throughout the cultivation. This can be especially useful in multi-cellular organisms, where the expression or inactivation of particular genes is only desired past an initial growth and development period [37, 40].

The ability to effectively remove an arbitrary sequence from an organism's genome with precision renders Cre-lox systems an ideal option for the removal and recycling of selection markers. Especially when dealing with fungal genetics and not yet fully understood secondary metabolic pathways, it is common to delete and/or heterologously express different combinations of enzymes in order to better understand their function. Furthermore, the complete recostruction of heterologous pathways in host organisms for the production of cell factories can require the transformation of dozens of genes. For instance, heterologous biosyenthesis of hydrocodone and noscapine in yeast requires the manipulation of 23 and more than 30 genes, respectively [42, 43]

In order to assure the integration or deletion of the gene(s) of interest, a selection marker is always required, of which the use of multiple is impractical and leads to an increasingly expensive growth medium [40, 44]. In addition, the presence of selection markers in industrial strains is usually not

desired for the biosafety concerns, as well as consumers viewpoints [39].

The Cre-Lox system have been reportedly used with success in bacteria, yeasts, filamentous fungi, plants and also mammals [39]. An example of a successful use of Cre-Lox systems for recycling of selection markers has been reported by Zhang et al., where a self-excising cassette was used in *Aspergillus oryzae*, allowing to successfully remove the *AdeA* gene from *Aspergillus nidulans*, used as selection marker. This construct was then used for the heterologous expression of an oxidoreductase and a transporter in order to induce overproduction of kojic acid. Interestingly, it was also reported that the initial assembly of the plasmid containing the cassette failed due to the fungal promoter used for Cre-recombinase being leaky and causing restriction to take place in *E. coli*. This problem was however easily solved with the introduction of an *Aspergillus oryzae* intron [40].

Cre-Lox recombination has also reportedly successfully been used in the basidiomycetous yeast *Xanthophyllomyces dendrorhous* as a proof of concept, in order to remove a G418 (geneticin) selection marker surrounded by LoxP sites [39]. In this particular study, *X. dendrorhous* was transformed with a linear Cre-expressing vector lacking any form of replication origin, which rendered it genetic unstable. This instability was exploited to ensure the vector would not remain in the cell long after excision of the G418 selection marker was complete, instead being lost during subsequent cell replication cycles. This system reported a 100% efficiency in removing the G418 selection markers in single-gene loci.

Further successful use of Cre-Lox systems in other basidiomycete yeasts has also been reported in *Rhodosporoium toruloides* to recycle the hygromycin B selection marker [22], as well as *Ustilago maydis* for conditional excision of genes under different developmental stages [45].

## 6 Tetracycline induced expression systems

Controlled expression of heterologous genes is a highly desirable traits in the host, as transgenic models with constitutive promoters lack control over timing of gene expression and may result in toxicity to mutants in the early stages of growth, limiting the study of transgene function in the later growth stages. Such a system is not suitable for investigating compensatory responses or development-dependent effects of the heterologously expressed genes [46]. Inducible promoters are able to solve this issue by allowing transcription of the gene of interest to begin only once an inducer molecule is added, a repressor removed or both. For this purpose, it is important for the gene regulated by the inducible system to have no basal level expression in the uninduced state, also known as being "not leaky", and for the inducer/repressor molecules to not cause toxicity or interfere with growth/metabolism of the host considerably [46, 47]. To the best of our knowledge, however, no inducible promoter systems for *C. oleaginosus* suitable for controlling the expression of an in-vivo genome editing tool such as Cre-Lox systems has been described at the time of writing. This task can instead be theoretically completed using a tetracycline controlled expression system, a versatile tool for controlling gene expression in various organisms, allowing for the observation of phenotypic changes by regulating gene activity in a temporally defined way [48–50]. This system originates from a mechanism of resistance of Gram-negative bacteria towards the antibiotic tetracycline, where the promoter *Ptet* expresses the repressor TetR and the protein TetA, responsible for pumping the antibiotic out of the cell. The reason for the expression of both the TetR repressor and the TetA protein by the same promoter is due to the fact that an overproduction of TetA by the cell can become lethal due to a non-specific transfer of charged molecules outside the cell, disrupting the cytoplasmic membrane potential. To avoid this from happening, TetR is able to bind to [MgTc]+ (a positively charged magnesium-tetracyclin complex) and while in this state it cannot bind to two operator sites (TetO1,2), allowing transcription of more TetR and TetA to continue. When the amount of tetracyclin decreases, TetR is able to bind to tetO1,2 and inhibit transcription [48, 49].

Different tetracyclin-based expression systems have been derived from this original bacterial defence mechanism in order to artificially control the expression or repression of specific genes of interest in eukaryotes. Further modifications have also been performed in order to better suit the system to its target host. Transcription or repression is controlled with the addition of either tetracycline (tet) or its analog doxycycline (dox), which are membrane permeable and also lack known targets in eukaryotes [49].

Currently there are two options for the general setup of such systems, one labelled TetOFF, where

transcription is induced by absence of tetracyclin or its analogs, and the other labelled TetON, where the addition of the antibiotic will trigger transcription [49].A schematic representation of both the TetOFF and TetON system is provided in Figure ??.

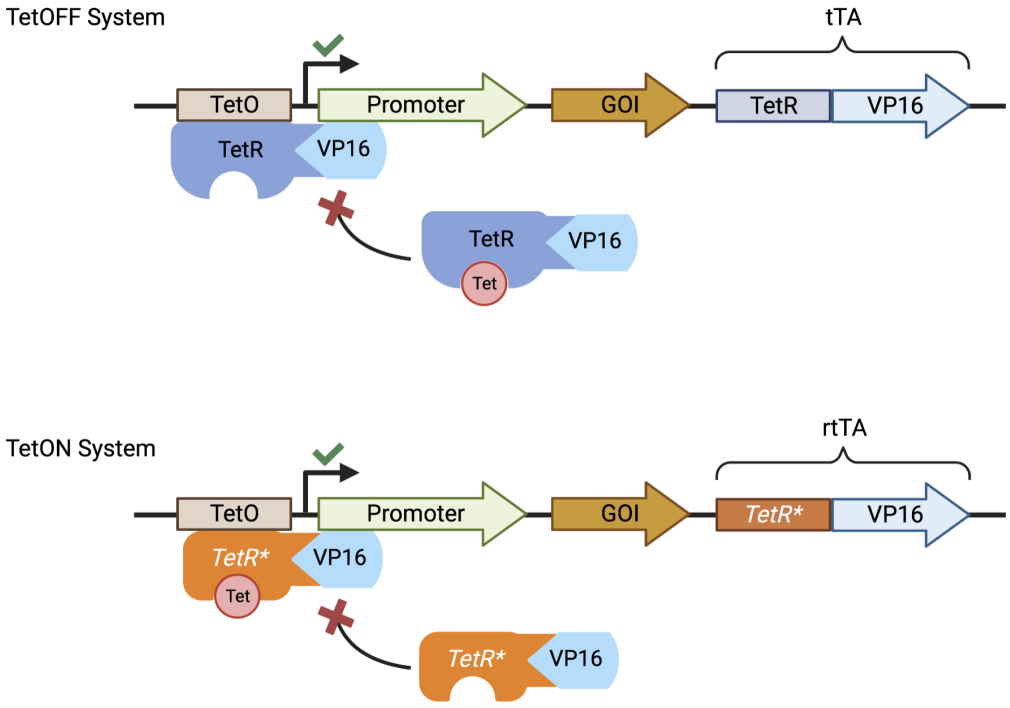


Figure 2: Representation of mechanism of action of tetracycline-induced TetOFF and TetON systems.

The TetOFF system is constructed by placing the tet operator (tetO) upstream of the gene of interest and a modified TetR repressor called tTA under the control of a constitutive promoter. The tTA protein is a fusion protein that consists of the TetR repressor domain and a VP16 activation domain from the herpes simplex virus. In the absence of tetracycline, the TetR repressor domain is able to bind to the tetO sequence and the VP16 domain initiates transcription. In the presence of tetracycline or its analogs, the tTA protein undergoes a conformational change that causes it to dissociate from the tetO operator sites, thereby preventing activation of gene expression. When tetracycline or its analogs are removed, the tTA protein can once again bind to the tetO operator sites, leading to activation of gene expression [49].

The TetON system on the other hand is a variant of the tetracycline-regulated gene expression system that allows for the induction of gene expression in response to tetracycline or its analogs. In this system, a mutated *TetR** repressor is used, which is able to bind to tetO sequences only when in the presence of tetracyclin or its analogs. Fusion of the mutated *TetR** repressor and the VP16 viral domain constitutes the rtTA protein, for reverse tetracycline-controlled transactivator. When

tetracycline or its analogs are added to the system, they bind to the rtTA protein, which is then able to bind to the tetO operator site and allows the VP16 activation domain to activate transcription of the target gene [49]. The TetON system has several advantages over the TetOFF system. For example, it allows for tighter regulation of gene expression, as the rtTA protein has a higher affinity for the tetO operator sequence than the non-mutated TetR repressor. Additionally, the TetON system allows for the temporal control of gene expression, as the timing and duration of tetracycline administration can be adjusted to control the level of gene expression [49].

Zarnack et al. [50] worked on adapting a Tet-controlled system system for use in *Ustilago maydis*, a biotrophic phytopathogenic basidiomycete fungus that causes corn smut disease, with the aim of achieving control of a pheromone response factor. These efforts initially failed due to a non-codon optimised version of TetR showing premature polyadenylation and the VP16 domain showing what appeared to be cytotoxic traits. Codon optimisation of TetR, the removal of cryptic enhancer sequences from the promoter and the use of an acidic minimal activation domain served to solve this issue. This resulted in the creation of a TetOFF system for *U. maydis*, while a TetON system for this fungus still remained unavailable [50].

A study by Ingole at al. [49] managed to create a tight working TetON system in *U. maydis* in 2022 by fusing Mql1 to the codon optimised TetR in order to overcome basal leaky expression. Mql1 is the closest ortholog of the transcriptional co-repressor Ssn6 from yeast, which was transcriptionally fused to an unmodified TetR, creating a fusion protein that binds to TetO in absence of tetracycline and prevent leaky expression in the uninduced state. The system also made use of a native minimal promoter with 10 copies of the tetO binding sites and was successfully used to tightly regulate the expression of fluorescent proteins and the toxic gene *Bax1* in both solid and liquid media. Confocal microscopy and immunoblotting confirmed the tight regulation of gene expression, with no expression observed in the uninduced state. The results demonstrate the potential of this system for controlled gene expression in *U. maydis* and possibly other fungi [49]. In fact, this TetON system will be tested in *C. oleaginosus* throughout this project as a tool for controlling the expression of cre-recombinase (see Chapter 5) and excise selection markers from the genome.

# 7 Problem statement

The oleaginous yeast *C. oleaginosus* has shown clear potential for use in industrial fermentation of biomasses into fatty acids. The ability of this microorganism to withstand common fermentation inhibitors and simultaneously produce high value lipids suitable for multiple purposes has been highlighted across literature multiple times.

At Aalborg University Esbjerg, research is being conducted on *C. oleaginosus* to explore the potential of redirecting its primary metabolic pathway from lipid biosynthesis to the production of bioquinone polyketides as electrolytes in a red-ox flow battery. The long-term goal is to hijack the molecular toolbox of this microorganism for this new application.

However, in order to carry out this research, a molecular toolbox, complete with a reliable transformation protocol, is indispensable. The research conducted in this report will therefore focus on the development of such a toolbox, which will include a transformation protocol, recyclable selection markers and targeted genomic integration.

If successful, this could open up new possibilities for the use of this microorganism in the production of electrolytes for redox flow batteries, a technology with significant potential for energy storage applications. This research has the potential to impact not only the fields of biotechnology and energy storage, but also many other industries that rely on the production of fatty acids.

The following section of the report will therefore attempt to answer the following question:

"*How can multiple exogenous genes of interest be successfully integrated into the genome of C. oleaginosus and is it possible to excise selection markers in-vivo using a Cre-recombinase based system?*"

# 8 Materials and methods

The following chapter is set out to present and describe the procedures adopted to study the oleaginous yeast *C. oleaginosus* and to develop a suitable molecular toolbox. Detailed information about the methods and protocols adopted will also be provided.

## 8.1 Vectors

This subchapter is dedicated to the vectors relevant for the experiments carried out. An exhaustive list is provided in Table 2, while the maps are provided below.

Table 2: Vectors used and attempted to be assembled during the experiment

| Plasmid name | Antibiotic resistance | Origins of replication | Extra features |
|---|---|---|---|
| *pRF-HU2-Hyg-yfp* | Kanamycin Hygromycin B | oriV | yfp |
| *pFLEXI-NPTII* | Kanamycin G418 | CEN/ARS oriV | - |
| *pUC57-NTC* | Kanamycin | pUC ori | NTC |
| *pFLEXI-NTC* | Kanamycin NTC | CEN/ARS oriV | yfp - LoxP sites |
| *pUC57-NTY* | Kanamycin NTC | pUC ori | yfp |
| *pUC57-HYC* | Kanamycin Hygromycin B | pUC ori | TetON-Cre-Lox system |

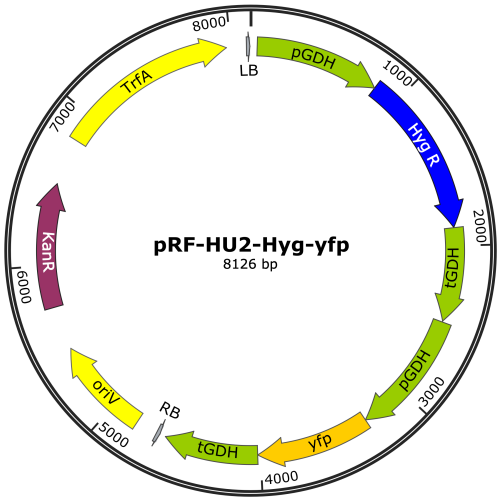


Figure 3: *pRF-HU2-Hyg-yfp*

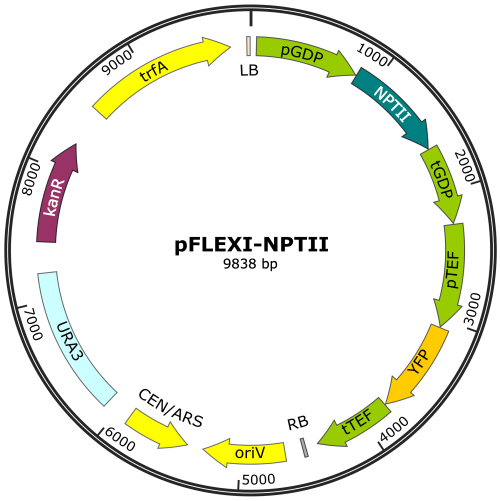


Figure 4: *pFLEXI-NPTII*

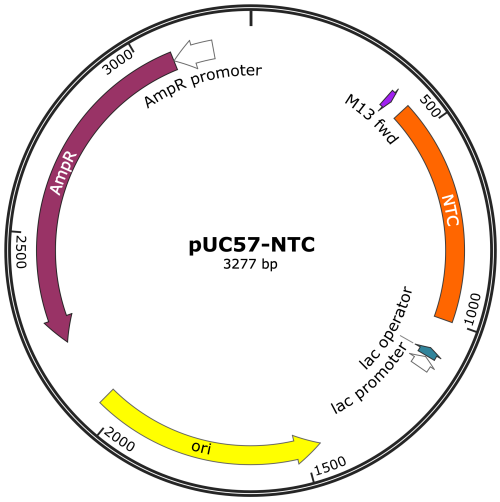


Figure 5: *pUC57-NTC*

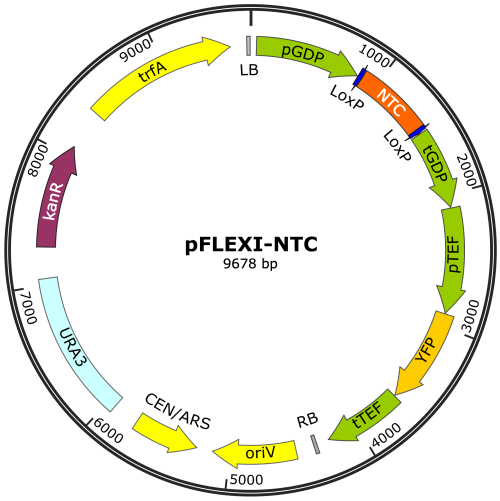


Figure 6: *pFLEXI-NTC*

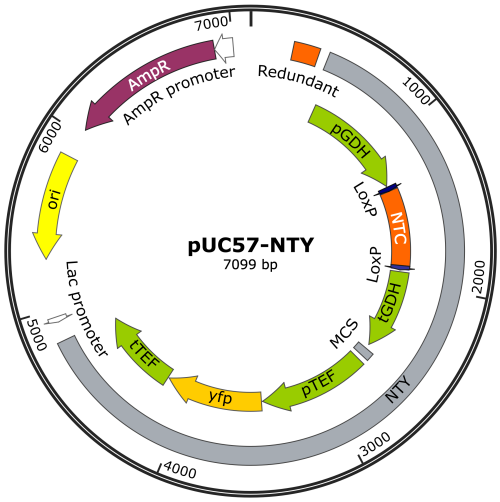


Figure 7: *pUC57-NTY*

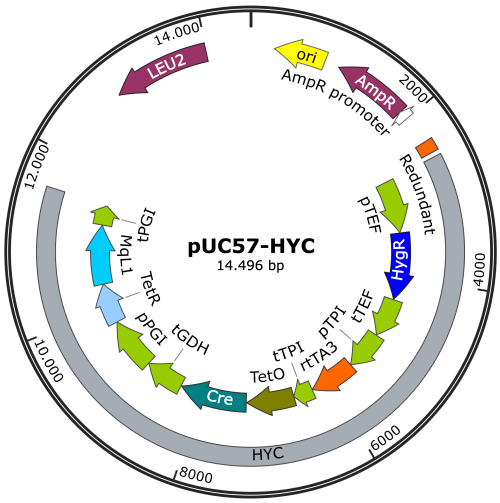


Figure 8: *pUC57-HYC*

## 8.2 Adaptation of the only existing electroporation protocol

Even though *C. oleaginosus* could be reliably transformed through *A. tumefaciens* at Aalborg University Esbjerg before [51], electroporation was chosen as the preferred method for this report due to the much shorter time required for the transformation of a single sample. More specifically, the starting point was the electroporation protocol used by Koivuranta et al. for *C. oleaginosus* [2], which used a Bio-Rad (Hercules, CA, United States) GenePulser at the settings: 1800 V, 1000 Ω and 25 µF. However, the only electroporator machine currently available at Aalborg University Esbjerg is a Bio-Rad (Hercules, CA, United States) MicroPulser, which works sligtly differently than the Gene Pulser. Most importantly, the parameters for the MicroPulser which can be controlled are voltage (V) and pulse length (ms), instead of voltage (V), resistance (Ω) and capacitance (µF). It is possible to convert resistance and capacitance to pulse length and vice versa using the formula:

$$t[ms] = R[\Omega] \cdot C[mF]$$

Using this formula returns a pulse length of 25 ms, while the maximum allowed by manual settings on the MicroPulser is just 4 ms. In spite of this, it was decided to replicate the protocol from literature as closely as possible. A first attempt was therefore performed with the following protocol.

A 50 mL 2xYPD (Recipe in Appendix 12) culture of *C. oleaginosus* was prepared in a 250 mL baffled conical flask and incubated overnight at 30°C and 200 rpm. Once the OD600 reached 20, the culture was centrifuged at 5000 rpm for 4 min and the cells washed with 5 mL of ice-cold EB buffer (recipe in Appendix 12). The pellet was the resuspended with 5 mL of IB buffer (recipe in Appendix 12) and incubated at 30°C and 200 rpm for 30 min. Next, the culture was once again centrifuged at 5000 rpm for 5 min and the pellet washed with 5 mL of ice-cold EB buffer and resuspended in 1.5 mL of EB buffer. Of this suspension, three samples were made using 400 µL each. The plasmid DNA added was obtained from a miniprep of three 50 mL *pRF-HU2-Hyg-yfp E. coli* cultures, grown on LB media + 200 ng/µL kanamycin (recipe in Appendix 12) at 37°C and 200 rpm for 3 days. More specific information of the samples is provided in Table 3.

The cell suspension and minipreps were mixed in 0.4 cm cuvettes and incubated on ice for 15 min, before being subject to electroporation though the MicroPulser at 1800 V and automatic pulse length. For all samples, however, the instrument displayed "Arc", signalling the activation of the arc prevention and quenching system. This situation persisted also when attempting lower voltages. Arcing usually occurs when the sample is too conductive, in this case either due to too many cells, impurities in the suspension or a combination of both. It was therefore decided to investigate the effect of different

Table 3: The three samples produced in the first attempt to replicate the already existing electroporation protocol.

| Sample | *C. oleaginosus* suspension [µL] | *pRF-HU2-Hyg-yfp* plasmid DNA [µg] |
|---|---|---|
| WT | 400 | 0 (0µL) |
| S | 400 | 7.2 (50µL) |
| K | 400 | 13.0 (90µL) |

parameters on the sample, in order to avoid arc-over events.

From here, a series of trials was performed in order to further study the parameters influencing the outcome of performing electroporation on *C. oleaginosus* cultures and adapt the protocol developed by Koivuranta et al. [2] to the equipment available on the Esbjerg campus. The main variables it was decided to operate on were the cuvette size, cell concentration, concentration and type of antibiotic on the Petri dishes, suspension buffer for the cells and pulse voltage. A summary of all trials performed is provided in Table 4, while a more exhaustive explanation for all trials, including samples produced, preparation and reasoning is provided in Appendix 12.

Table 4: Overview in of the trials performed in order to study the parameters influencing the outcome of C. oleaginosus electroporation and to replicate the protocol performed by Koivuranta et al. [2]. Further information about the individual parameters, samples preparation and choice of experiments is presented in the Appendix 12. *Dilutions performed on a 5 mL overnight culture, instead of the 50 mL one described by the original protocol.

| Trial | Antibiotic used [ng/µL] | Cell dilution from original protocol | Suspension buffer(s) | Pulse voltage [kV] | Cuvette size [cm] |
|---|---|---|---|---|---|
| 1 | - | 1:1 - 1:20 | EB buffer | 1.1 - 1.3 | 0.1 + 0.4 |
| 2 | - | 1:50 - 1:200 | EB buffer + Tris-Cl | 1.2 - 1.8 | 0.1 |
| 3 | - | 1:10 - 1:100 | $H_2O$+ Sorbitol | 1.5 - 2.0 | 0.1 + 0.4 |
| 4 | Hygromycin B 200 | 1:25 - 1:100 | EB buffer + Tris-Cl $H_2O$ + Sorbitol | 1.2 - 1.8 | 0.1 |
| 5 | Hygromycin B 200 + 250 | 1:75 | EB buffer + Tris-Cl $H_2O$ + Sorbitol | 1.2 - 1.7 | 0.1 |
| 6 | Hygromycin B 250 | 1:1 - 1:50* | EB buffer + Tris-Cl $H_2O$ + Sorbitol | 1.2 - 1.7 | 0.1 |
| 7 | Hygromycin B 200 + 250 | 1:50* | Tris-Cl | 1.3 | 0.1 |
| 8 | Hygromycin B 200 + 250 | 1:25-1:50* | Tris-Cl | 1.2 - 1.3 | 0.1 |

### 8.3 Screening for antibiotic resistance

Attempts to adapt the Koivuranta et al. [2] electroporation protocol and study the parameters that influence it were unsuccessful in clearly separating wild type/control samples from samples containing the target plasmid *pRF-HU2-Hyg-yfp*. This suggests that the colonies observed on the target plates were mostly false positives and that selection through the antibiotic hygromycin B was not enforced. Because of this conclusion, it was decided to further investigate the resistance of *C. oleaginosus* to four different antibiotics, phosphinothricin (BASTA), geneticin (G418), hygromycin B (hyg B) and nourseothricin (NTC), and determine a MIC. For this purpose, a standardised version of the electroporation protocol used during the last trial carried out in Section 8.2 was used. The procedure is summarised in the following paragraph:

A 5 mL overnight culture of *C. oleaginosus* was prepared by growing the cells in 2xYPD at 30°C and 200 rpm. Once the culture reached an OD600 of 10-11, the cells are harvested by centrifugation at 5000 rpm and 4°C for 5 minutes. The resulting pellet is washed with 5 mL of EB buffer (recipe in Appendix 12) and resuspended in 5 mL of IB buffer (recipe in Appendix 12). The culture is then incubated at 30°C and 200 rpm for 1 hour. After this incubation period, the culture is centrifuged again at 5000 rpm and 4°C for 5 minutes and the pellet is washed 3 times with demineralised water. The pellet is then resuspended in 1 mL of 10 mM tris-Cl. A 1:25 dilution is then made from this suspension with more 10 mM tris-Cl, from which 50 µL are pipetted into a 0.1 cm cuvette to be electroporated. 2 µL of a control plasmid are also added to the cuvette in order to better simulate the presence of the target plasmid. The cuvettes are then incubated on ice for 15 minutes. Electroporation is carried out at 1.3 kV with automatic pulse length and, immediately after the pulse, 1 mL of warm 2xYPD media is used to resuspend the content of the cuvette and transfer it to 50 mL falcon tubes. The tubes are then incubated at 30°C and 200 rpm for 4 hours. From these cultures, 60 µL of suspension was plated on 55mm YPD plates, supplemented with the chosen antibiotic. The Petri dishes are then incubated at 30°C for three days.

Overall, the efficacy of the four antibiotics was tested, in collaboration with a bachelor student semester report [52]. The final tested antibiotic concentrations were in the range between 50 ng/µL and 400 ng/µL. NTC and hyg B, the antibiotics relevant to this report, were tested at the concentrations of 10, 50, 100 and 200 mg/L for the former and 50, 100, 200 and 400 mg/L for the latter.

A second trial was also performed using only NTC, using the concentrations 73, 175, 200 and 225 ng/µL and 5 replicates per concentration. Furthermore, in contrary with the standardised protocol, 125 µL of the 4 h subcultures were plated on standard 100 mm Petri Dishes instead of 250 µL.

## 8.4 Construction of cassette with antibiotic resistance gene

In order to further boost the chance of successfully transforming *C. oleaginosus* though electroporation, it was decided to switch the vector chosen to deliver the antibiotic resistance gene. More specifically, rather than the low-copy plasmid *pRF-HU2-Hyg-yfp* a linear DNA cassette was picked instead, containing the *yfp* gene and a selection marker for either hygromycin B or NTC, both surrounded by GDP promoter and terminator. A linear vector can be replicated by PCR in a relatively short amount of time, allowing to achieve much higher concentrations of DNA compared to a whole low-copy plasmid without the need to grow and maintain *E. coli* cultures.

### 8.4.1 Amplification of cassette with hyg B resistance gene

A linear vector containing *yfp* and the hyg B resistance gene is already contained in the *pRF-HU2-Hyg-yfp* plasmid in between the border sites meant for ATMT. This fragment can thereby be amplified by PCR using the primers CS.Fw and CS.Rv (primer sequences can be found in Appendix 12)

### 8.4.2 Construction of plasmid with NTC resistance gene

A cassette containing the NTC resistance gene and *yfp* surrounded by promoters and terminators readable by *C. oleaginosus* was not already present in the laboratory and therefore had to be assembled. This was attempted using the plasmid *pFLEXI-NPTII*, which already contains *yfp* surrounded by a TEF promoter and terminator and therefore only needed the *G418* gene to be replaced with *NTC*. Assembly of this construct was attempted both with homologous recombination (HR) in BY4743 and in-vitro ligation using DNA ligase followed by transformation in *E. coli*. The first step consisted in PCR amplification of the *NTC* gene from the plasmid *pUC57-NTC* using two sets of primers. The first set, Nrsr.Fw and Nrsr.Rv, was used to produce a fragment with matching ends suitable for HR, while the second, DD.LoxP.NourR.Fw and DD.LoxP.NourR.Rv, was used for ligation. The full sequence of all primers can be found in Appendix 12. Homologous recombination was attempted first, by digesting the plasmid *pFLEXI-NPTII* with BcuI and BamHI FastDigest (Thermo Fisher Scientific ®) restriction enzymes following the manufacturer provided protocol and incubating the reaction mix at 37°C for 30 min. The product was then run on gel electrophoresis for 50 min at 90 V and the main band obtained at ∼9500 bp was excised and the DNA extracted using a QIAquick Gel Extraction Kit (QIAGEN ®). In order to the proceed with homologous recombination in *S. cerevisiae*, a 5 mL overnight culture of BY4743 on 2xYPAD was prepared and incubated at 30°C and 200 rpm. The next day, $2 \cdot 10^{10}$ cells from the overnight culture were inoculated in a 250 mL baffled conical flask, together with 50 mL of

more warm 2xYPAD and incubated at 30°C and 200 rpm for 4 hours. The cells were then recovered by centrifugation at 5000 rpm for 5 min, washed twice with 25 mL of demineralised water and resuspended in 1 mL of more demineralised water. Next, 100 µL of this suspension were transferred into sterile 1.5 mL microcentrifuge tubes for each desired sample. Together with 100 µL of cell suspension, each trial performed contained also 360 µL of transformation mix, composed of 240 µL of 50% (w/v) PEG 3350, 36 µL of 1 M lithium acetate, 50 µL of single stranded salmon sperm DNA (denatured at 95°C for 5 min, then chilled on ice) and 34 µL of $H_2O$ mixed with the target DNA. A full list of all the produced samples is provided in Table 5.

Table 5: Amount of $H_2O$ and target DNA added in the samples produced for assembly of the *pFLEXI-NTC* plasmid through homologous recombination in BY4743. The trials labelled 1 and 2 are replicates, while the series labelled K and M were performed with different *NTC* PCR products

| Sample | $H_2O$ [µL] | *NTC* PCR product [ng - µL] | *pFLEXI* backbone [ng - µL] | *pFLEXI-NPTII* uncut [ng - µL] |
|---|---|---|---|---|
| WT | 34 | - | - | - |
| + | 32 | - | - | 104 - 2 |
| - | 29 | | 70 - 5 | - |
| K1 | 27 | 512 - 2 | 70 - 5 | - |
| K2 | 27 | 512 - 2 | 70 - 5 | - |
| M1 | 27 | 478 - 2 | 70 - 5 | - |
| M2 | 27 | 478 - 2 | 70 - 5 | - |

This experiment was performed twice. However, since the obtained results were not deemed satisfactory, it was decided to attempt the assembly of the *pFLEXI-NTC* plasmid through DNA ligation.

First, amplification of the *pFLEXI* backbone was attempted through PCR using the primers couples pFlexi.Backbone.DD.Fw; pFlexi.Backbone.DD.Rv and loxP.pFlexi.Backbone.Fw; loxP.pFlexi.Backbone.Rv (Sequence of all primers is provided in Appendix 12). This however, failed to give any significant results under multiple conditions. It was therefore decided to obtain the backbone though digestion in the same way as for homologous recombination, i.e. with the restriction enzymes BcuI and BamHI and recovery of the cut plasmid though gel electrophoresis. The *NTC* PCR product obtained from the primers DD.LoxP.NourR.Fw and DD.LoxP.NourR.Rv was also digested using BcuI and BamHI Fast-Digest (Thermo Fisher Scientific ®) restriction enzymes to produce matching ends with the backbone. The digestion product was also purified from the removed ends through gel electrophoresis at 90 V for 50 min. Assembly of the target plasmid was then attempted using T4 DNA ligase kit (ThermoFisher Scientific ®) and following the instructions provided by the manufacturer. In total six samples were produced, a wild type, one containing only the *pFLEXI* backbone, one containing only the *NTC* gene and three replicates containing both. A complete list of all samples is provided in Table 6.

Table 6: Amount of $H_2O$ and target DNA added in the samples produced for assembly of the *pFLEXI-NTC* plasmid through T4 DNA ligation. The samples A, B and C are replicates of each other.

| Sample | $H_2O$ [µL] | *NTC* PCR product [ng - µL] | *pFLEXI* backbone [ng - µL] |
|---|---|---|---|
| WT | 17 | - | - |
| BB | 15 | - | 9.6 - 2 |
| INS | 15.7 | 89.3 - 1.3 | - |
| A | 9.5 | 89.3 - 1.3 | 29.8 - 6.2 |
| B | 9.5 | 89.3 - 1.3 | 29.8 - 6.2 |
| C | 9.5 | 89.3 - 1.3 | 29.8 - 6.2 |

For each sample, 5 µL of the reaction mixture were mixed with 50 µL of heat shock competent XL-1 blue *E. coli* cells in sterile Eppendorf tubes. For the trials A, B and C, this was performed three times per sample, yielding a total of 9 replicates. This mixture was then incubated on ice for 15 min, then at 45°C for 45 second and on ice again for 2 min. Afterwards, 1 mL of prewarmed SOC recovery media was added to each sample and the tubes were incubated at 37°C and 200 rpm for 1 h. For each trial, 250 µL of transformation mixture were plated on LB plates supplemented with 25 mg/L kanamycin.

As the results from this experiment were not satisfactory, the trials were performed again using the same steps and protocols, but different quantities and proportions of DNA fragments were added. A complete list of all samples and the quantities of DNA added to each is provided in Table 7.

Table 7: Amount of $H_2O$ and target DNA added in the samples produced for the second attempt at assembling the *pFLEXI-NTC* plasmid through T4 DNA ligation. The samples A, B and C are replicates of each other.

| Sample | $H_2O$ [µL] | *NTC* PCR product [ng - µL] | *pFLEXI* backbone [ng - µL] | *pFLEXI-NPTII* uncut [ng - µL] |
|---|---|---|---|---|
| WT | 17 | - | - | - |
| BB | 15 | - | 154 - 6 | - |
| INS | 15.7 | 29 - 2.0 | - | - |
| A | 9.5 | 29 - 2.0 | 154 - 6 | - |
| B | 9.5 | 29 - 2.0 | 154 - 6 | - |
| C | 9.5 | 29 - 2.0 | 154 - 6 | - |

Like the previous experiment, the ligation mixture was transformed into XL-1 Blue *E. coli* through heat shock. Only samples A and B were duplicated.

## 8.5 Electroporation of *C. oleaginosus* with linearised *pUC57-NTY* and *pUC57-HYC*

As the assembly of the *pFLEXI-NTC* plasmid failed, it was decided to order two plasmid constructs from GenScript®, one containing *yfp* with NTC as selection marker and the other an inducible TetON-Cre-recombinase system using hyg B as a selection marker. These two cassettes were labelled NTY for NTC + *yfp* and HYC for hyg B + TetON-Cre, including the respective promoters and terminators. Both cassettes were delivered on standard *pUC57* plasmids. Maps of the NTY and HYC cassettes can be found in Figures 9 and 10, respectively, while the full plasmid maps can be found in the Appendix.

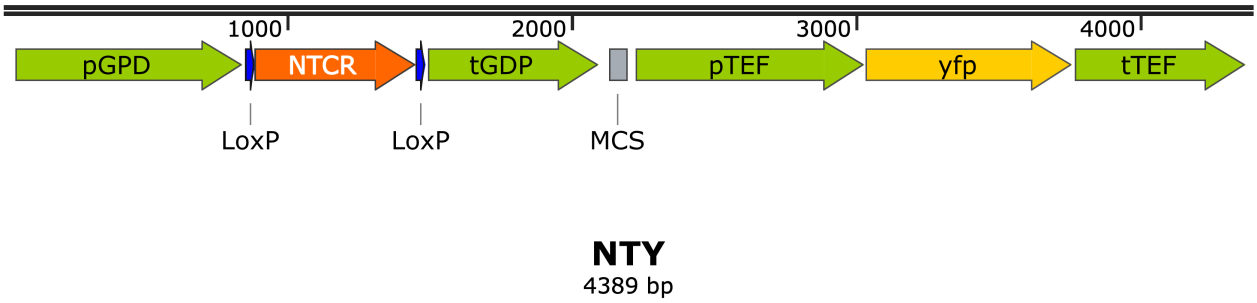


Figure 9: Map of the features present on the NTY cassette. *C. oleaginosus* native GPD and TEF promoter/terminator sequences highlited in green are used to express *yfp* and NTC selection marker (NTCR) surrounded by LoxP sites. A multiple cloning site (MCS) is also present in the centre to facilitate the insertion of other genes of interest on this cassette.

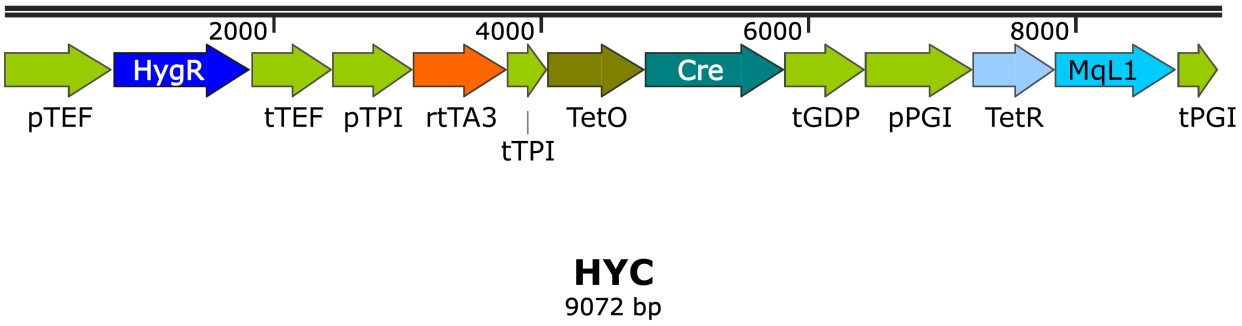


Figure 10: Map of the features present on the HYC cassette. TEF, TPI, GDP and PGI native promoter/terminator sequences from *C. oleaginsus* are used to express five genes on this cassette. The selection marker is *HygR*, which confers resistance to the antibiotic hygromycin B. Cre-recombinase (Cre) is positioned downstream of the Tet-operator TetO, which contains a truncated fragment of the GDP promoter. The modified Tet-controlled transactivator rtTA3 is coloured in orange, this fusion protein is responsible for binding to TetO and initiating trasncription in the presence of tetracycline. The TetR-MqL1 fusion protein is located at the end of the cassette and its expression controlled by the PGI promoter. This fusion protein containes the unmodified TetR protein, which is able to bind to TetO only in absence of tetracycline, and is fused to MqL1, a transcriptional corepressor. This fusion protein will then bind to the TetO site in absence of tetracycline (uninduced state) and prevent leaky expression of Cre-recombinase.

PCR of the NTY and HYC cassettes was attempted using four primer sets NTY.Fw, NTY.RV, HYC.Fw, HYC.Rv, NTY-CS.Fw, NTY-CS.RV, HYC-CS.Fw and HYC-CS.Rv. The primers in the first two sets were simply designed in order to amplify the cassettes from the respective *pUC57* plasmid, to be then be used directly for electroporation in *C. oleaginosus*. The second set on the other hand used the same primer sequences as the first, but with the addition of tails matching a *pSHUT* plasmid backbone, intended to assembled into a plasmid for *A. tumefaciens*. Nevertheless, amplification in all cases failed to produce fragments of the intended lengths, despite numerous attempts using TD-PCR, different annealing temperatures and elongation times. Because of this, it was decided to proceed with electroporation using the whole *pUC57-NTY* and *pUC57-HYC* plasmid, linearised by using the restriction enzymes BcuI and KpnI.

### 8.5.1 First transformation of NTY cassette

Transformation of linearised *pUC57-NTY* was attempted first. The plasmid was cut through restriction enzyme digestion using ThermoFisher®FastDigest BcuI following the manufacturer's instruction except for the incubation time at 37°C, which was prolonged from 5 min to 30 min. The cells were prepared similarly to the procedure described in Section 8.2 and 8.3. More specifically, a 5 mL overnight culture of *C. oleaginosus* in 2xYPAD was prepared and incubated at 30°C and 200 rpm until the OD600 reached 45. Only an amount of cells equaling to a 5 mL culture with OD600 of 11 (1.2 mL of the OD600 45 culture) were then collected by centrifugation at 5000 rpm for 5 min, washed with 5 mL of ice-cold EB buffer, resuspended in 5 mL of fresh IB buffer and incubated for 30 min at 30°C and 200 rpm. The prepared IB buffer from this trial onwards was frozen at -20°C and thawed only when needed. After the sub-incubation period, the culture was once again centrifuged at 5000 rpm for 5 min and then washed 3 times with 5 mL of $H_2O$. The cells were then resuspended in 1 mL of 10mM Tris-Cl and from this solution a 1:20 dilution was made to be electroporated. The individual samples were produced by mixing 50 µL of the diluted cell suspension and 2 µL of linearised plasmid DNA inside 0.1 cm cuvettes, which were then left on ice for 15 min before electroporation. As the whole digestion mix, together with buffer and restriction enzyme was intended to be used for electroporation, it was decided to once again test out the performance of all four previously tested electroporation buffers, namely $H_2O$, 10 mM Tris-Cl, 1 M sorbitol and EB buffer. A full list of all samples can be found in Table 8.

After electroporation, the content of the cuvettes was resuspended in 1 mL of 2xYPAD, transferred into individual Falcon tubes and incubated for 4 hours at 30°C and 200 rpm. After this, 250 µL of suspension was plated on Petri dishes supplemented with 200 ng/µL of NTC. In contrast to previous electroporation attempts, 300 ng/µL of cefoxitin was also added to the growth medium. This antibiotic

Table 8: Samples produced in the first trial of electroporation of C. oleaginosus using *pUC57-NTY* plasmid DNA linearised with BcuI

| Sample | Suspension buffer | Pulse voltage [kV] | Linear *pUC57-NTY* added [ng-µL] |
|---|---|---|---|
| W | $H_2O$ | 1.7 | 400 - 2 |
| EB | EB buffer | 1.2 | 400 - 2 |
| T | Tris-Cl | 1.3 | 400 - 2 |
| S | Sorbitol | 1.8 | 400 - 2 |
| W-WT | $H_2O$ | 1.7 | - |
| EB-WT | EB buffer | 1.3 | - |
| T-WT | Tris-Cl | 1.5 | - |
| S-WT | Sorbitol | 2.0 | - |

was used to induce a higher level of selection between transformants and non-transformants. It was observed that no apparent selection problems occurred during previous studies performed using ATMT [36, 51], where 200 ng/µL of hygromyicin B were used (found to be insufficient in this study). Cefoxitin was added to suppress the growth of *A. tumefaciens* post-transformation. It was speculated that no selection issues were observed with this method because the use of cefoxitin, although not specific to *C. oleaginosus*, is able to boost the effect of hygromycin B (and perhaps also NTC) and induce an improved level of selection between transformants and non-transformants.

Incorporation of the NTY cassette and by consequence the entire *pUC57-NTY* plasmid was verified through colony PCR using the primers DD.LoxP.NourR.Fw, DD.LoxP.NourR.Fw, NTY-Val1.Fw, NTY-Val1.Rv, NTY-Val2.Fw, NTY-Val2.Rv, pUC57-Val1.Fw, pUC57-Val1.Rv, pUC57-Val2.Fw and pUC57-Val2.Rv. After being replated on YPD media, a colony was inoculated into 300 µL of lysis buffer (recipe in Appendix 12). The suspension was vortexed, then centrifuged for 30 seconds at 13400 rpm and 1 µL of the supernatant was used as template for a single PCR reaction. Overall, the presence of five roughly evenly spaced out fragments of the *pUC57-NTY* plasmid was verified using dedicated primers (full list can be found in the Appendix).

### 8.5.2 Transformation of HYC cassette

Following the clear proof of the successful electroporation trial, three separate electroporation trials were performed using the HYC cassette on both *C. oleaginosus* wild type and a validated mutant carrying the NTY cassette. This was done in order to investigate the effect of parameters such as concentration of cells, pulse voltage, suspension buffer and concentration of cefoxitin on the overall transformation efficiency. A summary of the trials performed can be found in Table 12

For all three trials listed in Table 12, the cell preparation procedure was similar to the one used for

Table 9: Summary of the electroporation trials performed on C. oleaginosus and NTY mutants using HYC cassettes linearised with BcuI and KpnI

| Trial | Cell suspension | Pulse voltage [kV] | *C. oleaginosus* transformed | OD600 of ON culture | *pUC57-HYC* added [ng - µL] | Cefoxitin conc. [mg/L] |
|---|---|---|---|---|---|---|
| 1 | 1:20 $H_2O$ | 1.4 - 1.8 | NTY mutant | 40 | 400 - 2 (Lin. with BcuI) | 300 |
| 2 | 1:15 Tris-Cl | 1.3 | NTY mutant | 45 | 400 - 2 (Lin. with BcuI) | 175, 200, 300 |
| 3 | 1:15 Tris-Cl | 1.3 | WT | 46 | 400 - 2 (Lin. with KpnI) | 200, 300 |
| | | | NTY mutant | 9 | | |

electroporation of *C. oleaginosus* with the NTY cassette. The produced Petri dishes were incubated at 30°C and the growth of colonies was monitored from the 3rd day onwards. Mutants were validated through colony PCR by amplifying target regions on the HYC cassette using the primers Co-cPCR-Fw, Co-cPCR-Rv, HYC-Val1.Fw, HYC-Val1.Rv, HYC-Val2.Fw, HYC-Val2.Rv, HYC-Val3.Fw and HYC-Val3.Rv (sequences available in the Appendix 12).

### 8.5.3 Transformation of NTY cassette on HYC mutant

Using the same electroporation protocol described in Section 8.5.2, a validated *C. oleaginosus* mutant transformed with *pUC57-HYC* linearised with KpnI was transformed with *pUC57-NTY* linearised with BcuI, in order to create a strain carrying both the NTY and HYC cassettes and allowing for the testing of the TetON-Cre-Lox recombination system. This time the *C. oleaginosus* HYC mutant overnight culture was grown until an OD600 of ∼21 and the cell suspension post IB-buffer incubation was diluted 1:20 times before electroporation. Tris-Cl at a concentration of 10 mM was used as electroporation buffer, with 1.3 kV pulse voltage and 0.1 cm cuvettes used for all samples. Furthermore, in contrast to previous electroporation trials, in addition to a WT sample without any added plasmid DNA, an additional control sample was also produced, using a plasmid without selective markers or genes of interest utilisable by *C. oleaginosus*, complete with the FastDigest (Thermo Fisher Scientific ®) buffer used for restriction enzyme digestion. This was done in order recreate the conditions inside the cuvette for regular samples. A complete list of the samples produced is listed in Table 10.

Finally, each sample was plated on two sets of YPD media, both supplemented with 200 mg/L NTC and either 200 or 300 mg/L cefoxitin. Six colonies isolated from A, B and C samples after 3 days of growth were then restreaked on a YPD plate to create a library and subsequently validated through colony PCR. These mutants, labelled DM1 to DM6 were validated using the primers sVal.Fw and sVal.Rv for the presence of the NTC selection marker, but also using the primers HYC-Val1.Fw,

Table 10: Samples produced for the transformation of a validated C. oleaginosus HYC mutant with *pUC57-NTY* plasmid DNA linearised with BcuI.

| Sample | Control plasmid added [ng - µL] | Linear *pUC57-NTY* added [ng - µL] |
|---|---|---|
| WT | - | - |
| K | 75-2 | - |
| A | - | 200 - 2 |
| B | - | 200 - 2 |
| C | - | 200 - 2 |

HYC-Val1.Rv, HYC-Val2.Fw, HYC-Val2.Rv, HYC-Val3.Fw and HYC-Val3.Rv to verify that the HYC cassette had not been disrupted by the second round of electroporation.

## 8.6 Induction of TetON-Cre-Lox system

After verification of the integrity of both NTY and HYC cassette on mutants DM1 to DM6 (Section 9.4.3), induction of the TetON-Cre-Lox system was first attempted following the liquid media method described by Ingole et al. . For this purpose, 5 mL YPD overnight cultures of *C. oleaginosus* DM1 - DM6 mutants were prepared and supplemented with 100 mg/L of tetracycline. The cultures were then incubated at 30°C and 200 rpm overnight. The cells were then pelleted by centrifugation at 5000 rpm for 5 min and washed once with 25 mL of demineralised water. A portion of the centrifugation pellet was then transferred in 150 µL of lysis buffer using an inoculation loop, vortexed and centrifuged for 1 min at 13400 rpm. Subsequently, 1 µL of supernatant was used as template for colony PCR using the primers sVal2.Fw and sVal2.Rv. As this relatively expedited approach was inconclusive, it was decided to investigate the effect of different tetracycline concentrations, as well as the supplementation of cefoxitin. As the addition of this antibiotic proved essential for the success of electroporation and effective selection, it was hypothesised that its supplementation to tetracycline may ease the penetration of the latter into the cell and activate the Cre-Lox system. For this purpose, it was opted to create 5 mL overnight YPD cultures supplemented with 100 mg/L, 250 mg/L, 500 mg/L and 750 mg/L of tetracycline and 0 mg/L, 100 mg/L, 200 mg/L and 300 mg/L of cefoxitin. Furthermore, only the mutant DM3 was chosen for this trial as out of DM1 - DM6 it displayed the highest integrity of the HYC cassette after the second round of electroporation (as displayed in Section 9.4.3). A full list of the cultures produced, together with the labels given for future references is provided in Table 11.

Table 11: Samples produced in the second attempt to induce the TetON-Cre-Lox system, located on the HYC cassette in mutant DM3, continues to next page

| Sample | Tetracycline concentration [mg/L] | Cefoxitin concentration [mg/L] |
|---|---|---|
| A1 | 100 | - |
| A2 | 100 | 100 |
| A3 | 100 | 200 |
| A4 | 100 | 300 |
| B1 | 250 | - |
| B2 | 250 | 100 |
| B3 | 250 | 200 |
| B4 | 250 | 300 |

| | | |
|---|---|---|
| C1 | 500 | - |
| C2 | 500 | 100 |
| C3 | 500 | 200 |
| C4 | 500 | 300 |
| D1 | 750 | - |
| D2 | 750 | 100 |
| D3 | 750 | 200 |
| D4 | 750 | 300 |

The cultures were then incubated overnight at 30°C and 200 rpm. The following day an equivalent volume of 2 µL, 0.2 µL and 0.04 µL of each culture was plated on YPD and incubated overnight at 30°C in order to be able to isolate single colonies. Three colonies from each plate inoculated with 0.04 µL were then restreaked and used as template for colony PCR using the primer sets sVal2.Fw - sVal2.Rv and sVal3.Fw - sVal3.Rv.

## 8.7 Genome sequencing of DM3 and D33 mutants

As a complete proof of integration of the NTY and HYC cassettes in the genome and successful excision of the NTC selection marker after the addition of tetracycline, two mutants, labelled DM3 and D33 were chosen to be genome sequenced through Oxford Nanopore genome sequencing. As better explained in Section 9.4, DM3 was the only obtained mutant containing both the NTY cassette and a non-disrupted HYC cassette, while D33 was isolated from a DM3 sample treated with 750 mg/L of tetracycline and 200 mg/L of cefoxitin to induce expression of Cre-recombinase and exhibited the most promising behaviour during validation through colony PCR. Colonies of DM3 and D33 were inoculated in 5 mL YPD overnight cultures and incubated at 30°C and 200 rpm. The following day, these cultures were added to 50 mL of YPD in 250 mL baffled shake flasks and once again incubated overnight at 30°C and 200 rpm. The cultures were then centrifuged at 5000 rpm and 4°C for 5 min, washed once with 25 mL of $H_2O$. The pellet was resuspended in 3 mL of water, which was then equally split into 3 microcentrifuge tubes. The cells were once again pelletted by centrifugation at 13400 rpm for 1 min, the supernantant removed and the tube placed at -80°C overnight. The samples were then freeze-dried for 16-20 h by carving a hole in the side of the microcentrifuge tubes in order to allow moisture to escape. Finally, the now freeze-dried cells were used for gDNA ligation sequencing using the Native Barcoding Kit 24-V14 (SQK-NBD114.24) by Oxford Nanopore Technologies ®.

# 9 Results

In this section the results from the laboratory experiments will be presented. The same order as described in section 8 will be followed.

## 9.1 Electroporation of *C. oleaginosus*

Overall, nine different experiments were performed in order to adapt the electroporation protocol published by Koivuranta et al. [2] using the MicroPulser available at the Esbjerg Campus. For practicality, only pictures from the last trial (Table 21, details in Appendix 12) will be reported, as they are considered representative of most of the results obtained with electroporation of *C. oleaginosus* and plating on Hygromycin B. The plates can be found in Figure 11.

The first picture of Figure 11 displays four samples, each with a respective control (no *pRF-HU2-Hyg-yfp* plasmid DNA added), plated on YPD media supplemented with 200 ng/µL of hyg B. In contrast, the second picture shows samples with identical parameters but plated on YPD supplemented with 250 ng/µL of hyg B. This difference in antibiotic concentration has a significant impact on the ability of *C. oleaginosus* to survive after electroporation.

However, there is no significant difference between the main and control samples for both concentrations of antibiotics. This suggests that the electroporation protocol used may be ineffective due to factors such as insufficient voltage, inadequate plasmid vector concentration, or the inability of *C. oleaginosus* to incorporate circular vectors into its genome. Moreover, there is no noticeable difference between samples with the same antibiotic concentration and amount of added plasmid DNA. For example, there is no significant difference between samples 1, 2, 3, and 4. This indicates that differences in cell dilution and pulse voltage (ranging from 1:25 to 1:50 and 1.2 kV to 1.3 kV, respectively) do not have a significant effect on the number and appearance of colonies on the plates.

However, it is important to note that the lack of replicates makes it difficult to observe small yet existing differences across different parameters, if present at all.

As mentioned before, the behaviour of the samples of this trials can be considered quite representative for all performed trials mentioned in section 8.2. No significant difference was observed for the effect of parameters such as pulse voltage, electroporation buffer and cell dilution on the amount of colonies observed on Petri dishes after electroporation. Nevertheless, it was observed how these factors exhibited a clear correlation between each other, namely the use of a higher cell dilution and electroporation buffers such as 1 M sorbitol and $H_2O$ allowed to reach considerably higher pulse voltages compared to more concentrated cell suspensions and 10mM Tris-Cl and EB buffer as electroporation buffers. These

factors were kept into consideration later on in the project when a different transformation vector was used for electroporation of *C. oleaginosus*.

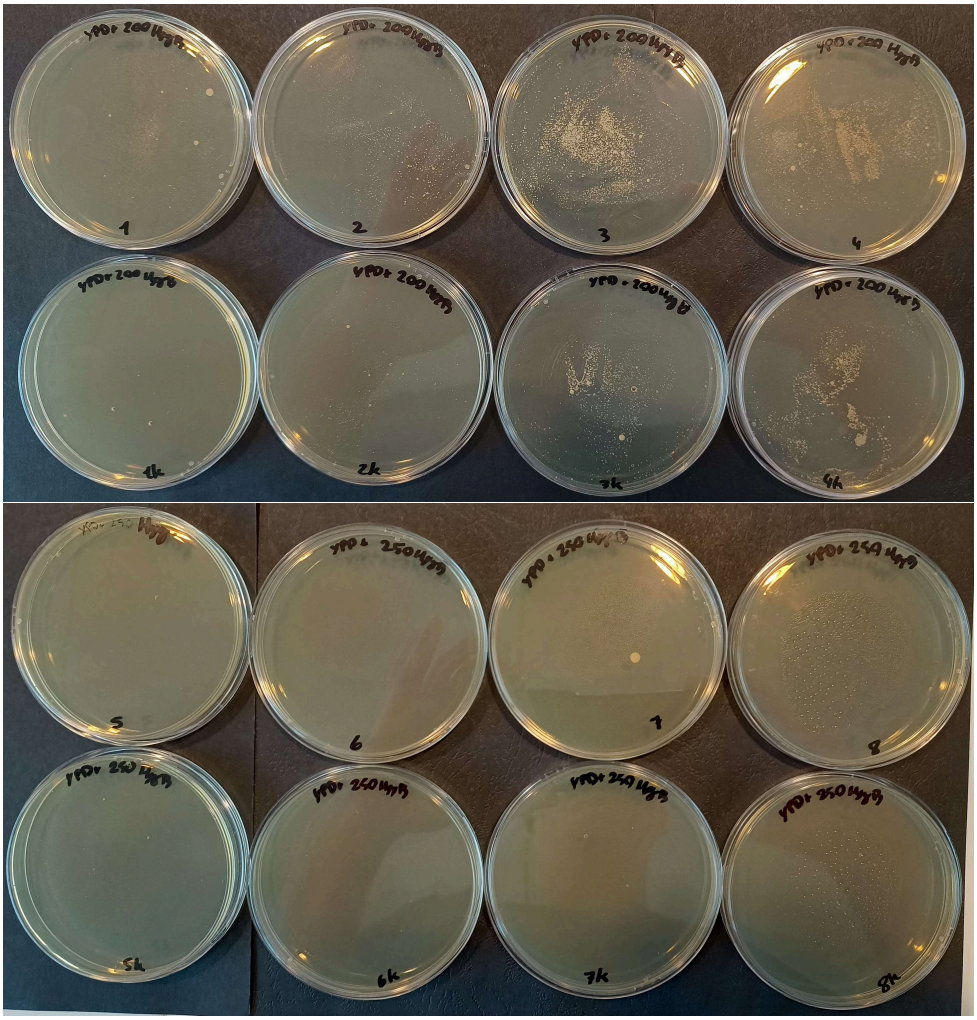

Figure 11: Results of the last trial of electroporation, more details on the individual samples and preparation is available in Table 21. Samples labelled with "k" are control samples and are therefore replicates of the respective number to which the target plasmid *pRF-HU2-Hyg-yfp* was not added. As one can see from the labels on the Petri dishes, samples in the first picture were plated with 200 ng/µL of Hyg B, while the ones on the second picture with 250 ng/µL. Other parameters varied between samples include the cell dilution (1:25 for odd-numbered samples and 1:50 for even-numbered samples) and the pulse voltage (1.2 kV for samples 1,2,5,6 and 1.3 kV for samples 3,4,7,8).

## 9.2 Antibiotic resistance testing

Throughout the first attempts at performing electroporation on *C. oleaginosus* it was noticed how the use of the antibiotic hygromycin B as selection marker did not allow for a clear separation between target and control samples. Moreover, a clear MIC could not be defined, rather the amount of antibiotic required to significantly reduce growth on Petri dishes post electroporation seemed to vary between

different trials. It was for these reasons that is was opted to simultaneously test the resistance of *C. oleaginosus* to the antibiotics hyg B, NTC, BASTA and G418, in order to assess if a clear cut-off concentration could be achieved. This particular study was performed in collaboration with a BSc student as part of a semester project [52]. The first protocol followed to prepare the cells, as described in Section 8.3 is a standard electroporation protocol using 10 mM Tris-Cl as electroporation buffer and a 1:25 dilution of the 1 mL post-sub-incubation cell suspension. Hyg B showed results very similar to the ones observed in section 9.1 due to the similar protocols, parameters and antibiotic concentration used, while BASTA and G418 failed to inhibit growth at all tested concentrations (data not shown). The only antibiotic which showed promising results was NTC, which showed a much clearer cut-off concentration than hyg B at 200 ng/µL. A comparison between cultures supplemented with hyg B and NTC as part of this experiment can be seen in Figure 12, where the eight figures show the amount of growth observed in presence of the two antibiotics at different concentrations. Both pictures were first uploaded on Bech's project report [52]. It can be noticed how at the concentration of 200 ng/µL only two colonies are present for the sample supplemented with NTC, while the sample supplemented with hyg B clearly displays a large amount of growing colonies. While a concentration of 400 ng/µL of hyg B did manage to successfully inhibit any growth of *C. oleaginosus*, it is believed that the use of such a high amount of antibiotic would ultimately become too expensive and unsustainable. In fact, such a concentration may also counter the effect of an active resistance gene of potential successful transformants, as a concentration of 200 ng/µL has been reported to be sufficient to induce selection after ATMT [36, 53].

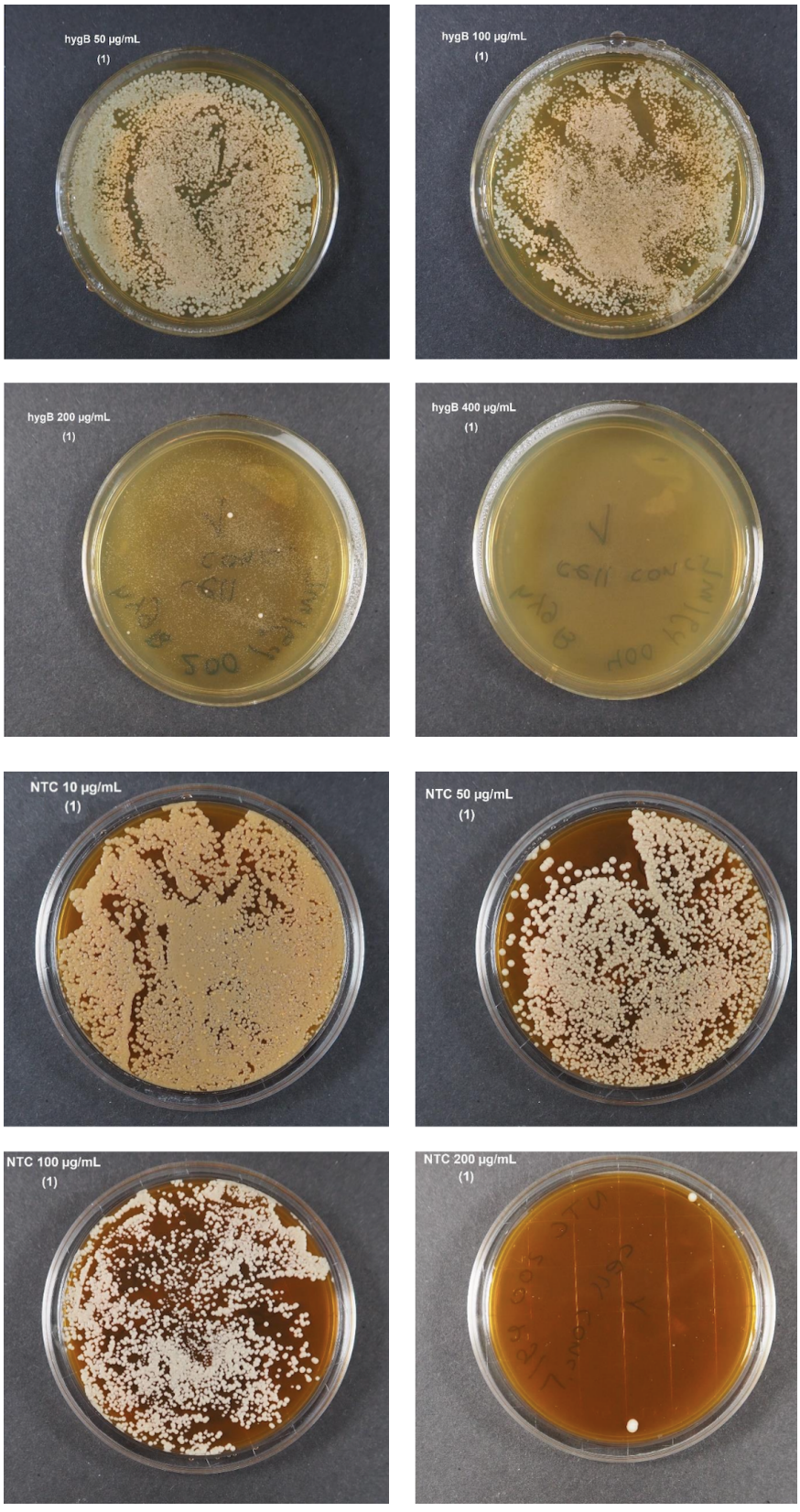


Figure 12: Comparison of the effect of the antibiotics Hyg B (above) and NTC (below) on the growth of *C. oleaginosus* after the electroporation protocol described in section 8.3 [52]

To identify a minimum inhibitory concentration (MIC) with increased accuracy for NTC, a further trial was conducted using NTC at concentrations of 73, 175, 200, and 225 mg/L. The results of this trial are presented in Figure 13. Although five replicates were performed for each concentration, only one replicate per series is shown for simplicity, as no significant differences were observed across replicates.

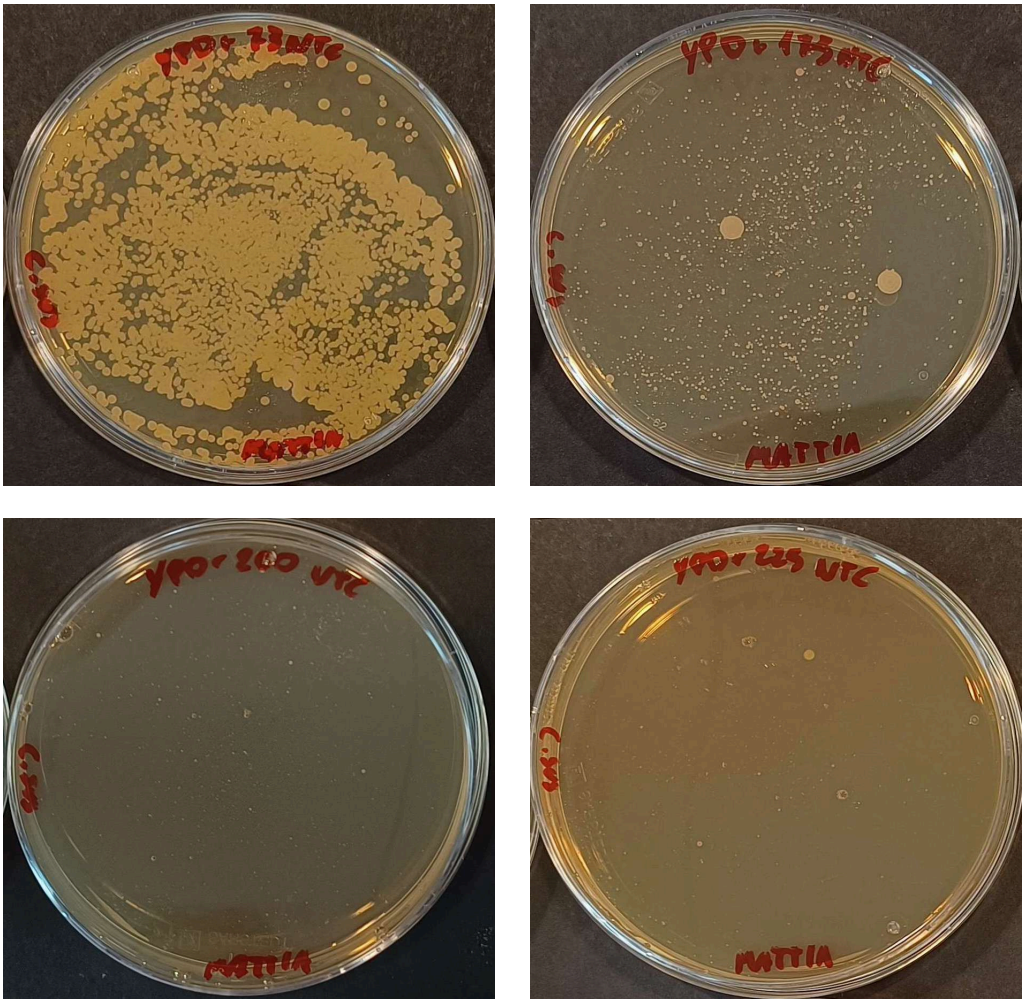

Figure 13: Samples supplemented with NTC, more specifically 73 mg/L (top-left), 175 mg/L(top-right), 200 mg/L (bottom-left) and 225 mg/L (bottom-right)

From the figures, it can be seen how a concentration of 200 ng/µL and higher, is able to nearly completely inhibit *C. oleaginosus* growth on the Petri dishes. The higher efficacy of NTC compared to hyg B made it a more desirable selection marker for the transformation cassette developed later in the project.

## 9.3 Assembly of cassette with antibiotic resistance gene

### 9.3.1 Amplification of cassette with *Hyg B* resistance gene

The first attempt at obtaining a linear transformation vector consisted in using the *pRF-HU2-Hyg-yfp* plasmid as a template in order to amplify through PCR the cassette containing the Hyg resistance gene and *yfp*, together with the respective promoters and terminators. This was done using the appositely designed primers CS.Fw and CS.Rv. The amplification of this cassette was attempted multiple times without success, changing parameters such as concentration of template, concentration of primers, annealing temperature and elongation time also failed to return the desired product. An example of such attempt can be seen in Figure 14, where different template and primers concentrations were tested, yet clearly either multiple or no bands can be observed and none at the expected length of ∼4.6 kpb.

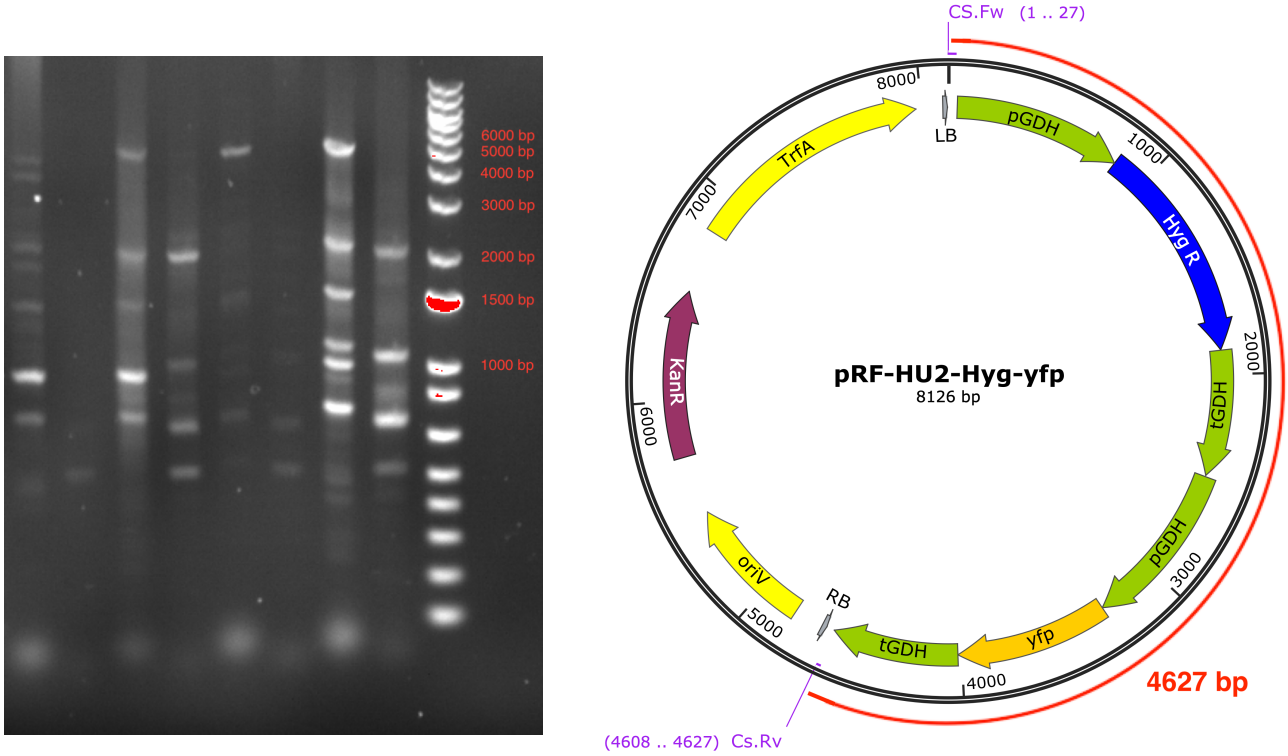


Figure 14: Attempt of amplification of the Hyg + yfp cassette from the plasmid pRF-HU2-Hyg-yfp using different concentrations of template and primers. Note how multiple bands are present in many of the samples, indicating a likely mis-binding of the primers. No bands of the expected length (4.627 kpb) is however present.

It was also attempted to extract the amplified DNA from the bands close to the expected product length of 4.6 kbp and use them as template for subsequent PCR reactions in an attempt to limit the possible binding sites of the primers. This attempt however yielded similar results to the ones shown

previously (data not shown).

### 9.3.2 Assembly of *pFLEXI-NTC* plasmid

As it was not possible to produce a linear vector from the plasmid *pRF-HU2-Hyg-yfp*, it was decided to attempt the assembly of a different plasmid using the backbone *pFLEXI* and the better performing NTC selection marker. The plasmid to be produced was named *pFLEXI-NTC* and was expected to perform better as a PCR template due to having different promoters and terminator sequences for *yfp* and the antibiotic selection marker, instead of the redundant *pGDH* and *tGDH* found in *pRF-HU2-Hyg-yfp*.

Assembly of the *pFLEXI-NTC* plasmid consisted in the ligation of two fragments. The first fragment was the NTC selection marker, which was amplified by PCR from the plasmid *pUC57-NoursR* using the primers NrsR.Fw and NrsR.Rv. The second fragment was the *pFLEXI* backbone itself, which was obtained by cleaving the selection marker out of another plasmid, *pFLEXI-NPTII*. These two fragments were then transformed into the *S. cerevisiae* strain BY4743 to assemble the *pFLEXI-NTC* plasmid through homologous recombination. This procedure was attempted twice, but no growth was observed, save for some colonies of positive control plates (data not shown). Because of this, it was decided to attempt the assembly of the plasmid through in-vitro ligation and subsequent transformation in *E. coli*. This process was attempted twice, the first attempt showed no growth whatsoever, while the second, which used different proportions of added fragments and a different *E. coli* stock, did show valid results. Pictures of the resulting samples can be found in Figure 15.

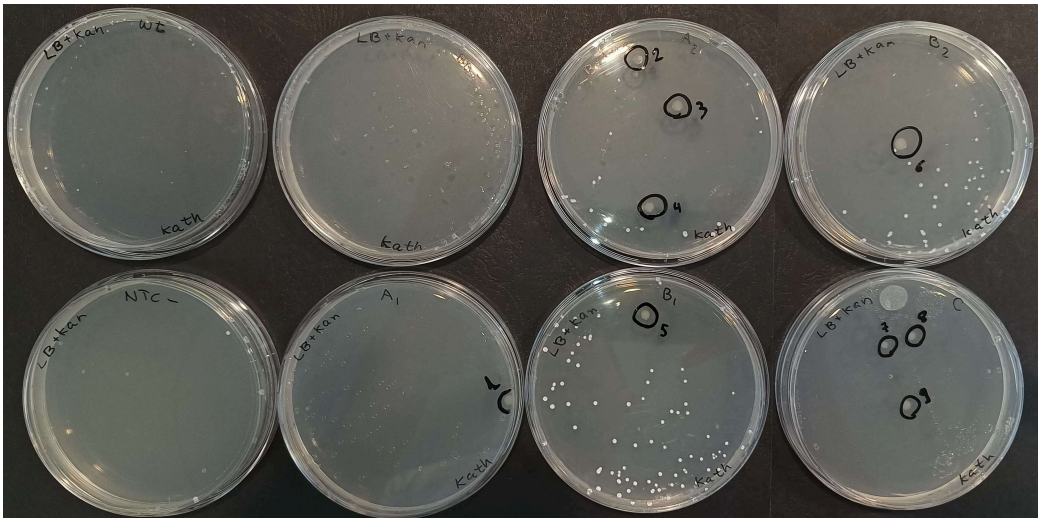


Figure 15: Pictures of the Petri dishes resulting from the second attempt at assembling the *pFLEXI-NTC* plasmid through ligation and subsequent transformation in *E. coli*. Note that the actual colonies are circled and numbered, while the other white dots seen on most plates are just air bubbles which formed during incubation.

In total, nine mutants could be isolated, which were cultivated overnight. The assembled plasmid was extracted and validated through restriction enzyme digestion with BglI. The resulting gel electrophoresis for constructs mutants 1, 2, 5 and 8 can be seen in Figure 16.

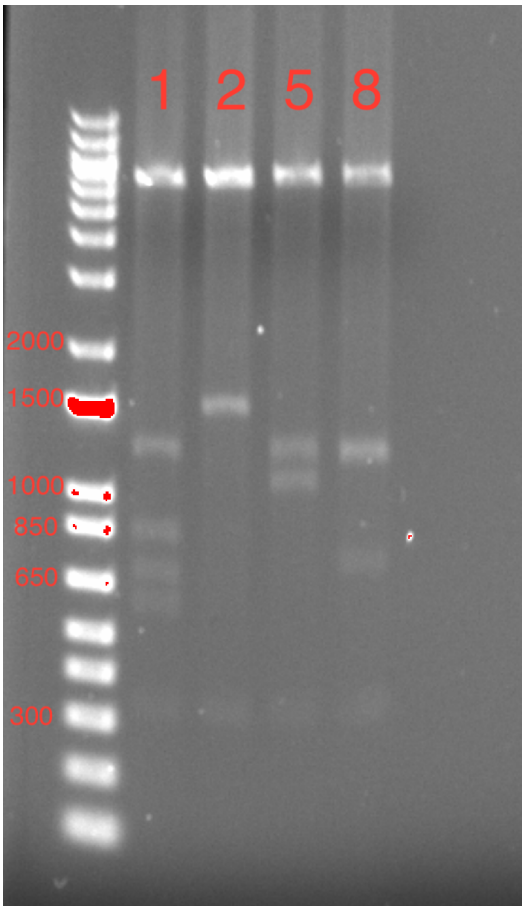


Figure 16: Validation of constructs 1, 2, 5 and 8 though digestion with BglI and run though gel electrophoresis. The expected bands for the correctly assembled plasmids are at 321, 718, 1181, 1158 and 6273 bp

A correctly assembled copy of *pFLEXI-NTC* digested with BglI is supposed to give bands at 321, 718, 1181, 1158 and 6273 bp. Despite the bands being very faded, it is possible to see that the bands at 6273 and 321 can be found for all tested constructs, while the ones at 718,1181 and 1158 cannot be seen for any. Instead, other bands are present, spanning from 650 bp all the way to 1500 bp in length. These were not deemed the possible result of an incomplete digestion, as no combination of the predicted fragments that results in the obtained extra bands exists. They could however be the result of contamination and to test this, a PCR validation was performed on all constructs using the primers pVAL.Fw and pVAL.Rv. Which bind on the *pFLEXI* backbone and on the terminator for NTC respectively, amplifying the NTC selection marker and relative *pGPD* promoter whole. The expected product length for this reaction is ∼2800 bp. The result of this amplification can be seen in Figure 17.

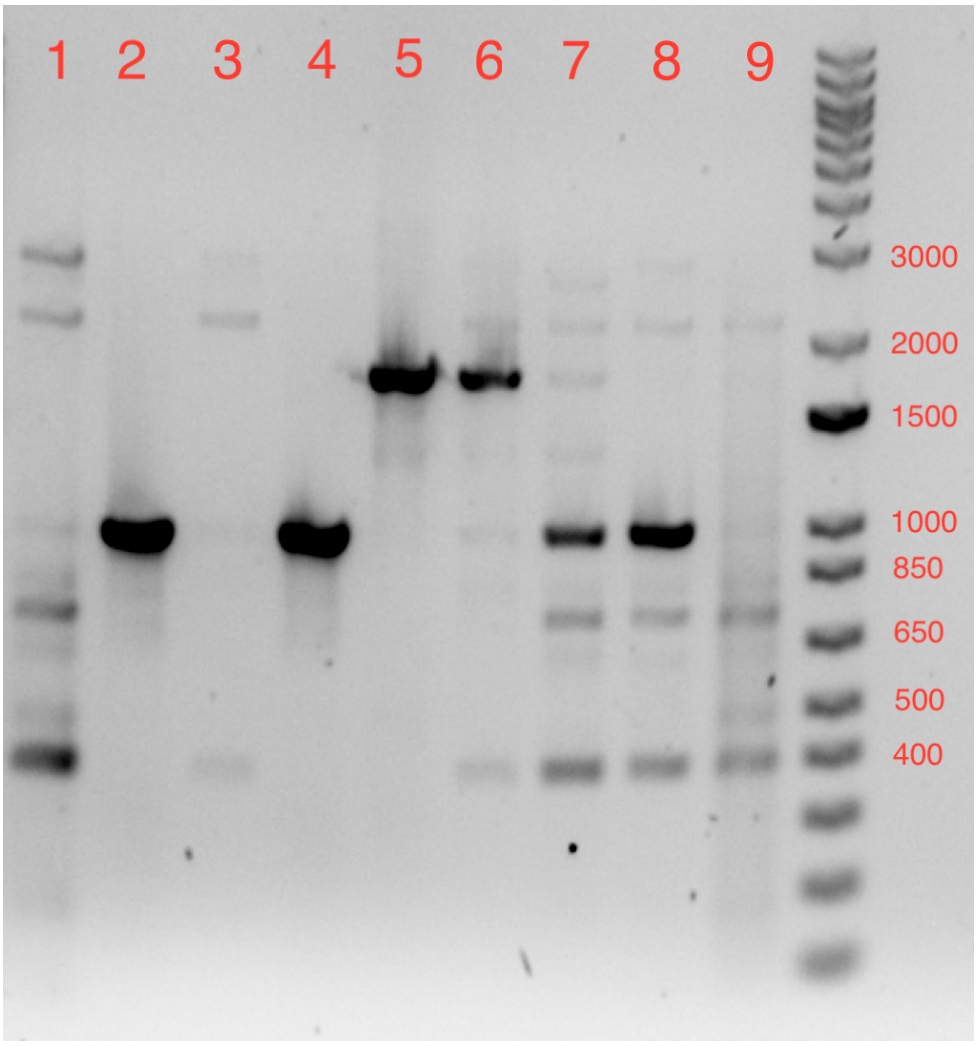


Figure 17: Validation of constructs 1 to 9 through PCR with *pVAL.Fw* and *pVAL.Rv* primers and run though gel electrophoresis. The expected band for the correctly assembled plasmid is ∼2800 bp

It can be clearly seen how different bands are present for most constructs, while a band close to the expected length of ∼2800 bp is only present in constructs 1, 3 and 8, although very faded, making it difficult to estimate its precise position. The more defined bands are present at either ∼1000 bp in the case of constructs 2, 3, 7, 9 or ∼1800 bp in the case of constructs 5 and 6. These are both far from the expected band, as well from the band hypothesised to originate in case the pFLEXI backbone happened to close back on itself (∼2000bp), despite having non-matching sticky ends.

The fact that the obtained bands could not be explained, led to the hypothesis that the starting *pFLEXI-NPTII* plasmid had not been assembled correctly. To verify this, four *pFLEXI-NPTII* constructs, of which construct 1 had been used to attempt assembly in both *S. cerevisiae* and in vitro through ligation, were digested with BglI and run though agarose gel. In addition, construct 1 of *pFLEXI::NPTII* was used as a template for PCR amplification using the same *pVAL.Fw* and *pVAL.Rv*

primers. The results of this validation can be seen in Figures 18 and 19.

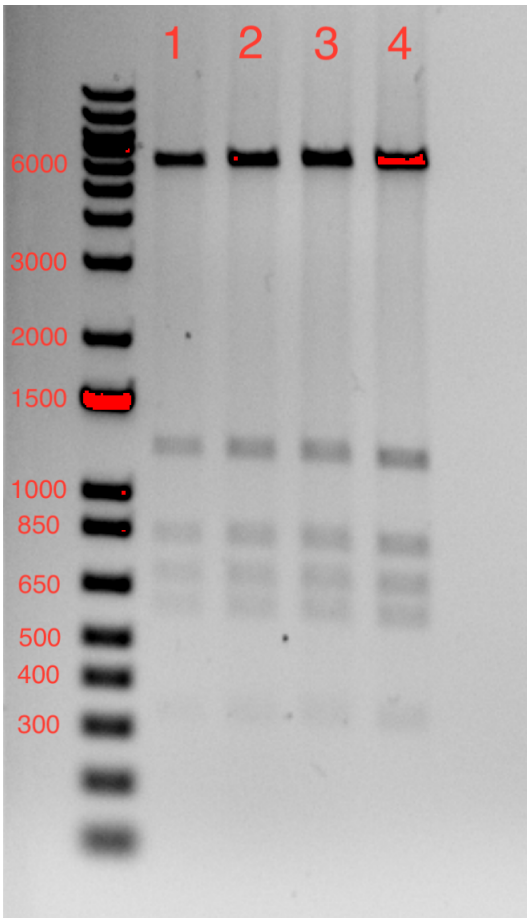


Figure 18: Digestion of four *pFLEXI-NPTII* constructs with BglI

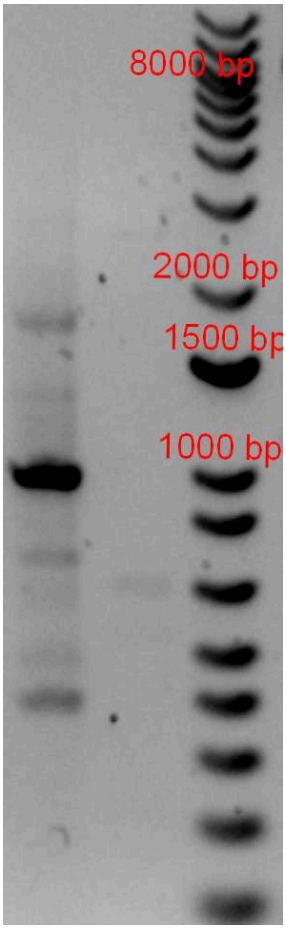


Figure 19: PCR using primers pVAL.Fw and pVAL.Rv and using *pFLEXI-NPTII* as template

Digestion of the *pFLEXI-NPTII* construct is supposed to yield bands at 6273, 1181, 811, 676, 576 and 321 bp, which match with the obtained lengths for the four constructs tested and shown in Figure 18. PCR validation with pVAL.Fw and pVAL.Rv is however supposed to yield a product once again at ∼2.8 kbp, while the main band observed in Figure 19 is ∼1000 bp long. This is consistent with the band observed in the validation of the assembled *pFLEXI-NTC* construct 2, 4, 7 and 8, shown in Figure 16. Such an outcome suggests that the *NPTII* was indeed extracted and substituted with the *NTC* selection marker, but the promoter and terminator sequences close to it might have been somehow compromised. Multiple attempts were then made at amplifying a cassette from the *pFLEXI-NTC* 1 to 9 using the primers NTCCS.Fw and NTCCS.Rv, which failed to give a product of the expected length (4200 bp), instead returning either bands at ∼650 bp, ∼2200 or no bands at all. The results of one of these PCR reactions can be seen in Figure 20.

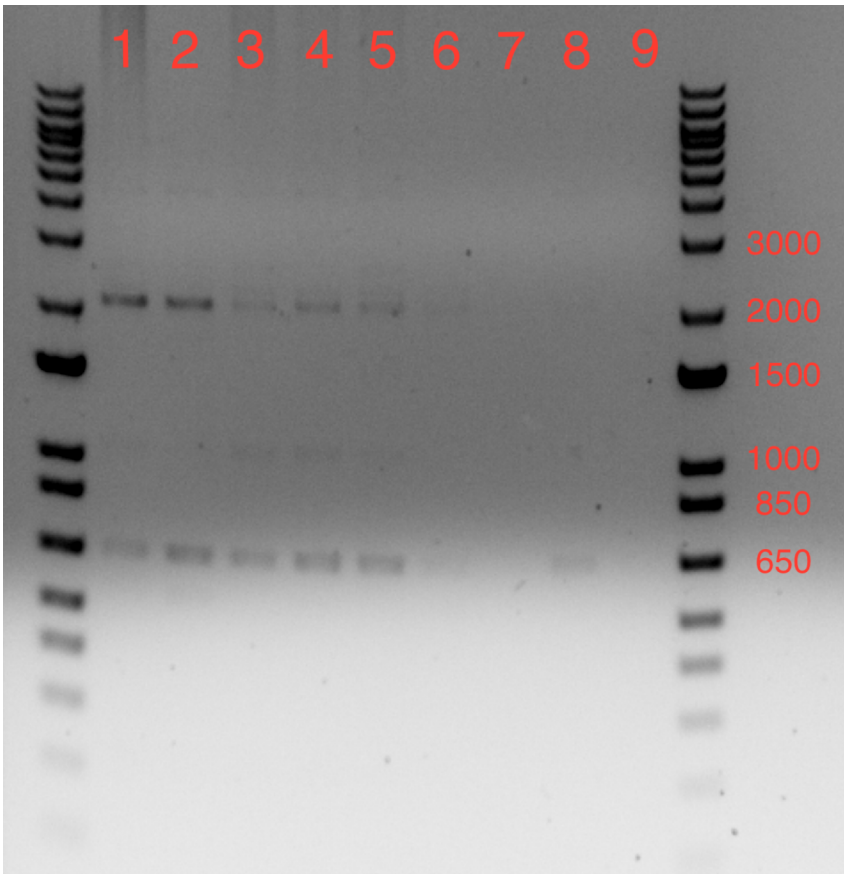


Figure 20: Validation of constructs 1 to 9 through PCR with NTCCS.Fw and NTCCS.Rv primers and run though gel electrophoresis. The expected band for the correctly assembled plasmid is ~4200 bp

These PCR validations led to the assumption that the obtained constructs were unsuitable to be used as PCR templates for electroporation linear vectors, most likely due to a prior mistake during the assembly of the *pFLEXI-NPTII* plasmid. Because of this, it was decided to order a cassette containing the NTC resistance gene and *yfp* already inserted on a *pUC57* vector, together with an inducible TetON system for Cre-recombinase using Hyg as a selection marker, both on a separate *pUC57* plasmid.

## 9.4 Electroporation of *C. oleaginosus* with linearised *pUC57-NTY* and *pUC57-HYC*

As the assembly of a plasmid carrying the NTC resistance gene could not be carried out, two *pUC57* plasmid constructs were ordered from GenScript®, already containing the already assembled NTY and HYC cassettes. As mentioned in section 8.5, the NTY cassette carried an NTC selection marker surrounded by LoxP stes and *yfp*, while the HYC cassette contained a hyg B selection marker and the tetracycline-induced expression system for Cre-recombinase, which included Cre-recombinase itself, the repressor fusion protein TetR-MqL1 and the transactivator rtTA3.

PCR amplification of these cassettes was attempted multiple times using primer sets both with and without tails and different annealing temperatures. These attempt always returned the results shown in Figure 21, where a clear band is obtained for both cassettes, just far from the expected lengths of 4369 bp for NTY and 9189 bp for HYC.

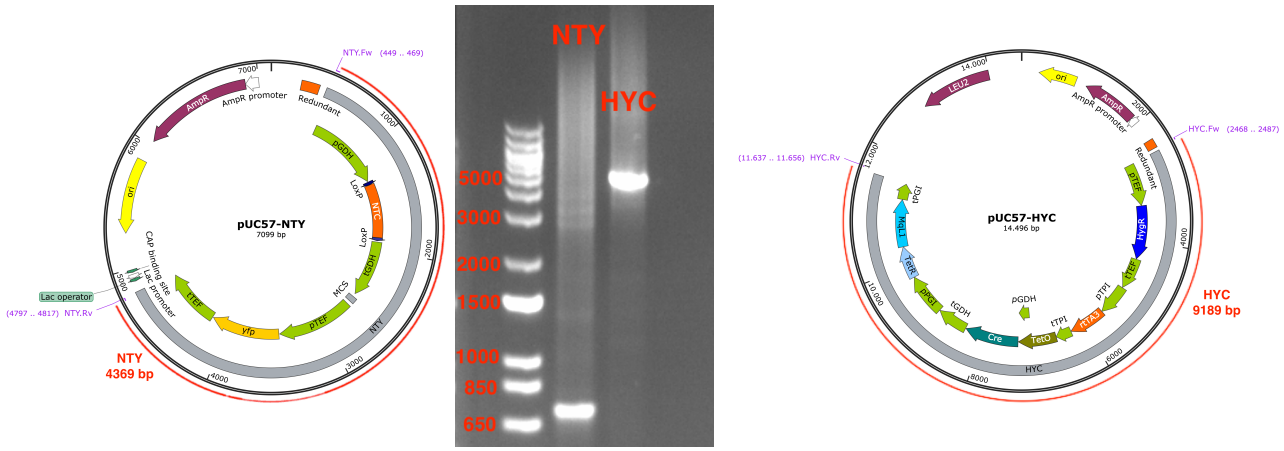


Figure 21: Attempt at PCR amplification of NTY and HYC cassette, in this instance using the tail-less primer sets NTY.Fw-NTY.Rv and HYC.Fw-HYC.Rv. It can be seen how clear and relatively distinct bands are observed for both reactions, but the observed length is far from the expected one for both fragments (∼700 bp vs ∼4369 bp for NTY and ∼5000 bp vs ∼9189 bp for HYC)

### 9.4.1 First transformation of NTY cassette

As PCR amplification of the NTY and HYC cassette failed, it was decided to proceed with electroporation of *C. oleaginosus* anyway, using the whole *pUC57-NTY* and *pUC57-HYC* plasmids linearised with BcuI as transformation vectors. The first plasmid to be transformed was *pUC57-NTY*, the Petri dishes resulting from this trial are shown in Figure 22.

First, it is possible to notice the efficacy of the combination of cefoxitin and NTC at suppressing the growth of *C. oleaginosus* compared to media only supplemented with NTC as shown in Section 9.2. Secondly, a total of 5 mutants labelled A to E could be isolated and were replated on a separate YPD plate. It can be seen how the only control sample which showed any growth whatsoever was the one with sorbitol (S-WT), where 2 colonies can be seen. This can however be explained by the lack of any DNA being added to the cell suspension during electroporation, which can alter the conductivity of the sample compared to its counterpart containing the linearised plasmid vectors. Nevertheless, mutants A to E were validated through colony PCR of the NTC resistance gene (data not shown) and later on of four roughly-equally-spaced fragments on the *pUC57-NTY* plasmid. This second validation was performed in order to verify whether the whole linearised plasmid had been integrated or only the fragment able to confer resistance to the antibiotic. The results of this validation are shown only for

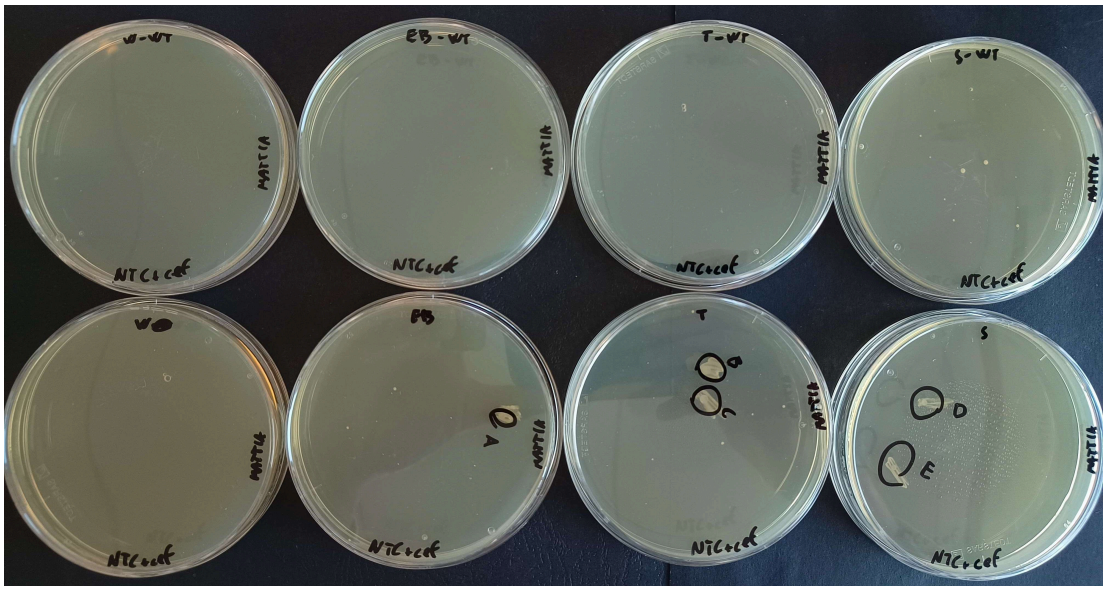

Figure 22: Samples produced during electroporation of *C. oleaginosus* with linearised *pUC57-NTY* plasmid DNA. The colonies circled and labelled A to E were replated on a YPD plate. This is also the reason of the unusual elongated shape, as the Petri dishes in the figure were further incubated for a day days before the picture was taken

mutant C for conciseness and can be found in Figure 23 and 24.

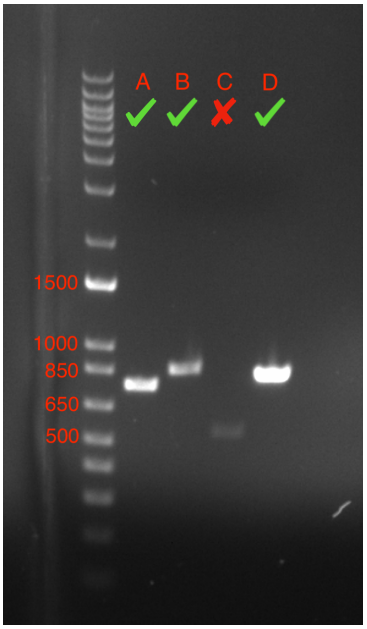


Figure 23: PCR amplification of fragments A, B, C and D from genomic DNA of *C. oleaginosus* mutant C transformed with linearised *pUC57-NTY*

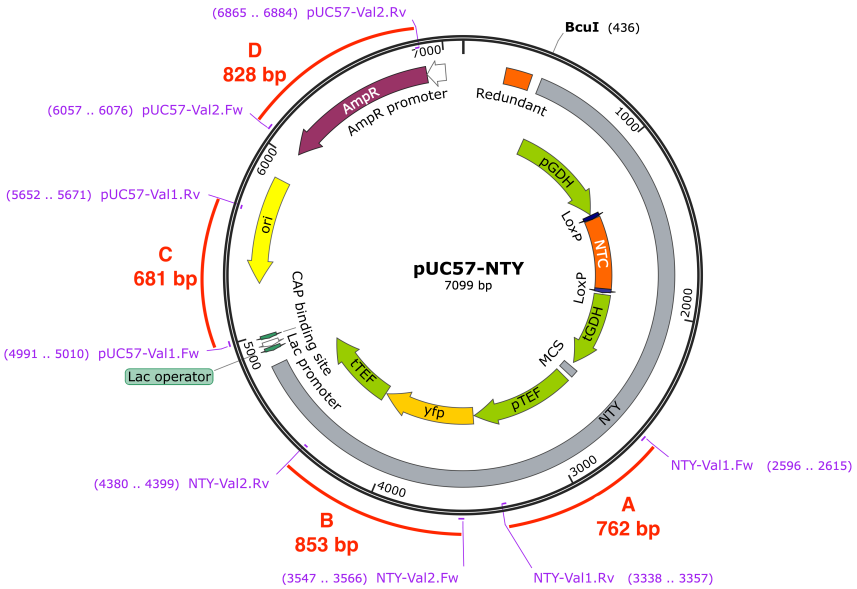


Figure 24: Map of the plasmid *pUC57-NTY* showing the location of the fragments A, B, C and D amplified though colony PCR and the BcuI restriction site used to linearise the vector

The expected lengths of the individual fragments is 762 bp for A, 853 bp for B, 681 bp for C and

828 bp for D. While A, B and C are present at the correct length and show a clear, distinct band on the agarose gel in Figure 23, fragment C does not. A more faded band is instead present for this fragment at $\sim$ 550 bp. This suggests a partial recombination of the linearised plasmid, where the extremities, the NTC selection marker and *yfp* recombined correctly, but the more central location of the vector did not. It is also possible that the primer design is not suitable to the amplification of the fragment C, leading to an incomplete product of a shorter length.

### 9.4.2 Transformation of HYC cassette

As described in Section 8.5.2, three separate trials were performed in order to better test out the efficiency of the newly developed electroporation protocol and potentially increase the number of transformants. For concision, the general results, as well as the total number of mutants isolated and validated are summarised in Table 12.

Table 12: Results of the three trials performed on C. oleaginosus WT and validated NTY mutants. Several mutants were isolated and validated through colony PCR of a fragment on the HYC cassette using the primers HYC-Val3.Fw and HYC-Val3.Rv. Note that the amount of mutants isolated from the individual combination of parameters is not a direct indication of the efficiency of the transformation protocol, as the number shown is the total number of colonies that could be isolated across a variable amount of replicates. Furthermore, in instances where numerous colonies grew close the each other and did not allow to properly isolate single mutants validation was not attempted. Therefore, a better indication of transformation efficiency can be the comparison between total colonies that could be isolated and the ones that could be validated.

| Trial | Cell suspension | C. oleaginosus transformed | OD600 of ON culture | Cefoxitin conc. [mg/L] | Mutants isolated - of which validated | |
|---|---|---|---|---|---|---|
| | | | | | After 3 days | After 6+ days |
| 1 | 1:20 $H_2O$ | NTY mutant | 40 | 300 | 5 - 5 | No growth |
| 2 | 1:15 Tris-Cl | NTY mutant | 45 | 175 | 8 - 6 | No growth |
| | | | | 200 | 6 - 5 | 8 - 7 |
| | | | | 300 | No growth | |
| 3 | 1:15 Tris-Cl | WT | 46 | 200 | 1 - 1 | 4 - 4 |
| | | | | 300 | - | 4 - 4 |
| | | NTY mutant | 9 | 200 | No growth | |
| | | | | 300 | | |

From the data displayed in the table, it can be noticed how the number of colonies that can be isolated post-transformation is still relatively low, but selection between transformants and non-transformants using the a combination of hygromycin B and cefoxitin can be achieved quite reliably, with a higher concentration of cefoxitin enforcing stricter selection, but also resulting in a much lower number of overall colonies growing on the Petri Dishes. This effect can be noticed in Figure 28, where the series of samples plated on 175 mg/L cefoxitin (Figure 25) displays on average more than triple the

amount of colonies compared to samples plated on 200 mg/L cefoxitin (Figure 26). The complete lack of colonies on the plates using 300 mg/L of cefoxitin in this trial is however most likely due to a human mistake during electroporation. It should also be noted that despite the control WT plates exhibiting a significant amount of growth in both Figure 25 and 26, compared to the target plates, most of the colonies isolated from the target plates were validated through colony PCR. This suggests that creating control samples solely by not inoculating the target DNA does not produce a sample similar enough to the targets. Therefore, it is not possible to accurately estimate the efficiency of selection through the combination of antibiotics using such control samples.

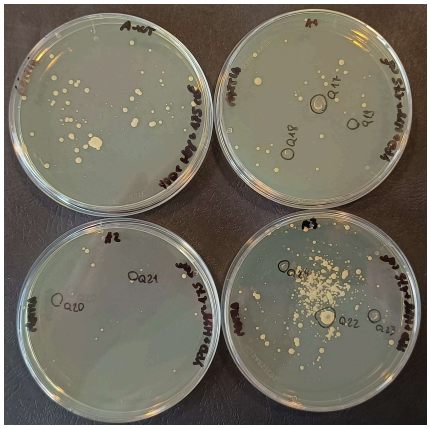

Figure 25: 175 mg/L cefoxitin

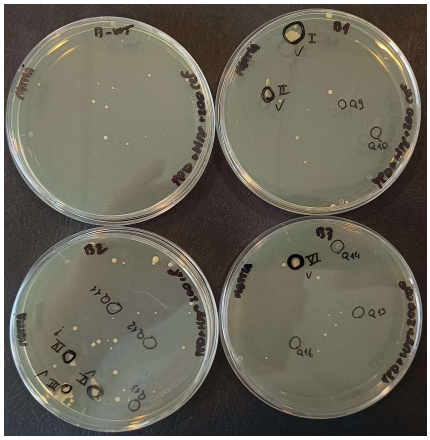

Figure 26: 200 mg/L cefoxitin

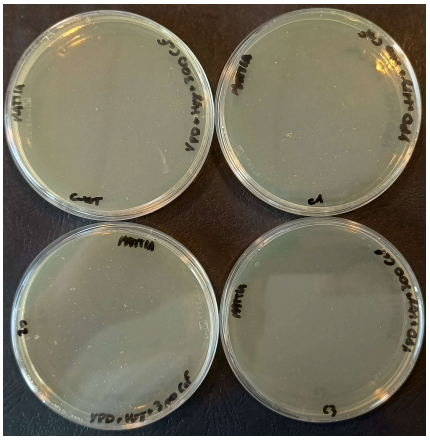

Figure 27: 300 mg/L cefoxitin

Figure 28: Petri Dishes obtained from trial 2 of transformation of NTY mutants with HYC cassette. The three figures represent the obtained samples for the three series using 175, 200 and 300 mg/L cefoxitin for selection of transformants (from left to right, respectively). Note the progressive decrease of number of colonies present with increasing antibiotic concentration.

Validation of the obtained mutants was performed through the amplification of four fragments on the HYC cassette by colony PCR. The location of these fragments, named A to D, is shown in Figure 30. The expected lengths are 579, 866, 1778 and 1563 bp, respectively.

Figure 34 displays the validation results of a single mutant that was transformed with the *pUC57-HYC* plasmid that was linearised with KpnI. The figure illustrates that the expected lengths of fragments A, B, and D, which are 579 bp, 866 bp, and 1563 bp respectively (Figure 30), have been amplified and are present. Specifically, the mutant that was tested in the third trial, as shown in Table 12, was utilized in conjunction with WT *C. oleaginosus*, 200 mg/L cefoxitin, and was isolated after 3 days. This confirms that the majority of the HYC cassette has been incorporated into the *C. oleaginosus* genomic DNA. While fragment C could not be amplified, other validated mutants transformed with KpnI linearised *pUC57-HYC* also failed to display this fragment. However, fragments A, B, and D could reliably be observed. This observation suggests that the repeating sequences in the TetO promoter,

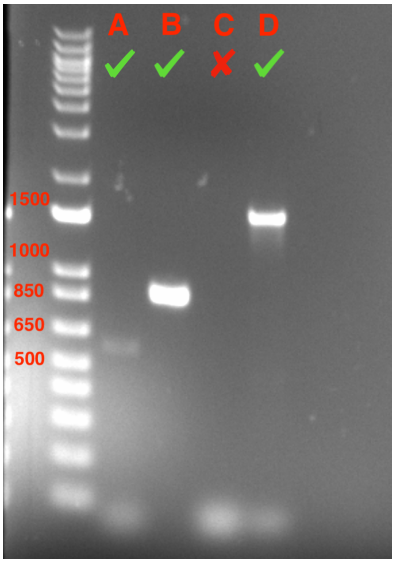


Figure 29: Fragments A, B, C and D obtained though colony PCR of a HYC mutant transformed with KpnI linearised *pUC57-HYC* plasmid DNA

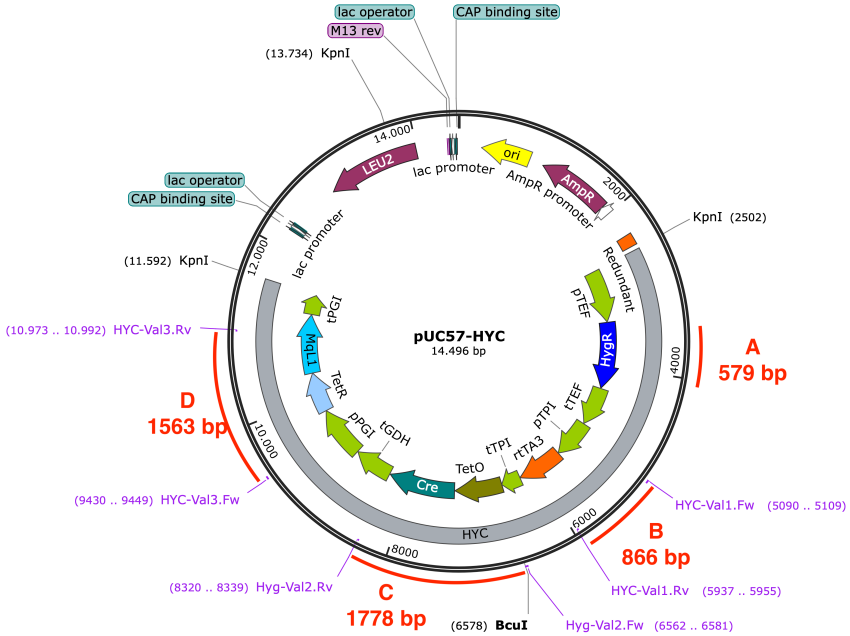


Figure 30: Map of the *pUC57-HYC* plasmid highlighting the location of the BcuI and KpnI restriction sites used for linearisation, as well as the fragments A, B, C and D amplified through colony PCR

which are located just downstream of the HYC-Val2.Fw primer binding site, may inhibit amplification of the fragment as a whole. Because of this, amplification of B or D fragments was used to validate other mutants obtained from the other three electroporation trials performed with the HYC cassette, where the presence of the amplified fragment at the correct location was considered sufficient proof of the incorporation of the cassette.

### 9.4.3 Transformation of NTY cassette on HYC mutant

The *C. oleaginosus* HYC mutant validated in Figure 34 was subsequently transformed with *pUC57-NTY* plasmid linearised with BcuI, in order to introduce the NTY cassette and be able to later test out the TetON-Cre-Lox recombination system. In total, five samples were generated, including two control samples (WT and K) and three target samples (A, B, and C). These samples were then plated on two sets of YPD plates that had been supplemented with 200 mg/L of NTC and either 200 or 300 mg/L of cefoxitin.

The control sample WT was prepared without any DNA added before electroporation, whereas K utilized a control plasmid that lacked any selection markers and was prepared using the same FastDigest®buffer used for restriction enzyme digestion of the actual target samples. This was done to create a control sample that could better replicate the conductivity of the target samples.

The target samples A, B, and C were replicates of each other, and were supplemented with *pUC57-NTY* plasmid DNA that had been digested with BcuI. The resulting plates can be seen in Figures 31 and 32, with the first being the set with 200 mg/L of cefoxitin and the latter the one with 300 mg/L.

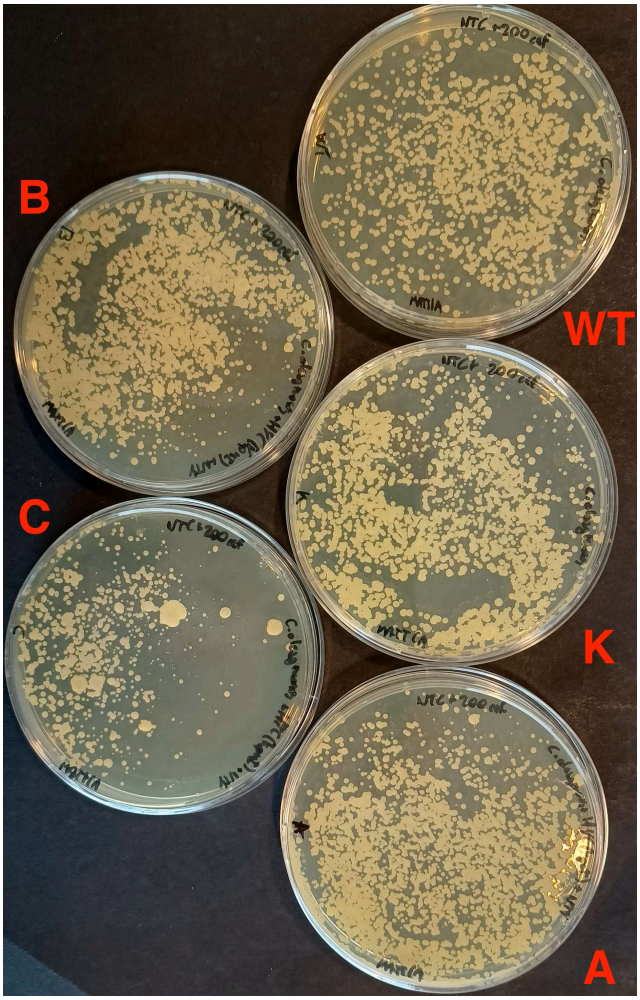


Figure 31: Plates obtained from transformation of *C. oleaginosus* HYC mutants with NTY cassette, 200 mg/L NTC + 200 mg/L cefoxitin

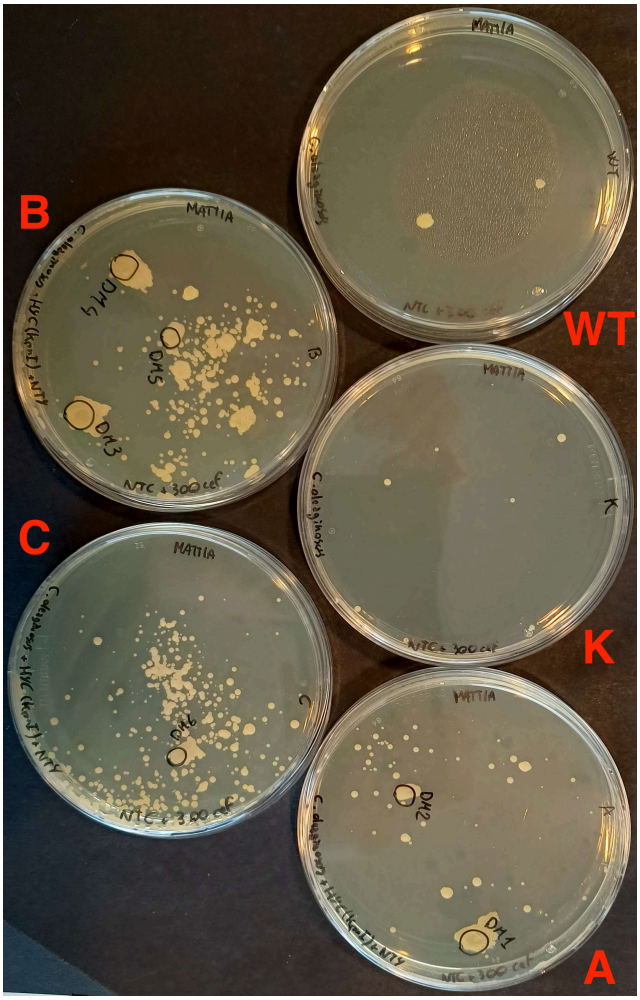


Figure 32: Plates obtained from transformation of *C. oleaginosus* HYC mutants with NTY cassette, 200 mg/L NTG + 300 mg/L cefoxitin

It is overall clearly visible how impactful the difference in concentration of cefoxitin is on the total number of colonies on both target and control plates. In fact, while barely any growth is present on both WT and K samples at 300 mg/L (Figure 32), a much larger amount of colonies is present on their counterparts plated on 200 mg/L cefoxitin (Figure 31), which is much more comparable with the number of colonies present of the target A, B and C samples at the same antibiotic concentration. This suggests how in this particular case the use of cefoxitin at 200 mg/L, in combination with 200 mg/L of NTC is not able to provide a sufficient level of selection between transformants and non transformants, which is instead possible when plating on 300 mg/L cefoxitin + 200 mg/L NTC. These results initially

appear to in contrast with what observed in Section 9.2, where a concentration of 200 mg/L of NTC without any cefoxitin supplementation was sufficient at almost completely suppressing growth of *C. oleaginosus* colonies after an almost identical cell preparation and electroporation protocol, with the main difference between the two sets of experiments being the OD600 of the overnight *C. oleaginosus* culture.

Nevertheless, six mutants, labelled DM1 to DM6, were isolated from the plates supplemented with 300 mg/L cefoxitin and the presence of the NTY cassette was verified through colony PCR, while the colonies on grown on 200 mg/L cefoxitin were discarded.

Colony PCR performed on DM1-DM6 mutants was carried out using the primer pairs sVal2.Fw-sVal2.Rv and sVal3.Fw-sVal3.Rv. The former was designed to amplify a 2138 bp fragment on the NTY cassette, comprising of the NTC selection marker and the relative pGDH promoter and tGDH terminator, while the latter pair instead amplified a 534 bp fragment within the NTC selection marker. Primers sVal2.Fw and sVal2.Rv failed to reliably amplify the intended 2138 bp sequence even on control samples and using the plasmid *pUC57-NTY* as template, therefore only sVal3.Fw and sVal3.Rv could be used to carry out the validation. The obtained fragment are displayed in Figure 33, where other control samples were also produced, namely "e" contained no added template DNA, WT used wild-type *C. oleaginosus* and M3 was a NTY mutant produced in Section 8.5.1, used as positive control.

It can be clearly seen how a band at the expected length is present for all samples, including "WT" and even "e", clearly suggesting a contamination. Further investigation suggested such contamination originated from chromosomal DNA of a produced *C. oleaginosus* DM mutant, which then made its way into the primer suspensions used for validation. Nevertheless, it can be observed how the negative control samples (e and WT) display a band of, albeit clear, much lower intensity compared to the positive control M3 and the first 5 DM mutants. This suggests how the contaminating DNA is present in small enough amounts compared to the intended template DNA, allowing to distinguish positive from negative amplification outcomes based on the intensity of the bands. The difference in intensity could be maximised by increasing the number of cycles during PCR from 34 to 36 and collecting a larger than necessary amount of cells from Petri dishes to be used for colony PCR. While not ideal, the bands obtained in Figure 33 were considered sufficient proof of successful integration of the NTY cassette in DM1 to DM5, while DM6 was considered not validated. In addition to validating the NTY cassette, it was decided to once again validate the integrity of the HYC cassette, in order to ensure no recombination events took place which resulted in the overlapping of the two cassettes. For this purpose, fragments B, C and D, which had already been used as proof of integration in Section 9.4.2, were once again amplified through colony PCR. The resulting fragments can be seen in Figure 34.

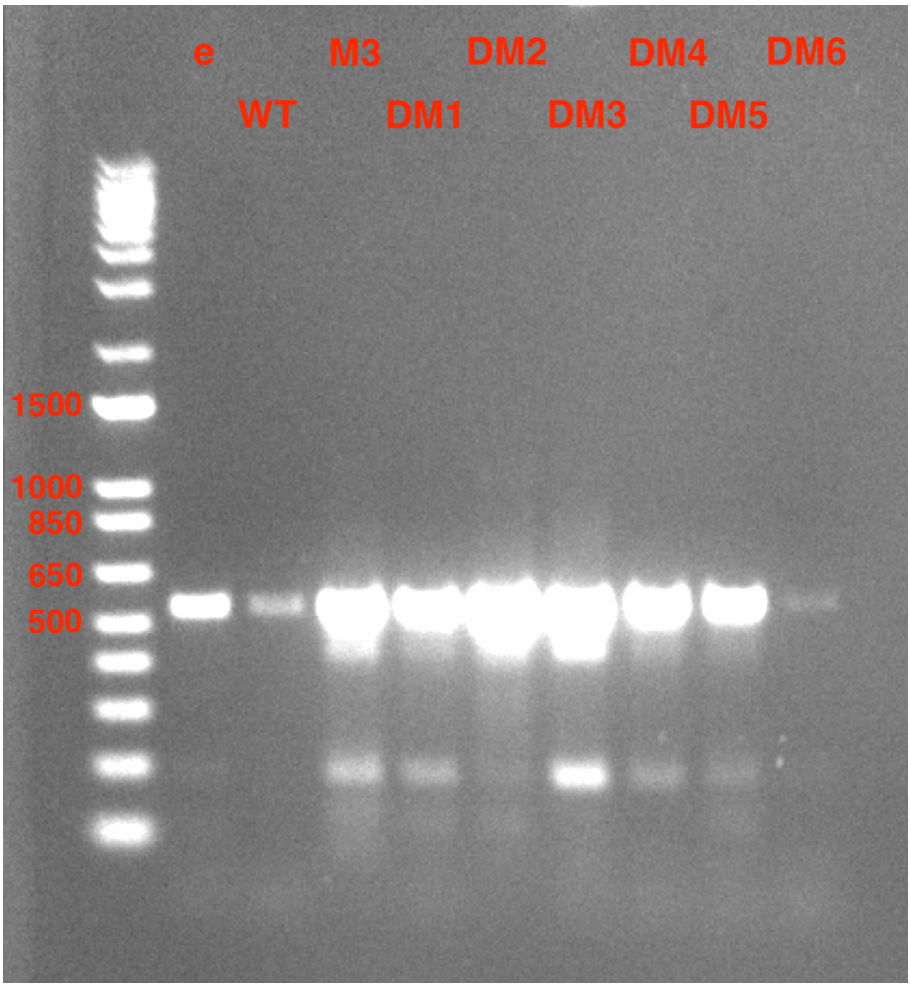


Figure 33: Colony PCR of a 534 bp fragment in the NTC selection marker on DM1 to DM6 mutants, sample "e" containing no template, wild-type "WT" and "M3" positive control

For each fragment series, two control samples were also produced in the form of WT (wild-type *C. oleaginosus*) and p (*pUC57-HYC* plasmid DNA).

It can be seen how for fragment B a band at the expected length of 866 bp is present for all samples, including the control WT sample, most likely due to the same contamination observed in Figure 33. While the amplification of this fragment appears inconclusive, for fragments C and D, on the other hand, only DM3 and the control p sample appear to give the expected bands at a considerably higher intensity compared to the contaminated WT sample and other mutants. Because of this, it was decided to pick mutant DM3 to test out the TetON-Cre-Lox selection marker recycling system.

### 9.5 TetON-Cre-Lox system induction

As previously mentioned in Section 5, 16 samples of mutant DM3 were produced and incubated with different concentrations of tetracycline and cefoxitin and after plating on Petri dishes, 3 colonies from

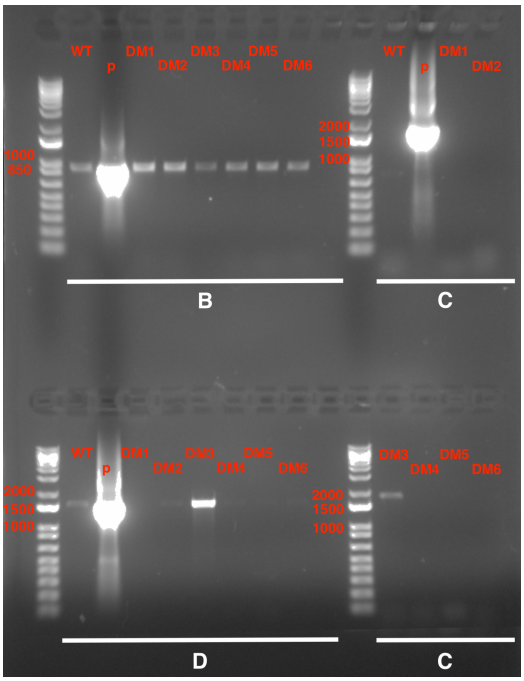


Figure 34: Fragments B, C and D obtained through colony PCR of mutants DM1 to DM6, as well as wild-type *C. oleaginosus* (WT) and *pUC57-HYC* plasmid DNA (p)

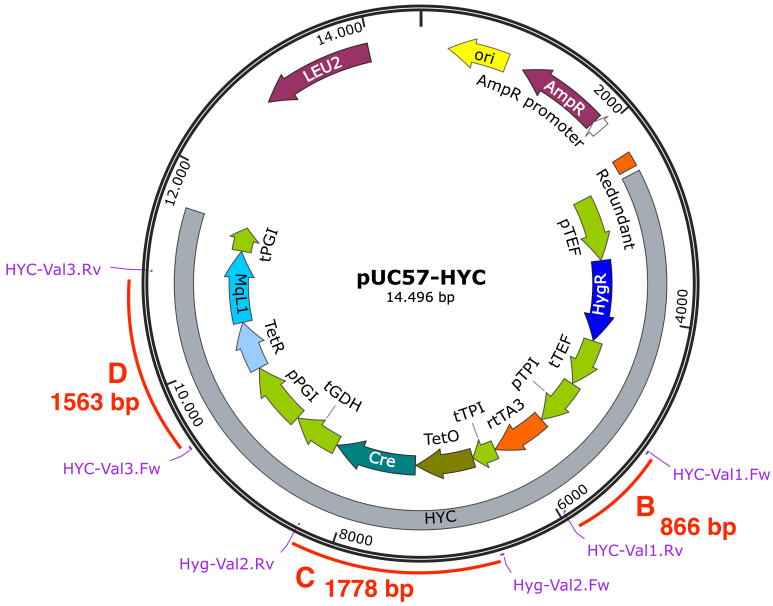


Figure 35: Map of the *pUC57-HYC* plasmid highlighting the location of the fragments B, C and D amplified through colony PCR

each sample were isolated and validated through colony PCR. Validation using the primers sVal2.Fw and sVal2.Rv proved once again inconclusive (data not shown), it was therefore only possible to proceed with primers sVal3.Fw and sVal3.Rv, amplifying a 534 bp fragment within the NTC selection marker. Validation through colony PCR of all 42 isolated colonies can be found in Figure 36. In addition, the DM3 mutant and wild-type (WT) *C. oleaginosus* were used as positive and negative controls, respectively.

Once again, it can be seen how contamination is present, as the WT sample shows a band at the expected length that is very similar in intensity to the one of the positive control DM3. Nevertheless, it can also be seen how some samples such as B21, B32 and C32 do not present a visible band, suggesting a possible removal of the NTC selection marker from the genome. Furthermore, although not fully clear, compared to the control samples, the intensity of the observed bands appears on average to progressively drop from samples of the series A towards the series D, which corresponds to an increasing concentration of tetracycline. At the same time, an increasing concentration of cefoxitin (corresponding to the second digit of all samples) does not appear to have any significant impact on the presence and/or intensity of the observed bands. Similarly, no significant similarity in appearance is present for colonies isolated from the same sample (identified by the same letter and first digit). Because of this relatively

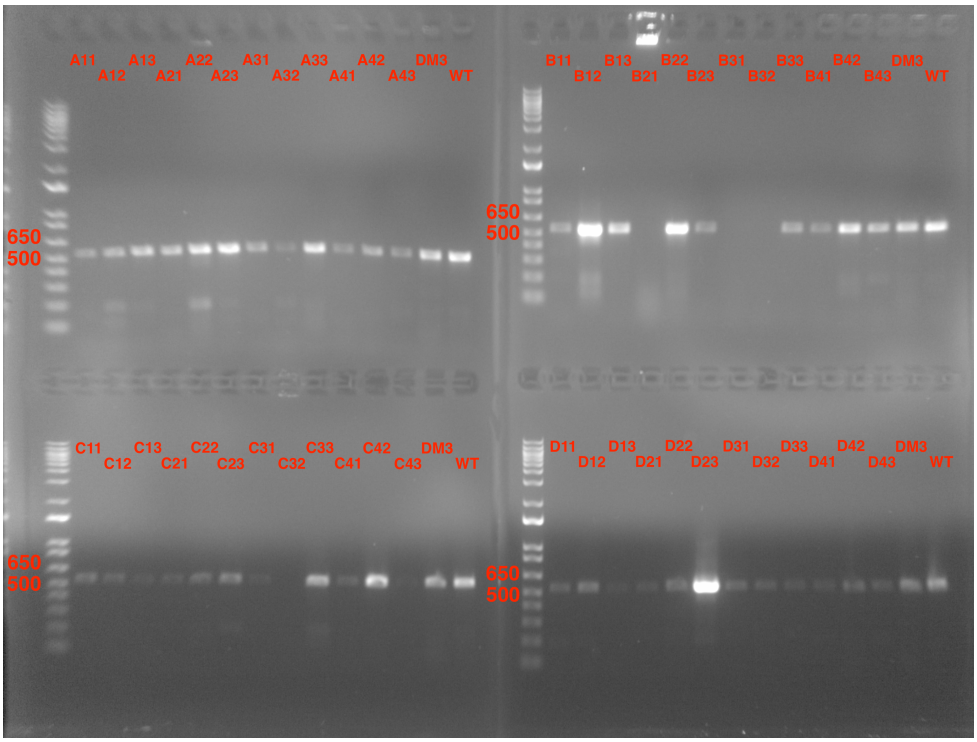


Figure 36: Amplification of a 534 bp fragment within the NTC selection marker from all 42 samples produced using different tetracycline and cofoxitin concentrations. Untreated DM3 mutant and wild-type *C. oleaginosus* were used as positive and negative controls, respectively.

inconclusive result, it was decided to attempt this validation once again on the samples which produced the bands with lowest intensity in order to rule out any potential human mistakes. For this purpose, samples B31, B32, B41, C21, C31, C32, D13 and D33 were chosen. The resulting amplification can be found in Figure 37. Furthermore, *C. oleaginosus* wild-type (WT), mutant DM3 (DM3), plasmid *pUC57-NTY* (p) and water (e) were all used as controls.

For this validation round, a band at the correct length is present for all samples and there is no distinguishable differences between WT, DM3, "p" and all the target mutants outside of D33, while the control sample without template "e" interestingly produced the most intense and clear band. Because of the conflicting and seemingly illogical results of this and the prior PCR validation, is was decided to deem the results as inconclusive and thereby incapable to estimate the efficacy of the TetON-Cre-Lox selection marker recycling system.

## 9.6 Genome sequencing of DM3 and D33 mutants

Despite the inconclusive results of PCR validation on DM1-DM6 and A11-D43 mutants, it was opted to still proceed with genome sequencing of mutants DM3 and D33. More specifically, these were chosen as DM3 was the only mutant transformed with both cassettes and able to seemingly maintain the

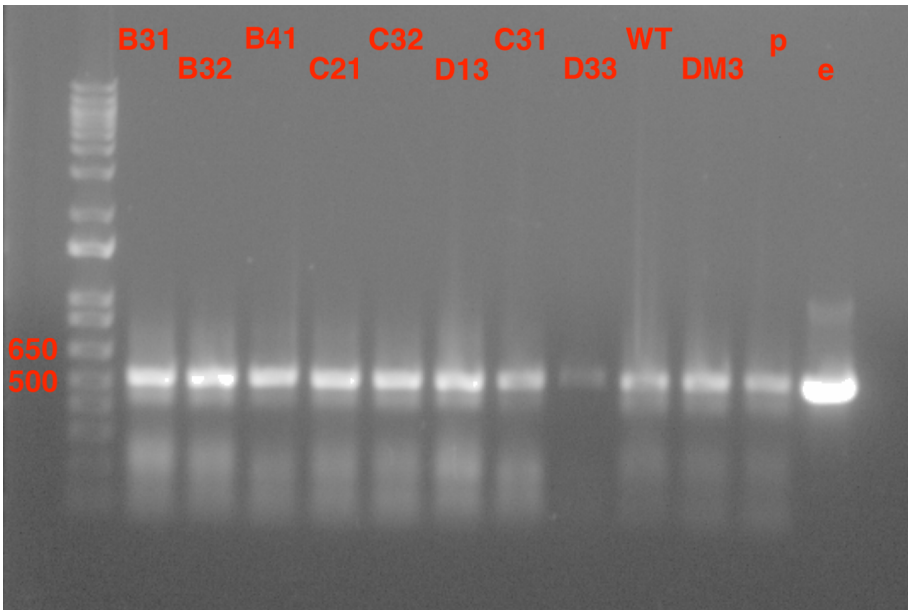


Figure 37: Second attempt at amplification of a 534 bp fragment within the NTC selection marker from samples showing the most faded bands in Figure 36. WT, DM3, *pUC57-NTY* and no template (e) are used as controls.

integrity of both, while D33 appeared to be the mutant most likely to have excised the NTC selection marker from its genome, as can be seen in Figure 37.

The sequencing process returned 15 contigs for DM3 and 12 contigs for D33 of lengths varying from 32 kbp to 4 Mbp. Overall, the total sequenced genome size amounted to 20,677,825 bp for DM3 and 20,382,664 bp for D33. Unexpectedly, it was not possible to find fragments of both the NTY and HYC cassette on either sequenced genome, therefore yielding no actual proof on integration. This is in contradiction to the results of PCR validations performed prior to sequencing for both cassettes. In addition to this, BLAST search of any tested sequence from any contig and both mutants is identified as fragments of larger *Yarrowia lipolytica* shotgun contigs. This strongly suggests that the sequenced samples are indeed instances of *Y. lipolytica*. The obtained genome sizes of (∼)20.7 Mbp and (∼)20.4 Mbp are also closer to the 20.5 Mbp reference genome of *Y. lipolytica* [54], compared with the 19.8 Mbp estimated genome size of *C. oleaginosus* [55]. It is unclear whether wild-type *Y. lipolytica* may have been mistakenly swapped with actual *C. oleaginosus* mutants prior to sequencing, or if some similar swap might have taken place at any point throughout or even before the start of laboratory work.

It was not possible to further look into this issue due to the limited time available to complete this project.

# 10 Discussion

Given the results of the genome sequencing of DM3 and DM33 mutants, as well as the ambiguous colony PCR validations, it is difficult to evaluate the overall success of the experiment and claim whether the original intended goals were achieved. While it was possible to achieve convincing proof of successful transformation trials using linearised *pUC57-NTY* and *pUC57-HYC* plasmid DNA, there is a concrete possibility that the obtained transformants were indeed *Y. lipolytica* instances and not the intended oleaginous yeast *C. oleaginosus*.

Nevertheless, throughout the execution of multiple electroporation trials, it was possible to study the effects of each individual parameter in order to achieve a fine-tuned equilibrium between transformation efficiency and selection of transformants. Most importantly, it was observed how parameters such as OD600 of the overnight culture, concentration of selective antibiotic (NTC and hyg B), concentration of non-selective antibiotic (cefoxitin) and, to a lower degree, electroporation buffer, influenced the survival rate of the cells after being electroporated and plated on solid selective media. Particularly, the OD600 of the culture most likely directly correlates to the development of a thicker cell wall and/or accumulation of intracellular lipids. Therefore, when approaching the later stages of the log phase or early stationary phase, it is possible that the cells tend to become more robust, which allows them to survive an electric shock that otherwise would be fatal. After electroporation, the concentration of both selective and non-selective antibiotics proved to be greatly influential in the ability of the cells to survive. In particular, it can be speculated that cefoxitin, despite being an antibiotic used for bacteria, is able to slow down cell wall regeneration long enough to allow NTC or hygromycin B to permeate the cell and enforce selection. On the other hand, factors such as pulse voltage, cell dilution and the type of cuvette used did not seem to have any particular effect on either cell survival rate or selection, but proved very influential in affecting the conductivity of the electroporation sample and therefore need to be considered in order to avoid short-circuiting and arc-over events. Overall, given the relatively similar cell morphology between *Y. lipolitica* and *C. oleaginosus*, it is possible that a very similar transformation protocol to the one developed in this report may be used to successfully transform *C. oleaginosus*, with only relatively minor adjustments to parameters such as voltage and antibiotic concentration.

It is also necessary to take into account the design of the NTY and HYC cassettes on the used yeast. More specifically, in case the organism used was indeed *Y. lipolytica*, it should be then considered that promoter and terminator sequences on the transformed fragments are native to *C. oleaginosus*. Furthermore, the ORFs used are not codon optimised for *Y. lipolytica*. The combination of non-native promoters and codon availability might therefore have greatly affected the expression level of

the exogenous genes or even the ability of *Y. lipolytica* mutants to express them at all. This can become particularly influential for selection, as a higher expression level of selection markers will result in the ability to tolerate a higher concentration of antibiotic and thereby potentially achieving cleaner selection between transformants and wild-type.

Regarding the ability to perform Cre-Lox recombination and excise the NTC selection marker, it is possible for codon availability and non-native promoters to have affected the efficiency and efficacy of the overall system. This is especially true due to the use of both the rtTA3 tetracycline-controlled transactivator and the TetR-MqL1 fusion protein. The function of the former is to induce transcription, while the latter is designed to prevent leaky transcription from taking place. In ideal conditions, both proteins are expressed and the presence or absence of tetracycline controls which of the two is able to bind and induce/prevent transcription. Different promoters were used for rtTA3 and TetR-MqL1, both strong constitutive promoters native to *C. oleaginosus*. Expression of this system unchanged in *Y. lipolytica* might have resulted in higher expression of one regulator protein over the other or even complete lack of expression of either or both, potentially resulting in a leaky constant expression of Cre-recombinase or a permanent uninduced state even when tetracycline is added. Both of these scenarios would therefore render the selection marker recycling system non-functional.

While such extreme scenarios are unlikely as *C. oleaginosus* and *Y. lipolyitica* are relatively similar microorganisms and promoter-terminator sequences should be at least partly interchangeable, the potential effects of using non-native promoters in transformation cassettes cannot be disregarded.

Aside from affecting the expressions of genes of interest once transformed, the switch from *C. oleaginosus* to *Y. lipolytica* might have had also affected the conservation of the HYC cassette after the subsequent transformation of the NTY cassette during the production of DM mutants. More specifically, these two transformation vectors share the promoter and terminator sequences pTEF, tTEF, pGDH and tGDH, which are respectively 800 bp, 600 bp, 800 bp and 600 bp long. The use of repeating sequences for transformation of *C. oleaginosus* was expected to not be a matter of concern due to the supposed high preference of this yeast for NHEJ. *Y. lipolytica* however, despite also having been shown to prefer NHEJ, is most definitely capable of carrying out HR with varying efficiency, mainly depending on the size of the homologous sequences [56]. The switch to *Y. lipolytica* might therefore at least partially explain the seeming disruption of the HYC cassette for 4 out of 5 successful DM mutants after the integration of the NTY cassette. While an 80% preference ratio for HR over NHEJ remains far higher than the 0-36% ratio reported in literature, it is possible for the presence of four homologous sequences in close proximity to each other to have affected the choice of DNA repair mechanism. Nevertheless, it should also be considered how the only information that could be gathered about the potential overlap

of the two cassettes was obtained through colony PCR and the failure to amplify a target alone cannot be considered conclusive proof of the disruption of said target from the genome. A conclusive proof of such theory would require further validation, preferably through sequencing of the affected genome area.

Another detail overshadowed by the fact that the sequenced mutants appear to be *Y. lipolytica* is the apparent complete lack of either transformed cassette on both the DM3 and D33 genomes. This does conflict with proofs of integration obtained with colony PCR, where although issues with template contamination were encountered for the NTY cassette, full integration of the HYC cassette could be proven without major issues. The clear distinction in number of colonies between target and control samples obtained during the transformation of the NTY in Section 9.4.3 further suggests proof of a successful integration. While it is possible for yeast to disrupt integrated exogenous genes after being transferred to non-selective media, it is believed to be highly unlikely for such an event to have taken place. A more likely explanation would instead be an accidental swap of samples before or during genome sequencing.

# 11 Conclusion and perspectives

Looking back to the project as a whole, it can be stated that it was most likely possible to obtain yeast transformants using a self-developed electroporation method, which resulted in the random genome integration of two cassettes. While convincing proof of integration was obtained through colony PCR, genome sequencing of mutants did instead point towards *Y. lipolytica* having been used rather than *C. oleaginosus* and no integration of either cassette having taken place.

While it is plausible for an accidental swap of samples to have taken place, resulting in the use of the wrong species for the experiment, the seeming lack of integration is in direct conflict with proof obtained through colony PCR and the difference in colony counts obtained on control and target samples.

Similarly, the testing of the TetON-Cre-Lox system for the excision of the NTC selection marker proved inconclusive and no definitive proof could be collected of either it working or not.

These uncertainties therefore do prevent from drawing any definitive conclusions on the success of the experiment, as well as the effectiveness of the developed electroporation protocol and selection marker recycling system.

Further research is needed in order to definitively verify the species of the produced mutants trough further sequencing of either the full genome or the ITS region. In case only the ITS region is sequenced, further validation through colony PCR will be required in order to verify the integration of the NTY and HYC cassettes paying particular care on avoiding template contamination.

In case the strain used is proven to be a *Y. lipolytica* instance, then it will be necessary to re-adapt the developed electroporation protocol in *C. oleaginosus*. A series of experimental trials will have to be executed in a similar fashion to the trials described in Section 8.2. This will allow to define the ideal electroporation parameters in order to achieve both successful transformants and a satisfactory level of selection. It is also likely that parameters more similar to the ones originally used by Koivuranta et al. [2] will be the most optimal. Once an effective transformation protocol has been developed, then it will be possible to again test out the TetON-Cre-Lox system. This selection marker recycling system was purposefully designed to be used in *C. oleaginosus*, therefore it is possible that when integrated in *Y. lipolytica*, as (allegedly) in the case of this experiment, it would perform inconsistently and provide unclear results. Given the template contamination present during colony PCR validation however, it is difficult to conclude whether this is indeed what happened.

Overall, in order to explore the potential use of *C. oleaginosus* for the production of polyketides and other compounds derived from acetyl-CoA and malonyl-CoA precursors, it will be crucial to establish a reliable transformation protocol and an effective selection marker recycling system for this yeast. With

these tools in place, it will be then possible to investigate the feasibility of heterologous production, taking advantage of the large pool of precursors available in *C. oleaginosus*.

Furthermore, using CRISPR/Cas9 will also allow to target and disrupt genes of interest starting from *Ku70*, in order to shift the preferred DNA repair mechanism from NHEJ further towards HR and allow for targeted gene integrations. This will in turn allow to interfere with the yeast's native fatty acid metabolism, both for the purpose of further boosting lipid biosynthesis, as well as for re-direction of precursors towards a heterologously expressed metabolic pathway.

In conclusion, this project failed to reach the intended goals of the development of a molecular toolbox for *C. oleaginosus*, most likely due to a accidental swap of samples. Nevertheless, it was possible to highlight the essential parameters to investigate in order to achieve a successful electroporation protocol. Furthermore a TetON-Cre-Lox selection marker recycling systems was developed, the testing of which proved inconclusive. The issues responsible for this (template contamination and sample swap) are however easy to eliminate.

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# 12 Appendix

## 12.1 Growth mediums and buffers

### YPD

- 10 g/L yeast extract
- 20 g/L peptone
- 20 g/L dextrose (glucose)
- 20 g/L agar (if required)

Autoclave at 105°C for 25 min to avoid Maillard reaction.

### 2xYPD

- 20 g/L yeast extract
- 40 g/L peptone
- 40 g/L dextrose (glucose)

Autoclave at 105°C for 25 min to avoid Maillard reaction.

### 2xYPAD

- 20 g/L yeast extract
- 40 g/L peptone
- 40 g/L dextrose (glucose)
- 20 g adenine hemisulfate

Autoclave at 105°C for 25 min to avoid Maillard reaction.

### LB

- 10 g/L tryptone
- 5 g/L yeast extract

- 10 g/L NaCl

Autoclave at 121°C for 15 min

**EB buffer**

- 10 mM Tris-HCl
- 270 mM sucrose
- 1 mM $MgCl_2$

Bring pH to 7.5 and autoclave for at 121°C for 15 min

**IB buffer**

- 25 mM DTT (Dithiothreitol)
- 20 mM HEPES free acid (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

Bring to volume in YPD broth, filter sterilise and store at -20°C. Thaw in warm water bath before use and freeze again after use.

**Lysis buffer**

- 0.2 M NaCl
- 0.1% Triton X-100
- 0.2% SDS
- 10.2 mM Tris-HCl
- 48 mM EDTA)

## 12.2 Primers

Table 13: List of all primers used throughout the project, complete with sequences, purpose of use and outcome.

| Name | Sequence | Function | Outcome |
|---|---|---|---|
| CS.Fw | TTGACGCTTAGACA ACTTAATAACACA | Amplification of hyg B resistance gene + yfp cassette from *pRF-HU2-Hyg-yfp* | Failed |
| CS.Rv | AAAGGTACCATCCG CTGACA | | |
| NrsR.Fw | TCAACTTGATCAAC AACTAGATGACCAC CCTCGACGAC | Amplification of NTC resistance gene from *pUC57-NTC* with tails matching pFLEXI backbone | Successful |
| NrsR.Rv | CTACAACCTAGAAA GGGATCGGGGCAG GGCATCG | | |
| DD.LoxP.NourR.Fw | ACTAGTATAACTTC GTATAATGTATGCT ATACGAAGTTATAT GACCACCCTCGAC GACA | Amplification of NTC resistance gene from *pUC57-NTC* with tails adding LoxP sites and BcuI + BamHI restriction sites | Successful |
| DD.LoxP.NourR.Rv | GGATCCATAACTTC GTATAGCATACATT ATACGAAGTTAT GGGGCAGGGCATC GACATGT | | |
| pVAL.Fw | ACCAAGAAGCGAA AAACCGC | Amplification of a 2602 bp fragment on *pFLEXI-NTC* | Failed |
| pVAL.Rv | ATTTGTCAGCACG GGTAGCA | | |
| NTCCS.Fw | GTACCTCGCGAAT GCATCTA | Amplification of cassette containing NTC resistance gene and yfp from *pFLEXI-NTC* | Failed |
| NTCCS.Rv | AACTGAAGGCGG GAAAGCTA | | |

| | | | |
|---|---|---|---|
| NTY.Fw | GGCGCGCCAACA CTAGATACT | Amplification of cassette containing NTC resistance gene and yfp from *pUC57-NTY* | Failed |
| NTY.Rv | AGTCGTGATAATG CGGAGTCA | | |
| HYC.Fw | TGTAAAACGACG GCCAGTGA | Amplification of cassette containing hyg B resistance gene and TetON-Cre-Lox system | Failed |
| HYC.Rv | AGATACGAGGCG CGTGTAAG | | |
| NTY-Val1.Fw | TAGACCGGGATC CGAAGAGT | Amplification of a 762 bp fragment from NTY cassette | Successful |
| NTY-Val1.Rv | CGGAGGATGTTG GATGGAGG | | |
| NTY-Val2.Fw | GGCCACAAGTTC TCGGTCTC | Amplification of a 853 bp fragment from NTY cassette | Successful |
| NTY-Val2.Rv | GACCAGTGCTAA GCCTGACT | | |
| pUC57-Val1.Fw | TAATTGCGTTGC GCTCACTG | Amplification of a 681 bp fragment from *pUC57* backbone | Failed |
| pUC57-Val1.Rv | CGCCTACATACC TCGCTCTG | | |
| pUC57-Val2.Fw | TGAGGCACCTAT CTCAGCGA | Amplification of a 828 bp fragment from *pUC57* backbone | Successful |
| pUC57-Val2.Rv | TTCCGTGTCGCC CTTATTCC | | |
| Co-cPCR-Fw | GATGTAGGAGGG CGTGGATA | Amplification of a 579 bp fragment from hyg B resistance gene | Successful |
| Co-cPCR-Rv | GATGTTGGCGAC CTCGTATT | | |
| HYC-Val1.Fw | GCAGCTAAACTT GACGGAGG | Amplification of a 866 bp fragment from HYC cassette | Successful |
| HYC-Val1.Rv | TCGAGCGAAAAA CCCTGCT | | |

| | | | |
|---|---|---|---|
| Hyg-Val2.Fw | TGCACTTGAGGC GGTTACTA | Amplification of a 1778 bp fragment from HYC cassette | Failed |
| Hyg-Val2.Rv | CCGTCCTCAAGG AGACGAAC | | |
| HYC-Val3.Fw | AAAGTGTTCGGT CCGGTAGC | Amplification of a 1563 bp fragment from HYC cassette | Successful |
| HYC-Val3.Rv | CGGTCGTACAGG ATGCCAAT | | |
| sVAL.Fw | TAGTAGCACGAGC AGATGCG | Amplification of a 1346 bp fragment from NTY cassette including NTC resistance gene | Failed |
| sVAL.Rv | ATGACCGCTGGAG TTGAAGG | | |
| sVAL2.Fw | TGGGCGCGCCAA CACTAGAT ACTCA | Amplification of a 2138 bp fragment from NTY cassette including NTC resistance gene | Failed |
| sVAL2.Rv | ACCCTGCCAGCT GCGAATCCGT | | |
| sVAL3.Fw | CCGCCTACCGCTA CCGCACCT | Amplification of a 534 bp fragment within NTC resistance gene | Successful |
| sVal3.Rv | ACATGTAGAGGG CCTGCTCGCCG | | |

## 12.3 Development of an electroporation protocol

This subchapter provides a detailed explanation of the different trials performed to adapt the *C. oleaginosus* electroporation protocol developed by [2] to the Bio-Rad (Hercules, CA, United States) MicroPulser available on the Esbjerg campus.

As introduced in Chapter 8.2, the first attempt to replicate the above mentioned electroproation protocol failed due to the anti-arc system kicking in. To further investigate the parameter that caused this, another experiment was performed with a modified version of the previously described protocol. One 50 mL YPD cultures of *C. oleaginosus* was prepared and grown overnight until an OD600 of 20 was reached. The cells were then collected by centrifugation at 5000 rpm for 5 minutes, washed with 5 mL of ice-cold EB buffer and incubated for 30 min on IB buffer at 30°C and 200 rpm. Differently from the previous procedure, the culture was washed 3 times with EB buffer and resuspended in 1 mL of EB buffer. Three more suspensions were then produced, by pipetting 200 µL, 100 µL and 50 µL of the original suspension into separate sterile Eppendorf tubes and bringing the volume to 1 mL, effectively producing four dilutions of the original suspension (namely 1:1, 1:5, 1:10 and 1:20). 300 µL and 80 µL of these cell suspensions were pipetted into both 0.4 cm and 0.1 cm cuvettes respectively, without any added plasmid DNA. After incubating the cuvettes on ice for 15 min, the samples were electroporated at 1.8 kV and automatic pulse length, with the aim of detecting the minimum voltage at which no arcing event would take place. A summary of the samples produced is reported in Table 14.

Table 14: Samples produced in the first experiment to study the parameters influencing arcing of electroporation

| Sample | Cuvette size [cm] | Dilution from original protocol |
|---|---|---|
| S1 | 0.1 | 1:1 |
| S2 | 0.1 | 1:5 |
| S3 | 0.1 | 1:10 |
| S4 | 0.1 | 1:20 |
| B1 | 0.4 | 1:1 |
| B2 | 0.4 | 1:5 |
| B3 | 0.4 | 1:10 |
| B4 | 0.4 | 1:20 |

As the obtained voltages were considered too low compared with the original protocol, a new trial was planned using the same cell preparation and electroporation methods but utilizing 10-fold dilutions of 1:50, 1:100, and 1:200 in 0.1 cm cuvettes only. In addition, 10 mM Tris-Cl (one of the three components of EB buffer) will also be tested as a suspension buffer alongside EB. A summary of the samples is reported in Table 15

Table 15: Samples produced in the second experiment to study the parameters influencing arcing of electroporation

| Sample | Suspension buffer | Dilution from original protocol |
|---|---|---|
| EB50 | EB buffer | 1:50 |
| EB100 | EB buffer | 1:100 |
| EB200 | EB buffer | 1:200 |
| T50 | Tris-Cl | 1:50 |
| T100 | Tris-Cl | 1:100 |
| T200 | Tris-Cl | 1:200 |

The obtained voltage was deemed still too low, because of this it was decided to explore the use of other suspension buffers from literature with the aim of achieving a higher voltage without arcing. For this purpose, demineralised water and 1 M sorbitol were used. The protocol used to prepare the cells was once again similar to the one developed in the previous experiments, where a 50 mL culture of *C. oleaginosus* on YPD was prepared and incubated at 30°C overnight until an OD600 of 20 was observed. The cells were then centrifuged at 5000 rpm, washed once with 5 mL of ice-cold EB buffer and resuspended in 5 mL of IB buffer. This suspension was then incubated for 30 min at 30°C and 200 rpm, then centrifuged at 5000 rpm for 5 min once again and washed 3 times with demineralised water rather than EB buffer. The obtained pellet was resuspended in 1 mL of demineralised water and used to create 1:10, 1:50 and 1:100 dilutions using both more demineralised water and 1 M sorbitol. These

six suspensions were then transferred to both 0.1 cm and 0.4 cm cuvettes, to which 50 mL and 250 mL of suspension were added, respectively. The cuvettes were incubated on ice for 15 min, then subject to electroporation at a starting voltage of 2.0 kV and automatic pulse time. The voltage was progressively lowered by 0.1 kV after every pulse, until a level was reached where the sample wouldn't attempt to arc anymore. A list of all samples for this experiment is provided in Table 16.

Table 16: Samples produced in the third electroporation attempt to study the parameters influencing arcing of electroporation.

| Sample | Suspension buffer | Dilution from original protocol | Cuvette size [cm] |
|---|---|---|---|
| W10-S | $H_2O$ | 1:10 | 0.1 |
| W50-S | $H_2O$ | 1:50 | 0.1 |
| W100-S | $H_2O$ | 1:100 | 0.1 |
| W10-B | $H_2O$ | 1:10 | 0.4 |
| W50-B | $H_2O$ | 1:50 | 0.4 |
| W100-B | $H_2O$ | 1:100 | 0.4 |
| S10-S | Sorbitol | 1:10 | 0.1 |
| S50-S | Sorbitol | 1:50 | 0.1 |
| S100-S | Sorbitol | 1:100 | 0.1 |
| S10-B | Sorbitol | 1:10 | 0.4 |
| S50-B | Sorbitol | 1:50 | 0.4 |
| S100-B | Sorbitol | 1:100 | 0.4 |

Based on the results from this experiment, electroporation of *C. oleaginosus* with *pRF-HU-Hyg-yfp* plasmids DNA was once again attempted. A 100 mL overnight culture of *C. oleaginosus* on 2xYPD media (recipe in Appendix 12) was prepared and incubated at 30°C and 200 rpm overnight until an OD600 of 17.7 was reached. The cells were recovered by centrifugation at 5000 rpm for 5 min and washed with 5 mL of ice-cold EB buffer. Next, the pellet was resuspended with 5 mL of warm IB buffer and incubated for 30 min at 30°C and 200 rpm. The cells were once again harvested by centrifugation at 5000 rpm for 5 min and washed 3 times with 5 mL of demineralised water buffer, then resuspended in 1.77 mL of more demineralised water. From this, 1:25, 1:50 and 1:100 dilution were made both using EB buffer, demineralised water, 10 mM Tris-Cl and 1 M sorbitol. These 12 suspensions were tested in 0.1 cm cuvettes only. After mixing the cell suspensions and plasmid DNA inside the cuvettes, these were incubated on ice for 15 min, then electroporated. The electroporation voltage was set manually and the pulse time was left to autmatic. A more detailed list of all produced samples is displayed in Table 17.

Table 17: Samples produced in the second attempt to replicate the original electroporation protocol

| Sample | Suspension buffer | Dilution from original protocol | Pulse voltage [kV] | *pRF-HU2-Hyg-yfp* added [ng - µL] |
|---|---|---|---|---|
| A1 | $H_2O$ | 1:25 | 1.8 | 80 - 2 |
| A2 | $H_2O$ | 1:50 | 1.8 | 80 - 2 |
| A3 | $H_2O$ | 1:100 | 1.8 | 80 - 2 |
| B1 | EB buffer | 1:25 | 1.2 | 80 - 2 |
| B2 | EB buffer | 1:50 | 1.2 | 80 - 2 |
| B3 | EB buffer | 1:100 | 1.2 | 80 - 2 |
| C1 | Tris-Cl | 1:25 | 1.3 | 80 - 2 |
| C2 | Tris-Cl | 1:50 | 1.3 | 80 - 2 |
| C3 | Tris-Cl | 1:100 | 1.3 | 80 - 2 |
| D1 | Sorbitol | 1:25 | 1.7 | 80 - 2 |
| D2 | Sorbitol | 1:50 | 1.7 | 80 - 2 |
| D3 | Sorbitol | 1:100 | 1.7 | 80 - 2 |

After electroporation, the content of the cuvettes was resuspended in 1 mL of 2xYPD media, transferred to 50 mL falcon tubes and incubated for 4 h at 200 rpm and 30°C. Finally, 300 µL from each sample were plated on YPD plates supplemented with 200 ng/µL of hygromycin B. A wild-type control sample was also created by directly inoculating 5 µL of the 1:50 Tris-Cl dilution, together with 200 µL of $H_2O$. The plates were then incubated at 30°C for 3 days.

A similar experiment to this one was performed immediately next, in order to better investigate the effect of the addition of plasmid DNA to the cuvettes during electroporation. The preparation of cells was once again similar to previous trials: a 50 mL overnight culture of *C. oleaginosus* on 2xYPD was prepared and incubated at 30 °C and 200 rpm until an OD600 of 20 was obtained. The pellet was then centrifuged at 5000 rpm for 5 min, washed once with 5 mL of ice-cold EB, resupended in 5 mL of IB, incubated for 30 min at 30°C and 200 rpm, collected once again by centrifugation at 5000 rpm for 5 minutes, washed three times with 5 mL of demineralised water and resuspended in 1 mL of more water. From this, four 1:75 dilutions were made using demineralised water, EB buffer, 10 mM tris-Cl and 1 M sorbitol. Each of these dilutions was then used to produce one sample with 2 µL of added plasmid DNA and a respective wild-type sample without the plasmid DNA. A collective list of all produced sample is shown in Table 18. The cell suspensions and plasmid DNA (if added) were mixed in 0.1 cm cuvettes and incubated on ice for 15 min before being electroporated.

After electroporation, the content of the cuvettes was diluted with 1 mL of 2xYPD, transferred into falcon tubes and incubated for 4 h at 30°C and 200 rpm. After this period, 300 µL from each sample were plated on two sets of YPD plates supplemented with 200 ng/µL and 250 ng/µL of hygromycin B. The plates were then incubated at 30°C for 3 days.

Table 18: Samples produced in the fourth electroporation attempt to study the parameters influencing arcing of electroporation.

| Sample | Suspension buffer | Dilution from original protocol | Pulse voltage [kV] | *pRF-HU2-Hyg-yfp* added [ng - µL] |
|---|---|---|---|---|
| W | $H_2O$ | 1:75 | 1.5 | 80 - 2 |
| EB | EB buffer | 1:75 | 1.2 | 80 - 2 |
| T | Tris-Cl | 1:75 | 1.3 | 80 - 2 |
| S | Sorbitol | 1:75 | 1.7 | 80 - 2 |
| WTW | EB buffer | 1:75 | 1.7 | - |
| WTEB | EB buffer | 1:75 | 1.2 | - |
| WTT | Tris-Cl | 1:75 | 1.3 | - |
| WTS | Sorbitol | 1:75 | 1.7 | - |

The results from this trial suggested that a too large amount of cells was present, potentially attenuating the effect of the antibiotic on the plates. Because of this, it was decided to carry out an experiment with larger dilutions and also a starting *C. oleaginosus* overnight culture of 5 mL instead of 50 mL, which was incubated overnight at 30°C and 200 rpm until an OD600 or 11 was achieved. Similarly to previous trials, the cells in this culture were pelleted by centrifugation at 5000 rpm for 5 min, washed once with 5 mL of ice-cold EB buffer, resuspended with 5 mL of IB buffer, incubated at 30°C and 200 rpm for 30 min, centrifuged once again at 5000 rpm for 5 min, washed 3 times with 5 mL of demineralised water. The pellet that was obtained was divided into two parts. One part was dissolved in 0.5 mL of demineralised water, while the other part was dissolved in 0.5 mL of 10 mM Tris-Cl. These two solutions were used to make three additional sets of dilutions, which were 1:5, 1:10, and 1:50. These dilutions were made using the corresponding solution as a buffer. From each dilution, two samples of 50 µL were taken and placed in separate 0.1 cm cuvettes. *pRF-HU2-Hyg-yfp* plasmid DNA was added to one of the two cuvettes, and this cuvette was used as a test sample. The second cuvette, without the addition of plasmid DNA, was used as a wild-type control sample. The cuvettes were incubated on ice for 15 min, then electroporated. A more exhaustive list of all samples produced is reported in Table 19.

Table 19: Samples produced in the third attempt to replicate the original electroporation protocol.

| Sample | Suspension buffer | Dilution of original 1mL suspension | Pulse voltage [kV] | *pRF-HU2-Hyg-yfp* added [ng - µL] |
|---|---|---|---|---|
| T1 | Tris-Cl | 1:1 | 1.2 | 80 - 2 |
| T2 | Tris-Cl | 1:5 | 1.3 | 80 - 2 |
| T3 | Tris-Cl | 1:10 | 1.3 | 80 - 2 |
| T4 | Tris-Cl | 1:50 | 1.3 | 80 - 2 |
| S1 | Sorbitol | 1:1 | 1.3 | 80 - 2 |
| S2 | Sorbitol | 1:5 | 1.6 | 80 - 2 |
| S3 | Sorbitol | 1:10 | 1.7 | 80 - 2 |
| S4 | Sorbitol | 1:50 | 1.7 | 80 - 2 |
| WT-T1 | Tris-Cl | 1:1 | 1.2 | - |
| WT-T2 | Tris-Cl | 1:5 | 1.3 | - |
| WT-T3 | Tris-Cl | 1:10 | 1.3 | - |
| WT-T4 | Tris-Cl | 1:50 | 1.3 | - |
| WT-S1 | Sorbitol | 1:1 | 1.3 | - |
| WT-S2 | Sorbitol | 1:5 | 1.6 | - |
| WT-S3 | Sorbitol | 1:10 | 1.7 | - |
| WT-S4 | Sorbitol | 1:50 | 1.7 | - |

The content of the cuvettes was then diluted with 1 mL of 2xYPD media, transferred into 50 mL falcon tubes and incubated for 4h at 30°C and 200 rpm. Finally, 125 µL aliquotas from these suspensions were plated on YPD plates supplemented with 250 ng/µL of hygromycin B. The plates were then incubated for 3 days at 30°C.

The next experiment aimed to examine how the length of time after electroporation affected the number of colonies that formed on plates. The procedure was similar to the previous trial, but this time only a 1:50 dilution of 10 mM Tris-Cl solution was used. Four different recovery times were tested, 30 minutes, 1 hour, 2 hours, 3 hours, and 4 hours. For each recovery time, two samples were prepared, one with plasmid DNA and one without, which served as a control. A complete list of all samples is provided in Table 20.

After electroporation, the content of the cuvettes was resuspended in 1 mL of 2xYPD and incubated at 30°C and 200 rpm for the specified period of time. After this, 125 µL from each sample were plated on YPD plates supplemented with 250 ng/µL of hygromycin B. The plates were then incubated at 30°C for 3 days.

This entire trial was also repeated using a control plasmid that did not contain the gene for resistance to hygromycin B. This plasmid was added to the wild-type control samples in the same amount and concentration as the regular samples. This was done to eliminate any potential impact that the plasmid DNA or elution buffer might have on the conductivity of the suspensions in the cuvettes. The results

Table 20: Samples produced in the fifth electroporation attempt to study the parameters influencing arcing of electroporation.

| Sample | Suspension buffer | Dilution of original 1 mL suspension | Pulse voltage [kV] | pRF-HU2-Hyg-yfp added [ng - µL] | Recovery time [h] |
|---|---|---|---|---|---|
| A | Tris-Cl | 1:50 | 1.3 | 60 - 2 | 0.5 |
| B | Tris-Cl | 1:50 | 1.3 | 60 - 2 | 1 |
| C | Tris-Cl | 1:50 | 1.3 | 60 - 2 | 2 |
| D | Tris-Cl | 1:50 | 1.3 | 60 - 2 | 3 |
| E | Tris-Cl | 1:50 | 1.3 | 60 - 2 | 4 |
| WT-A | Tris-Cl | 1:50 | 1.3 | - | 0.5 |
| WT-B | Tris-Cl | 1:50 | 1.3 | - | 1 |
| WT-C | Tris-Cl | 1:50 | 1.3 | - | 2 |
| WT-D | Tris-Cl | 1:50 | 1.3 | - | 3 |
| WT-E | Tris-Cl | 1:50 | 1.3 | - | 4 |

from both repetitions were however very similar. More specifically, no significant growth was observed on any plate. Because of this it was decided to perform a further trial, using both 200 ng/µL and 250 ng/µL. The preparation of the cells for this trial was once identical to the previous experiment. Two dilutions were produced this time, 1:25 and 1:50, both using 10 mM tris-Cl. A list of all the produced samples is presented in Table 21.

Table 21: Samples produced in the sixth electroporation attempt to study the parameters influencing arcing of electroporation.

| Sample | Suspension buffer | Dilution of original 1 mL suspension | Pulse voltage [kV] | *pRF-HU2-Hyg-yfp* added [ng - µL] | Concentration of Hyg B [ng/µL] |
|---|---|---|---|---|---|
| 1 | Tris-Cl | 1:25 | 1.2 | 176 - 2 | 200 |
| 1k | Tris-Cl | 1:25 | 1.2 | - | 200 |
| 2 | Tris-Cl | 1:50 | 1.2 | 176 - 2 | 200 |
| 2k | Tris-Cl | 1:50 | 1.2 | - | 200 |
| 3 | Tris-Cl | 1:25 | 1.3 | 176 - 2 | 200 |
| 3k | Tris-Cl | 1:25 | 1.3 | - | 200 |
| 4 | Tris-Cl | 1:50 | 1.3 | 176 - 2 | 200 |
| 4k | Tris-Cl | 1:50 | 1.3 | - | 200 |
| 5 | Tris-Cl | 1:25 | 1.2 | 176 - 2 | 250 |
| 5k | Tris-Cl | 1:25 | 1.2 | - | 250 |
| 6 | Tris-Cl | 1:50 | 1.2 | 176 - 2 | 250 |
| 6k | Tris-Cl | 1:50 | 1.2 | - | 250 |
| 7 | Tris-Cl | 1:25 | 1.3 | 176 - 2 | 250 |
| 7k | Tris-Cl | 1:25 | 1.3 | - | 250 |
| 8 | Tris-Cl | 1:50 | 1.3 | 176 - 2 | 250 |
| 8k | Tris-Cl | 1:50 | 1.3 | - | 250 |

After electroporation, the content of the cuvettes was suspended in 1 mL of 2xYPD media,transferred to 50 mL falcon tubes and incubated at 30°C and 200 rpm for 4 h. From each sample, 125 µL were

plated on the respective YPD plates supplemented with hygromycin B.