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Development of a dedicated Golden Gate assembly platform for the nonconventional yeast *Rhodotorula toruloides*

Bachelor's Thesis (12 ECTS)

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Abstract:

In this study, Golden Gate assembly platform was adapted to develop an efficient assembly system for the nonconventional yeast *Rhodotorula toruloides*. The DNA fragments were assembled with predesigned 4-nt overhangs, building three transcriptional units, a selection marker, and insertional units for genome targeting. The platform was validated by deleting the *KU70* gene, increasing the genome integration efficiency, and by overexpressing the carotenoid production pathway. The total carotenoids increased by 210%. The dedicated GGA platform fills a gap in the advanced genome engineering toolkit of *R. toruloides*, enabling efficient design of complex metabolic pathways.

Keywords:

Rhodotorula toruloides, synthetic biology, metabolic engineering, nonconventional yeast, carotenoid, Golden Gate assembly, microbial cell factory

CERCS: T490 Biotechnology

Golden Gate assembly geenimodifitseerimise platvormi väljatöötamine mittetraditsioonilisele pärmile *Rhodotorula toruloides*

Lühikokkuvõte:

Rhodotorula toluroides on mittetraditsiooniline õlirikas pärm, millel on hea potentsiaal saada üheks peamiseks rakuvabrikuks lignocelluloosete suhkrute konverteerimisel kõrgema väärtusega kemikaalideks. Antud uuringus kohandati Golden Gate'i (GGA) geneetilise modifitseerimise platvormi esmakordselt just *R. toruloides* jaoks. Antud metoodikas seotakse DNA fragmendid, kasutades eelnevalt disainitud 4-nt pikkuseid üleulatuvaid DNA järjestusi. Nii konstrueeriti kolm transkriptsiooniühikut, selektsioonimarker ja sisestusühikud genoomi sihtimiseks. Platvorm valideeriti *KU70* geeni kustutamise, genoomi integreerimise efektiivsuse suurendamise ja karotenoidide tootmisraja üleekspresseerimise kaudu. Karotenoidide tootmine suurenes seeläbi 210%. Spetsiaalne GGA platvorm täidab lünga *R. toruloides*'i geenitehnoloogia

tööriistakomplektis, võimaldades keeruliste metaboolsete radade tõhusamat disainimist ja genoomi integreerimist.

Võtmesõnad:

Rhodotorula toruloides, sünteetiline bioloogia, metaboolne inseneerimine, mittetraditsioonilised pärmid, karotenoid, Golden Gate assembly, rakuvabrik

CERCS: T490 Biotehnoloogia

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TERMS, ABBREVIATIONS AND NOTATIONS

ADH2 – Alcohol dehydrogenase 2

CRTE – Geranylgeranyl diphosphate synthase

CRTI – Phytoene dehydrogenase

CRTYB – Phytoene synthase

GGA – Golden Gate assembly

GPD – Glyceraldehyde-3-phosphate dehydrogenase

HR – Homologous recombination

NHEJ – Non-homologous end joining

NOS – Nopaline synthase

TU – Transcriptional unit

XYL1 – Xylose reductase

YPD – Yeast extract peptone dextrose medium

YPX – Yeast extract peptone xylose medium

YTK – Yeast Toolkit

INTRODUCTION

Overuse of carbon from fossil sources has led to depletion of natural resources, increased carbon dioxide emission, and caused damage to our environment. Therefore, the change to an economy that prioritizes carbon recycling and sustainable biotechnology processes for the production of fuel and chemicals is crucial.

Microbial cell factories play an important role in the transition to bioeconomy, as the necessary chemicals and biofuels can be produced without requiring traditional resources, such as land or favourable climate. A microorganism that can naturally produce several industrially relevant compounds, e.g. lipids and antioxidants in the form of carotenoids, is the nonconventional yeast *Rhodotorula toruloides*. This yeast has important advantages for biotechnological purposes, such as its ability to grow to high cell densities, to consume various sources of carbon and nitrogen, and to naturally co-produce industrially important molecules (lipids, carotenoids, and enzymes). However, the lack of advanced genome engineering tools is a serious drawback for its industrial use. Therefore, a dedicated synthetic biology platform that enables the quick and efficient assembly of pathways is an important step to consolidate *R. toruloides* as a biotechnology workhorse.

Golden Gate assembly (GGA) offers a seamless and efficient way of assembling DNA fragments in a one-pot reaction, making it easier to diversify and optimize pathways of interest. GGA is based on Type IIS restriction enzymes (REases), which have the ability to cut the DNA outside of their recognition sequence. By placing the recognition sites on the both ends of the DNA fragment, it is possible to carry out digestion and ligation at the same time, as the REase leaves overhangs exposed and able to anneal to the complementary sequence.

Standardization and adaptation of GGA to *R. toruloides* aims to fill the gap in the genome engineering toolkit of this yeast. The validation of the robustness and efficiency of this platform will be done by the deletion of the *KU70* gene, which has been proven to raise the frequency of heterologous DNA integration in *R. toruloides*, and the overexpression of the three key genes of the carotenoids biosynthesis pathway: geranylgeranyl diphosphate synthase (*CRTE*), phytoene dehydrogenase (*CRTI*), and phytoene synthase (*CRTYB*).

1 LITERATURE REVIEW

1.1 Importance of yeast in biobased economy

The rapid process of industrialization, while leading to economic prosperity, has been accompanied by severe environmental damage. The wide-spread use of petrol, as well as its treatment via high pressure/temperature methods, is one of the leading causes of the increased greenhouse gases emission and the destruction of the ozone layer (IPCC, 2014). Therefore, there is a pressing need for alternative sources of carbon and the production of fuel in a more sustainable manner. A biobased approach to the economy promises to be the alternative that can help diminish these problems.

Microbial factories are an essential element for the transition towards a biobased economy, as biofuels, pharmaceuticals, and fine chemicals can be produced from engineered or native organisms. Another advantage of cell factories is that, for most applications, they are not in competition with the food industry and do not require land or other resources needed for traditional agriculture. They are also not affected by differing locations or climate (Chen and Wang, 2017).

The wealth of information available, coupled with abundant ways of genetic manipulation, has made the baker's yeast *Saccharomyces cerevisiae* the most popular eukaryotic organism for cell factories (Kim et al., 2012). However, non-*Saccharomyces*, or, nonconventional, yeasts have several advantages over *S. cerevisiae*, such as higher tolerances for growth-inhibitory compounds found in low-value substrate streams or metabolic traits that would need to be extensively engineered in baker's yeast (Gellissen and Hollenberg, 1997; Wolf, 2012). Most nonconventional yeasts used for bioprocesses are also Crabtree negative, in contrast to *S. cerevisiae*, and favor respiration over fermentation, which makes them particularly suitable for the biosynthesis of chemicals other than ethanol, as well as protein production (Wagner and Alper, 2016). Another disadvantage of *S. cerevisiae* is that its preferred substrate is the hexose sugar glucose, and it needs metabolic engineering and stringent fermentation conditions to ferment pentose sugars such as xylose and arabinose (Chiang et al., 1981; Jeffries, 2006; Wei et al., 2013). Xylose consumption by microbial cell factories is especially important for biobased economy, as it is the main C5 carbohydrate in hemicellulose. Hemicellulose is the second major abundant

biopolymer in the plant biomass after cellulose, making it an important resource for bioeconomy (Chandel et al., 2013).

One of the most used nonconventional yeasts so far has been *Yarrowia lipolytica*. It can accumulate lipid content exceeding 20% of dry cell weight in some cases, and as such, is considered an oleaginous yeast (Ratledge, 2010). *Y. lipolytica* has long since been established as a biotechnological chassis for the production of various bioproducts (Ledesma-Amaro et al., 2015; Ledesma-Amaro and Nicaud, 2016; Madzak, 2015; Nicaud, 2012). It has been used as a model for protein secretion (Nicaud et al., 2002), peroxisome biogenesis (Titorenko et al., 2000), dimorphism (Domínguez et al., 2000), studies of mitochondrial complex I (Abdrakhmanova et al., 2004), among others. A disadvantage of *Y. lipolytica* is that it is not a natural consumer of xylose and has to be engineered to metabolize it (Ledesma-Amaro et al., 2016). Despite this, *Y. lipolytica* is expected to play an important role in creating microbial factories as this yeast has well established tool boxes and metabolic engineering methods, which allowed the all the heavily required modifications over the past decades.

1.2 *Rhodotorula toruloides*

Another nonconventional yeast that has been increasingly regarded as an important microorganism for the transition towards a biobased economy is *Rhodotorula* (formerly *Rhodosporidium*) *toruloides* (Park et al., 2017). It can naturally produce numerous industrially relevant compounds, e.g., lipids and compounds derived from lipids that can be used in production of fuels and chemicals as substitutes for petroleum (Passoth, 2017), as well as carotenoids that are used as A-vitamin precursors, colorants or antioxidants. It also naturally accumulates higher amount of lipids compared to *Y. lipolytica* and, therefore, has a high potential of becoming an industrially competitive lipid producer. Consequently, there have been several physiological studies that discovered industrially important features of this yeast: *R. toruloides* has the capacity to grow to high cell density (Li et al., 2007), and can be cultivated on various sources of carbon and nitrogen (Lopes et al., 2020; Xu and Liu, 2017), creating the opportunity to produce high-value products from low-cost substrates. One example of such high-value products are the carotenoids, as *R. toruloides* produces mainly γ-carotene, β-carotene, torulene and torularhodin (Mata-Gómez et al., 2014). Torulene and torularrhodin are not commercially important as β-

carotene, but due to their valuable antioxidant properties, the interest in their production has been increasing lately (Kot et al., 2018).

In the past decade, a slew of systems biology studies have shed light on the genomic organization and metabolic pathways of *R. toruloides*. Complete genome sequences of different strains have been determined, helping to identify pathways of interest for strain engineering, discover the response of the organism towards environmental changes, and ascertain the mechanism of lipid accumulation (Hu and Ji, 2016; Kumar et al., 2012; Morin et al., 2014; Zhu et al., 2012). Basic tools for genetic manipulation have been developed or studied, such as constitutive (Liu et al., 2016, 2013; Wang et al., 2016) and inducible promoters (Johns et al., 2016; Liu et al., 2015), and auxotrophic (Yang et al., 2008) and antibiotic-based (Lin et al., 2014) selectable markers.

Despite these advances, there is still a lack of advanced genome engineering tools, which plays an essential role in developing *R. toruloides* as a workhorse for biotechnological applications. This is attributed to several factors. First, *R. toruloides* has a very high GC content (~62%) (Sambles et al., 2017), which hinders PCR reactions due to higher chances of forming secondary structures. This, in turn, disrupts polymerase efficiency and accuracy, impedes gene synthesis, and makes it difficult to design specific primers without reaching an extremely high annealing temperature; subsequently, almost all DNA fragments being used for genome engineering have to be codon optimized (Lin et al., 2014). Second, there is a lack of known ARS elements, meaning plasmids cannot be used to express heterologous genes and, instead, have to be always integrated into the genome (Tsai et al., 2017a).

Furthermore, integrating heterologous elements into the genome of *R. toruloides* is quite difficult, as the default mechanism of repairing double strand breaks (DSBs) in the DNA is non-homologous end joining (NHEJ) (Krappmann, 2007). In the case of NHEJ, the DSBs are directly ligated without a homologous template, which usually introduces several insertions and/or deletions (indels) into the DNA. This is in direct contrast to homologous recombination (HR), where a homologous piece of DNA must be present to guide the repair of the DSB, but the end result is much more accurate (Pardo et al., 2009). NHEJ and homologous recombination (HR) operate competitively in the cell, therefore, one strategy to improve gene targeting efficiency in *R. toruloides* is to inhibit or eliminate the NHEJ pathway, forcing the heterologous DNA to be

integrated via HR. The KU70/80 heterodimer is the main component of the NHEJ pathway in eukaryotes, and exists in organisms ranging from fungi to human (Daley et al., 2005; Lieber, 2011). Deleting the gene *KU70* (part of the KU70/80 complex) improves gene deletion and integration frequency without negatively affecting the oleaginous and fast growing features of *R. toruloides* (Koh et al., 2014).

A transformation methodology is required to deliver the new DNA fragment for the deletion or integration of genes into the yeast genome. Transformation methods have been described for *R. toruloides*, such as: the most commonly used one, *Agrobacterium tumefaciens*-mediated transformation (ATMT) (Lin et al., 2014; Liu et al., 2013); electroporation using an electrical field to make the cell membrane more permeable (Liu et al., 2017; Takahashi et al., 2014); and the most commonly used chemical method, lithium acetate/polyethylene glycol (PEG)-mediated transformation (Tsai et al., 2017a, 2017b).

Besides the availability of sequenced genomes, promoters, markers, strains with increased HR, and transformation methods it is also needed the capability of assembling pathways, which will be further discussed.

1.3 Overview of different assembly platforms

A crucial part of utilizing *R. toruloides* for industrial applications is the ability to engineer the metabolic pathways in the cell in an efficient and standardized manner (Park et al., 2017). Conventional approaches to pathway construction are limited in terms of automation, as well as when building complex regulatory circuits and multi-gene metabolic pathways. There has been an advancement in synthetic biology in the past decade that has led to the creation of several robust gene assembly platforms that can be exploited for metabolic engineering and pathway optimization. Two of these platforms, Sequence and Ligation-Independent Cloning (SLIC) (Li and Elledge, 2007) and Gibson assembly (Gibson et al., 2009), have similar mechanisms, based on *in vitro* homologous recombination and single strand annealing. Gibson assembly, combined with yeast *in vivo* recombination, has been used to assemble fragments several hundred kilobases long (Noskov et al., 2012). For one-step assembly of multi-gene pathways and combinatorial DNA libraries, Circular Polymerase Extension Cloning (CPEC) has been developed, using complementary fragment annealing and PCR overlap extension (Quan and Tian, 2011). While neither of these three assembly

methods has been utilized so far in *R. toruloides*, SLIC and Gibson assembly have been successfully used in *Y. lipolytica* (Rodriguez et al., 2016).

SLIC and Gibson assembly platforms, compared to conventional methods, offer an efficient way of constructing multi-gene pathways in one step, while being sequence-independent and scar-less as well. The disadvantage is that the terminal homology sequence has to be designed for each flanking element independently, which significantly slows down the design process and can be error-prone. Assembly of constructs with repeated sequences, such as promoters, ribosome binding sites (RBS), and terminators can lead to constructs with elements that are extra, missing, or arranged in the wrong order. Furthermore, as the overlap sequence is specified between junction elements, they cannot be used to shuffle gene order and diversify pathway configurations (Gibson et al., 2009; Li and Elledge, 2007; Quan and Tian, 2011).

A method that offers combinatorial strategy to pathway construction is the BioBrick assembly. In this approach, elements such as promoters, genes, terminators, etc., each have flanking regions of four compatible restriction enzymes, which can be ligated in any order to create a new configuration. After ligation, the enzyme recognition site is destroyed, and the original flanking site can be reused in the next round of assembly. As such, the BioBrick method can be used to combinatorially construct and optimize multi-gene pathways. A limitation of this mechanism is the generation of a sequence scar every ligation event and the multiple rounds of assembly required to build a functioning transcriptional unit (Shetty et al., 2008). BioBrick approach has been successfully established in *Y. lipolytica* (Wong et al., 2017), but there has been no report of a similar method in *R. toruloides*.

1.4 Golden Gate assembly

An assembly platform sidestepping the limitations described above while providing a way to construct, diversify, and optimize multi-gene pathways is Golden Gate assembly (GGA) (Engler et al., 2009, 2008). GGA exploits the ability of Type IIS restriction endonucleases (REases) to cleave outside of their recognition sequence. The recognition sites are strategically placed distal to the cleavage site of inserts and cloning vectors, and, as such, the endonucleases can remove the recognition site from the assembly. Therefore, as the overhang sequence is not dictated by the REase, no scar sequence is introduced, and the specificity of each overhang allows simultaneous

orderly assembly of multiple fragments. Digestion and ligation can be carried out at the same time, since the restriction site is eliminated from the ligated product (**Fig. 1)**. Consequently, several DNA fragments can be seamlessly assembled in the desired order in one reaction (Weber et al., 2011).

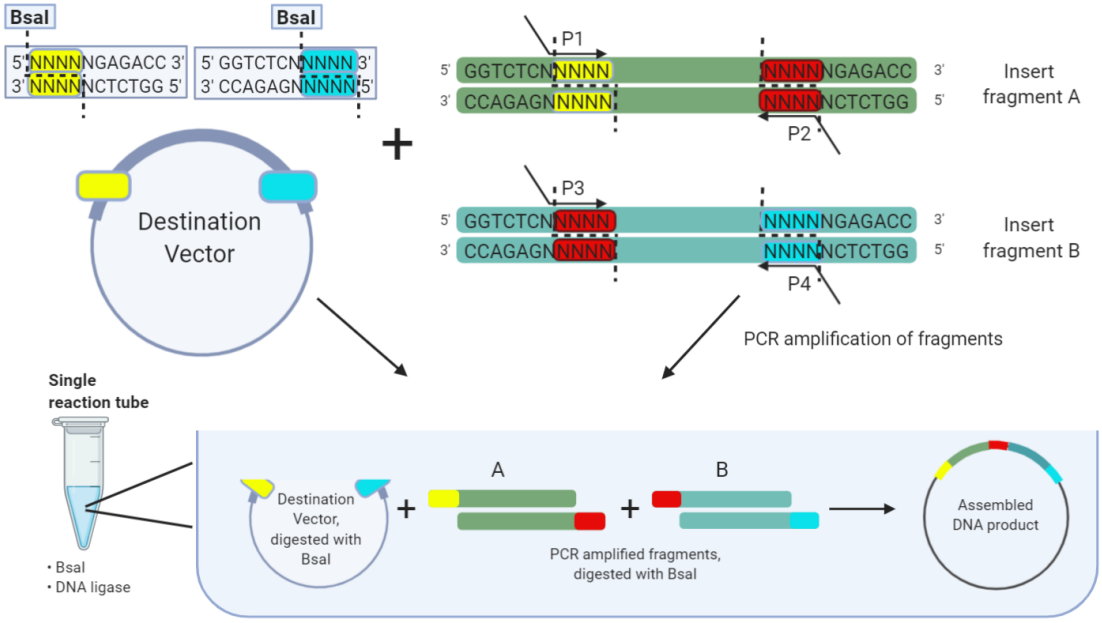

Figure 1. Golden Gate assembly. Insert fragments are amplified via PCR with their respective primers to introduce BsaI restriction sites and unique 4 base long overhangs. The purified amplicons, along with the destination vector, are incubated with the BsaI restriction enzyme and DNA ligase in a single reaction tube. After digestion by BsaI is done, the unique overhangs anneal and the DNA ligase ligates them into one final assembled DNA product. The image is adapted from https://international.neb.com/golden-gate/golden-gate. Created with BioRender.com

The Modular Cloning (MoClo) system, based on GGA, loses the advantage of scar-less cloning, but offers efficient hierarchical *in vitro* assembly of any eukaryotic multi-gene construct (Weber et al., 2011). One variant of MoClo, the Yeast Toolkit (YTK), expands upon this system by setting up three tiers of plasmids for storage or use (Lee et al., 2015). Parts, such as promoters, genes, terminators, etc., are stored in level I plasmids. At level II, level I plasmids are assembled together to create transcriptional units (TUs). Multiple TUs are assembled at level III into a multi-gene vector. The standardization of DNA fragments offers the opportunity to shuffle around parts in a one-pot reaction, making the diversification and optimization of engineered pathways a less laborious affair. YTK has been well adapted for the development of the GGA

platform in *Y. lipolytica* (Celińska et al., 2017), and *Kluyveromyces marxianus* (Rajkumar et al., 2019), but not yet to *R. toruloides*.

This work is aimed at the development of a *R. toruloides* YTK-dedicated platform in order to fill the gap of advanced metabolic engineering tools for this biotech workhorse. The proof of concept of such platform was done by the simultaneous deletion of *KU70* and overexpression of three native genes of the carotenoids pathway: geranylgeranyl diphosphate synthase (*CRTE*), phytoene dehydrogenase (*CRTI*), and phytoene synthase (*CRTYB*).

2 THE AIMS OF THE THESIS

To fully realize the engineering potential of the biotechnology workhorse *R. toruloides*, it is necessary to fix the lack of advanced genome engineering tools available for this non-conventional yeast. Golden Gate assembly is an efficient one-pot method of assembling several DNA fragments simultaneously, and so far has not been successfully adapted to the GC-rich genome of *R. toruloides*.

The aims of this thesis are:

- Establishing a dedicated GGA methodology in *R. toruloides.*
- Deleting the *KU70* gene to increase the rate of homologous recombination.
- Validating the efficiency of GGA platform by overexpressing the three native genes in carotenoids biosynthesis pathway.: geranylgeranyl diphosphate synthase (*CRTE*), phytoene dehydrogenase (*CRTI*), and phytoene synthase (*CRTYB*).

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Plasmids

The backbone plasmid used for the cloning of all level I parts is pSB1K3-RFP from the iGEM collection (BBa_J04450, http://parts.igem.org/Part:BBa_J04450). It is a high copy number plasmid carrying kanamycin resistance. For cloning of level II and level III plasmids, pSB1C3-RFP from the iGEM collection (BBa_J04450, http://parts.igem.org/Part:BBa_J04450), pGGA (New England Biolabs) or pGGA containing a red fluorescent protein (RFP, BBa_E1010, http://parts.igem.org/Part:BBa_E1010) (this study) were used. All three plasmids carry chloramphenicol resistance gene.

3.1.2 DNA sequences

The primer sequences used for removing internal BsaI cutting sites as well as to amplify all parts with their respective overhangs for the GGA can be found in **Supplementary Table 1**. The genomic DNA from *R. toruloides* CCT 0783 (Coleção de Culturas Tropicais, Fundação André Tosello, Campinas, Brazil; synonym IFO10076), herein called SBY29, was used as a template for the amplification of insertional region, promoters, and genes from the carotenoids pathway. The genomic extraction was performed according to a previously published protocol (Lõoke et al., 2011). For the insertional region, five hundred base pairs fragments upstream and downstream the *KU70* gene (DNA-dependent ATP-dependent helicase subunit 70, RHTO_06014) were amplified with primers 440/508 and 507/444 . The promoters from the genes glyceraldehyde-3-phosphate dehydrogenase (*GPD*, RHTO_03746), alcohol dehydrogenase 2 (*ADH2*, RHTO_03062), and xylose reductase (*XYL1*, RHTO_03963) were obtained by amplifying 786, 725, and 746 bp upstream the starting codon, respectively (Díaz et al., 2018; Sun et al., 2017). The genes geranylgeranyl diphosphate synthase (*CRTE*, RHTO_02504), phytoene dehydrogenase (*CRTI*, RHTO_04602), and phytoene synthase (*CRTYB*, RHTO_04605) were amplified and used for proof of concept aiming at improving carotene production. Nopaline synthase terminator (tNOS) from *Agrobacterium tumefaciens* (GenBank No. MF116010) was amplified using primers 199/200. The geneticin resistance gene (*G418*) was codon optimized for *R. toruloides* using an in house tool (https://github.com/SynBioUniTartu/R.toruloides)

and synthesized in two gBlock fragments due to high GC content by IDT (Leuven, Belgian). Both fragments of G418 were combined by overlapping PCR using primers 215/218 and further assembled with XYL1 promoter (pXYL) and tNOS by GGA, herein called marker (M).

3.1.3 DNA amplification by polymerase chain reaction (PCR)

All sequences over 3 kb were amplified via PCR using high-fidelity Phusion DNA Polymerase (Thermo Fisher Scientific). Otherwise, DreamTaq Green PCR Master Mix (2X) (Thermo Fisher Scientific) was used instead. All reactions were set up and performed according to the manufacturer's instructions. Following PCR, the reactions with Phusion polymerase were stained with 6X TriTrack DNA Loading Dye (Thermo Fisher Scientific). The samples were loaded on a 1% agarose gel and the electrophoresis was run at constant voltage of 120 V. The fragments sizes were estimated using GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher Scientific). For GGA assembly, the amplicons with correct size were excised from the gel and purified via FavorPrep™ GEL/PCR Purification Kit (Favorgen) following manufacturer's instructions. The fragments were quantified using Thermo Fisher's NanoDrop 1000 Spectrophotometer.

3.1.4 Construction of pGGA carrying a red fluorescence protein (RFP) for faster screening

pGGA was amplified using primers 497/498, while RFP was amplified using primers 499/500. The following mixture was set up for the subsequent Gibson Assembly (Gibson et al., 2009) reaction: 1 µL of pGGA, 3 µL of RFP, 10 µL Gibson Assembly Master Mix (New England Biolabs) in a final concentration of 2x, 6 µL of deionized H_2O. The sample was incubated at 50°C for 1 hour. Two microliters of the assembly reaction were taken for the consequent bacterial transformation.

3.1.5 Assembly of levels I, II, and III constructs by GGA

The level I Golden Gate comprised the storage of every single part (promoters, genes, marker, insertional region, and terminator) in a plasmid. Assembly reactions were set up in the following manner: 75 ng of pSB1K3, 150 ng of insert purified after electrophoresis, 2 µL of T4 DNA Ligase Buffer (New England Biolabs), 1 µL of T4 DNA Ligase (New England Biolabs), 1 µL of BsaI-HFv2 (New England Biolabs), up to 20 µL of nuclease-free H_2O. The mixture was incubated at 37°C for 1 hour, followed by 5 minutes at 60°C.

The level II of GGA was the assembly of a gene with promoter and terminator, resulting in a transcriptional unit (TU). The genes *CRTE*, *CRTI*, and *CRTYB* were combined with all three promoters (pXYL, pGPD, and pADH2), and tNOS using the pGGA plasmid as a vector. A total of nine TUs were generated. The reactions were done using 150 ng of each part of the TU and either used the same recipe as level I or by using the NEB Golden Gate assembly Mix (New England Biolabs) according to the manufacturer's instruction.

The final step of the GGA platform is to assemble the pathway with a selection marker and the insertional regions for guiding the pathway integration with simultaneous deletion of a target gene (**Fig. 2**). First, it was necessary to reinsert the flanking BsaI cutting sites of the TUs, marker, and insertional regions. This was done by PCR amplification followed by subcloning the aforementioned parts into pCR-XL-2-TOPO™ Vector (Thermo Fisher Scientific). The insertional regions were amplified by level I, while TUs and marker were amplified from level II using Platinum™ SuperFi™ Green PCR Master Mix (Thermo Fisher Scientific). After gel electrophoresis, bands with the correct sizes were cut out of the gel and purified using PureLink™ Quick Gel Extraction and PCR Purification Combo Kit (Thermo Fisher Scientific). The reaction for TOPO™ Cloning was assembled as follows: 1 µL of pCR-XL-2-TOPO™ Vector, X µL of insert required for 5:1 molar ratio of insert:vector, 1 µL of Salt Solution (Thermo Fisher Scientific), up to 6 µL of deionized H_2O. The mixture was incubated at 25°C for 1 hour. Two microliters of the cloning reaction were used for bacterial transformation. All other reactions mentioned above were performed according to the instructions provided in TOPO™ XL-2 Complete PCR Cloning Kit (Thermo Fisher Scientific).

The reaction to assemble level III GGA was prepared as follows: 75 ng of pGGA, mass of each insert (TOPO™ plasmid) required for 2:1 molar ratio of insert:vector, 2 µL of T4 DNA Ligase Buffer (New England Biolabs), 1 µL of NEB Golden Gate assembly Mix (New England Biolabs), up to 20 µL of nuclease-free H_2O. The reaction was performed as instructed by the official NEB GGA kit.

All assemblies from levels I, II, and III and TOPO™ subcloning were used for bacterial transformation. The constructions were screened by colony PCR and colonies containing the correct constructs were grown overnight using 4 mL of LB medium and selection antibiotics. One milliliter of culture was stored at -80°C and glycerol at 15% (v/v) and the re-

maining volume was used for plasmid purification using FavorPrep™ Plasmid DNA Extraction Mini Kit (Favorgen). All constructs were validated by DNA sequencing.

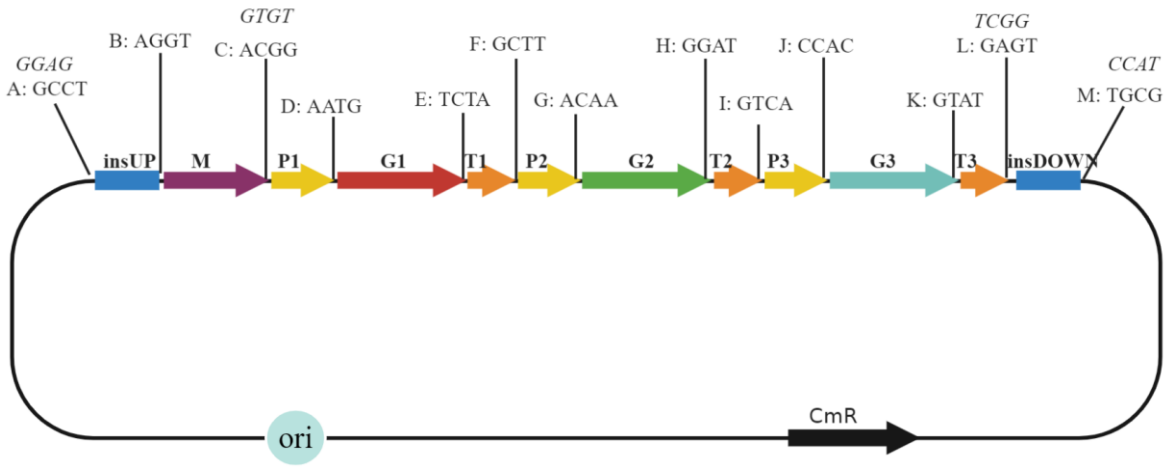

Figure 2. The Golden Gate assembly platform in a three-transcription unit (TU)-bearing format. InsUP/DOWN - sequence targeting insertion in the 5'/3' region; M - selection marker; P1/2/3 - promoter in transcription unit 1, 2, and 3, respectively; G1/2/3 - gene of interest in transcription unit 1, 2, and 3, respectively; T1/2/3 - terminator located in transcription unit 1, 2, and 3, respectively. The overhangs in *italic* are the changes made in the *Yarrowia lipolytica* GGA format to make it compatible with *R. toruloides*. Adapted from (Celińska et al., 2017). Created with BioRender.com

3.1.6 Bacterial transformation

Escerichia coli DH5α cells were taken out of -80°C freezer and left on ice to thaw. After thawing, 100 µL of the cell solution was added to the Eppendorf tube containing two microliters of GGA or TOPO reaction and resuspended gently. After keeping the mixture on ice for 30 minutes, it was heat shocked at 42°C for 2 minutes and put back on ice for another minute. 400 µL of LB media (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) was added to the transformation reaction and placed in a 220 rpm shaker at 37 °C for 1 hour. Next, the tube was centrifuged at 6000 rpm for 1 minute. Hundred µL of cells was plated on LB plates containing either 100 µg/mL kanamycin or 25 µg/mL chloramphenicol and incubated overnight at 37 °C.

3.1.7 Yeast transformation and screening

R. toruloides was transformed with the final level III construct. The strain was inoculated in 10 mL of YPD medium (glucose, 20 g/L; yeast extract, 10 g/L; peptone, 20 g/L) and

incubated overnight with a stirring speed of 160 rpm at 30 °C. The culture was diluted to an OD600 of ~0.2 in 10 mL of YPD and incubated againuntil an OD600 of ~0.8. The culture was harvested in a sterile 50 mL centrifuge tube at 3,000 *g* for 10 minutes. The cell pellet was resuspended in 25 mL of sterile water and centrifuged again. The cells were resuspended in one milliliter 100 mM Lithium Acetate (LiAc) (pH 7.5) and 500 µL of the suspension was transferred to a 1.5 mL microfuge tube. The cells were centrifuged at 3,000 *g* for 30 seconds and the LiAc was removed. In the following order, 240 µL of polyethylene glycol (PEG) 4000 (50% w/v), 36 µL of 1M LiAc (pH 7.5), 24 µL of sterile water were added. 10 µL of salmon sperm DNA (10 mg/mL), boiled at 100 °C for 10 minutes, was transferred to the tube before adding 50 µL of transforming DNA (0.1-10 µg). The cell pellet was mixed completely by vigorous vortexing. After incubating the tube at 30 °C for 30 minutes, 34 µL of dimethyl sulfoxide (DMSO) was added to the mixture, which was then heat shocked at 42°C for 15 minutes. The tube was centrifuged at 3000 *g* for 30 seconds and the supernatant was removed two times. The pellet was resuspended in 2 mL of YPD and transferred to a new 15 mL Falcon tube, which was incubated overnight with shaking at 30 °C. The following day the suspension was plated onto YPD plates containing G418 and left to grow at 30 °C for two days.

For screening, five colonies and the parental strain were grown in 10 mL of YPD and YPX (xylose, 20 g/L; yeast extract, 10 g/L; peptone, 20 g/L). After incubating at 30°C and 160 rpm for 48 hours, 1 mL of each culture was taken aseptically and centrifuged for 5 min at 3,000 *g*. The cell pellets were used for carotenoids extraction, identification, and quantification. Two candidates with the highest carotenoids content, herein called SBY91 and SBY92, were selected for further strain characterization in shake flasks.

3.1.8 Characterization of the strains in shake flasks

The strains SBY29 (control), SBY91 and SBY92 were characterized in terms of growth, sugar consumption, and carotenoids production. Glucose and xylose were used as a carbon source for the characterization of strains SBY91 and SBY92, respectively. Mineral medium, containing, per liter, 30.0 g carbon source, 0.8 g $(NH_4)_2SO_4$, 3.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7\ H_2O$, 1.0 mL vitamins solution and 1.0 mL trace metal solutions was used (Lopes et al., 2020). The carbon to nitrogen (C/N) ratio of the medium was 80 (mol/mol). The cells were incubated at 30 °C and a stirring speed of 160 rpm for 96 h. Samples for OD600, carotenoids and metabolites analyses were taken every 24 h.

3.1.9 Analytical methods

Concentrations of xylose, glucose, organic acids and glycerol were measured using High Pressure Liquid Chromatography (HPLC) (LC-2030C Plus, Shimadzu, Kyoto, Japan) equipped with HPX-87H column (Bio-Rad, CA, USA) and a refractive index detector (RID-20A, Shimadzu, Kyoto, Japan), at 45^{o}C and 5 mmol/L H_2SO_4 as mobile phase with isocratic elution at 0.6 mL/min.

Carotenoids were extracted and quantified using a modified method from Lee (2014). One milliliter of broth was centrifuged at 3000 *g* for 5 min and the cells were washed twice in distilled water, followed by resuspension in 1.0 mL of acetone. Cell lysis was done using acid-washed glass beads (400 – 650 μm) and the FastPrep homogenizer for 3 cycles (4 m/s for 20 s) (MP Biomedicals, CA, USA). Centrifugation at 15,000 *g* for 5 min was used and the acetone solution containing carotenoids was collected and kept at 4°C. The pellet was resuspended in 1 mL of acetone and a new round of lysis was carried out. These steps were repeated until the cell debris was colorless. The acetone-carotenoids containing solution from different extraction rounds were pooled together and evaporated in Concentrator Plus (Eppendorf, Hamburg, Germany). The extracted carotenoids were solubilized in a known volume of acetone and measured using Acquity Ultra Pressure Liquid Chromatography (UPLC) (Waters, MA, USA) equipped with a tuneable ultraviolet (TUV) detector (Waters, MA, USA) at 450 nm (modified from Weber, 2007) and C18 column (BEH130, 1.7 μm, 2.1 x 100 mm, Waters, MA, USA). A gradient elution from 80 to 100% of acetone in milli-Q water at a flow rate of 0.2 mL/min was used. Detected peaks were identified according to the known carotenoid retention time profile (Weber, 2007; Lee, 2014). A calibration curve using β-carotene (Alfa Aesar, MA, USA) was used for quantification.

3.2 RESULTS

3.2.1 Assembling level I, II, and III constructs with GGA

The goal of this work was to develop a standardized and versatile system of Golden Gate assembly (GGA) platform for *Rhodotorula toruloides*. A dedicated GGA platform enables fast construction of complex expression cassettes for metabolic engineering in *R. toruloides*. A set of thirteen 4-nucleotide (nt) overhangs from *Y. lipolytica* GGA system (Celińska et al., 2017) was initially used for this task. This set covered all three transcription units (TUs, containing P – promoter, G – gene, T - terminator), selection marker M, and upstream and downstream insertional units, insUP and insDOWN, respectively (**Fig. 2**).

For the assembly of level I, the parts library, the vector pSB1K3_RFP from the iGEM collection was used. For each position, the plasmid, along with the sequence to be used as the building block of the GGA, was PCR amplified using the corresponding primers containing the BsaI recognition site and the correct overhang (**Supplementary Table 1**). In the case of a successful round of GGA reaction and transformation, the original red colonies of the pSB1K3_RFP plasmid were changed to white ones, as the GGA building block was inserted instead of the RFP reporter gene. Therefore, allowing a quick and easy prescreening method.

The parts library (**Fig. 3A**) was successfully assembled and contained three strong promoters from the genes glyceraldehyde-3-phosphate dehydrogenase (*GPD*), alcohol dehydrogenase 2 (*ADH2*), and xylose reductase (*XYL1*); the three genes from the carotenogenic pathway, geranylgeranyl diphosphate synthase (*CRTE*), phytoene dehydrogenase (*CRTI*), and phytoene synthase (*CRTYB*) the terminator of nopaline synthase (tNOS); two insertional regions, 500 bp upstream and downstream the *KU70* gene (DNA-dependent ATP-dependent helicase subunit 70), insUP and insDOWN, respectively; the geneticin resistance gene (G418). All parts were verified by DNA sequencing.

The level II consisted of PCR amplifying parts from the library to add the BsaI cutting sites and the correct overhangs for the assembly of a TU by GGA. In total, nine TUs were assembled by having all three carotenogenic genes under the three different promoters (**Fig. 3B**). The TUs containing *CRTI* and *CRTYB* were assembled by GGA using manufacturer's instructions and pSB1C3_RFP plasmids **(Fig 4A).** For the remaining TUs, switching the

pSB1C3 plasmid to pGGA and changing the one-pot assembly methodology to a two-step protocol was required to produce a successful Golden Gate reaction (Larroude et al., 2019) (**Fig. 4B**). The RFP reporter gene from the iGEM collection (BBa_E1010, http://parts.igem.org/Part:BBa_E1010) was inserted into the NEB commercial pGGA plasmid for the purpose of easier screening. All nine TUs were assembled effectively and confirmed by sequencing.

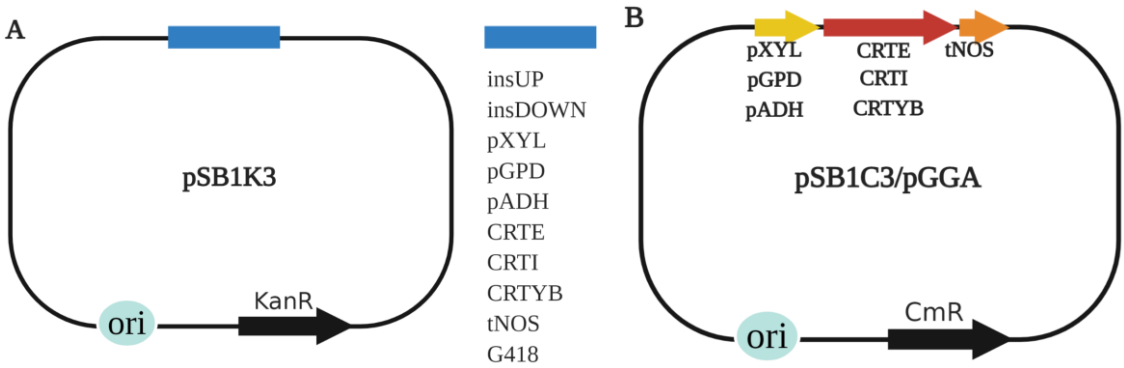

Figure 3. Level I parts library and construction of TUs in level II. A) All three promoters, four genes, two insertional units, and one terminator were cloned into pSB1K3 plasmid. B) Assembly of level II constructs, consisting of different combination of the promoters, genes, and terminator, by GGA. Legend: ori – origin of replication; KanR – kanamycin resistance gene; CmR – chloramphenicol resistance gene. Created with BioRender.com

For level III construction, it was required to assemble into pGGA the following PCR amplified parts: InsUP, Marker, TU1, TU2, TU3, and InsDOWN. The first several tries of assembling the level III were unsuccessful even by using pGGA plasmid, trying different concentrations of enzymes (BsaI and T7 or T4 DNA ligase) and buffer, different pre-assembly strategies, or even switching to the official NEB GGA kit. Suspecting that the similarities between some of the overhangs might be the reason for the continued failure to assemble the level III plasmid, new primers containing new overhangs were designed for some of the building blocks (**Fig. 2**). As this change also did not result in a positive outcome, it was decided to subclone each building block into the TOPO™ cloning system, as it allows ligating blunt PCR fragments without the need for any specific format. The subcloning of insUP and insD from level I worked by using the manufacturer's protocol, but for subcloning the TUs and marker it was required to optimize the protocol. Different ratios of TU fragment to TOPO™ vector were used, and a 5:1 molar ratio was found to be the most efficient (data not shown).

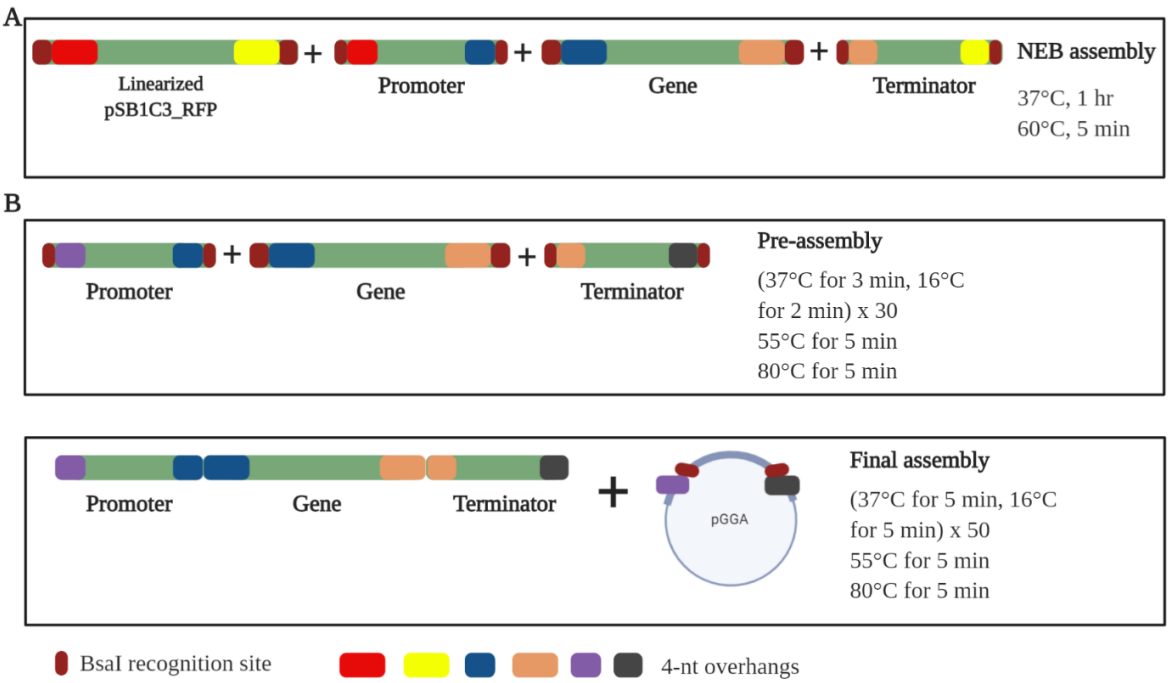

Figure 4. Difference in methodologies for level II assembly. A) Method for assembling a GGA reaction with three inserts using a linearized plasmid, according to the official NEB GG kit instructions. B) Two-step assembly protocol adapted from Larroude et al. (2019), using the commercial pGGA plasmid with built-in overhangs and BsaI recognition sites. Created with BioRender.com

A level III construct containing the *CRTE*, *CRTI*, and *CRTYB* genes under pXYL, pGPD, and pADH2, respectively, was assembled by GGA without the need of any pre-assembly, (**Fig. 5**). This pathway was used for the transformation of the parental strain (SBY29) and the results will be presented in **section 3.2.3**.

3.2.2 Deletion of the *KU70* gene

A deletion cassette (DC) for the deletion of *KU70* was constructed aiming at verifying if it is possible to improve transformation efficiencies by HR instead of NHEJ repair. The DC, containing ~500 base pairs upstream and downstream sequences of the *KU70* gene (insUP and insDOWN, respectively), promoter of the xylose reductase gene (pXYL), the geneticin resistance gene (*G418*), and the NOS gene terminator (tNOS), was assembled using Golden Gate assembly. Transforming the DC into *R. toruloides*, the correct insertion was confirmed by selective plating in YPD-G418 agar plates, PCR verification, and sequencing.

The ΔKU70 strain, hereby called SBY85, was transformed alongside parental strain SBY29 with a different deletion cassette (courtesy of Tobias Eichinger) containing nourse-

othricin (NAT) resistance gene. Nine colonies of transformed SBY29 and 54 colonies of transformed SBY85 grew on YPD-NAT agar plates, showing a 6-fold increase in integration efficiency of KU70 deficient strain.

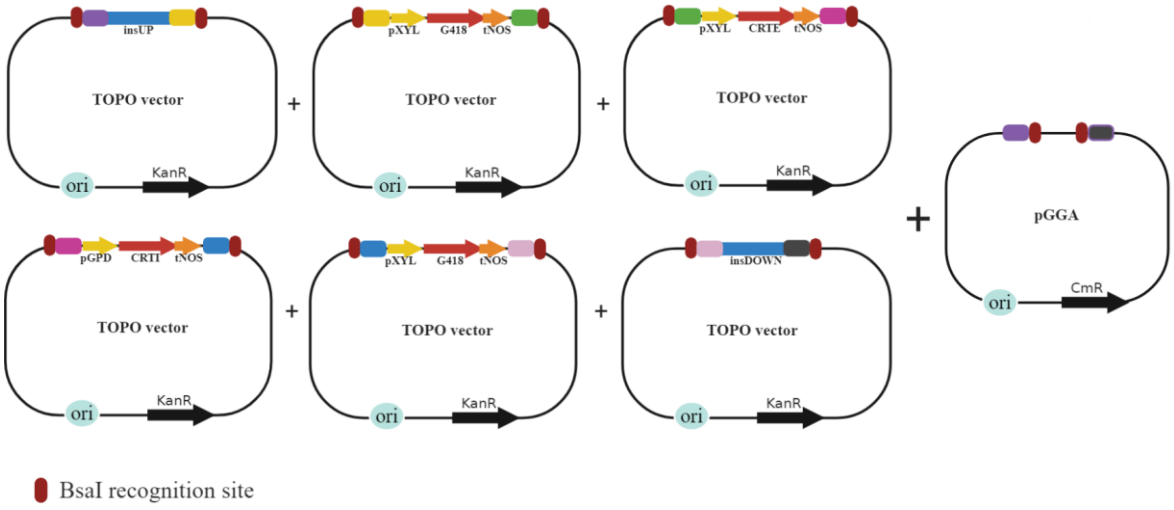

Figure 5. Assembly of level III construct. InsUP, insDOWN, Marker, and three TUs were all individually cloned into TOPO™ vectors, conserving their overhangs and BsaI recognition sites. The six TOPO™ vectors, along with pGGA, were used in a one-pot official NEB GG assembly reaction. Created with BioRender.com

3.2.3 Proof of concept

To validate the efficiency of the developed GGA platform, the final level III construct, containing three TUs, the selection marker, and the insertional units, was transformed into *R. toruloides* SBY29. The three genes in this assembly were geranylgeranyl diphosphate synthase (*CRTE*, RHTO_02504) in G1 position, phytoene dehydrogenase (*CRTI*, RHTO_04602) in G2 position, and phytoene synthase *(CRTYB*, RHTO_04605) in G3 position. As the genes code for the main enzymes in the carotenoid production pathway (**Fig. 6**), their overexpression was expected to lead to increased production of carotenoids, observable by different intensities of the orange (β-carotene) or pink (torulene and torularhodin) color development in transformed colonies. As the yeast produces those carotenes naturally it was not possible to detect by eye difference in the intensity of the coloration of the transformed colonies. Therefore, five colonies were randomly selected for further screening experiment.

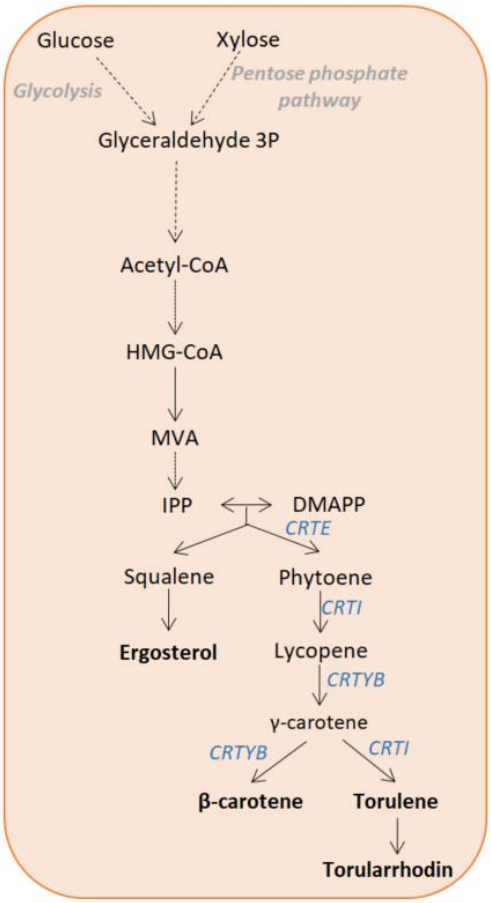

Figure 6. Carotenoid biosynthesis in *R. toruloides*. Geranylgeranyl diphosphate synthase (CRTE) catalyzes the formation of an important precursor of carotenoids, phytoene. Phytoene dehydrogenase (CRTI) and phytoene synthase (CRTYB) are both involved in the production of γ-carotene and downstream formation of β-carotene (CRTYB) and torulene (CRTI). Legend: 3P – 3-phosphate; CoA – coenzyme A; HMG – 3-hydroxy-3-methylglutaryl; MVA – mevalonic acid; IPP – isopentenyl pyrophosphate; DMAPP – dimethylallyl pyrophosphate. Figure courtesy of Marina Julio Pinheiro.

Colonies 8, 13, 20, 28, and 29 were selected from the plate and grown for 48 h in YPD (**Fig. 7**) and YPX media (**Fig. 8**), resulting in the possibility to see different intensities in both broth and pellets. Colony 28 showed higher intensity than the parental strain when grown in YPD, while colonies 13, 20, and 29 seemed to have more carotenoids in YPX. Surprisingly, colony 28 had impaired growth in YPX, as can be seen by (**Fig. 9A**). Carotenoids quantification by HPLC showed that colony 28 and colony 29 had the highest increase in total carotenoids compared to the parental strain (**Fig. 9B**). As such, they were chosen to study for further characterization. Colony 28 and 29 from now on will be referred to as SBY91 and SBY92, respectively.

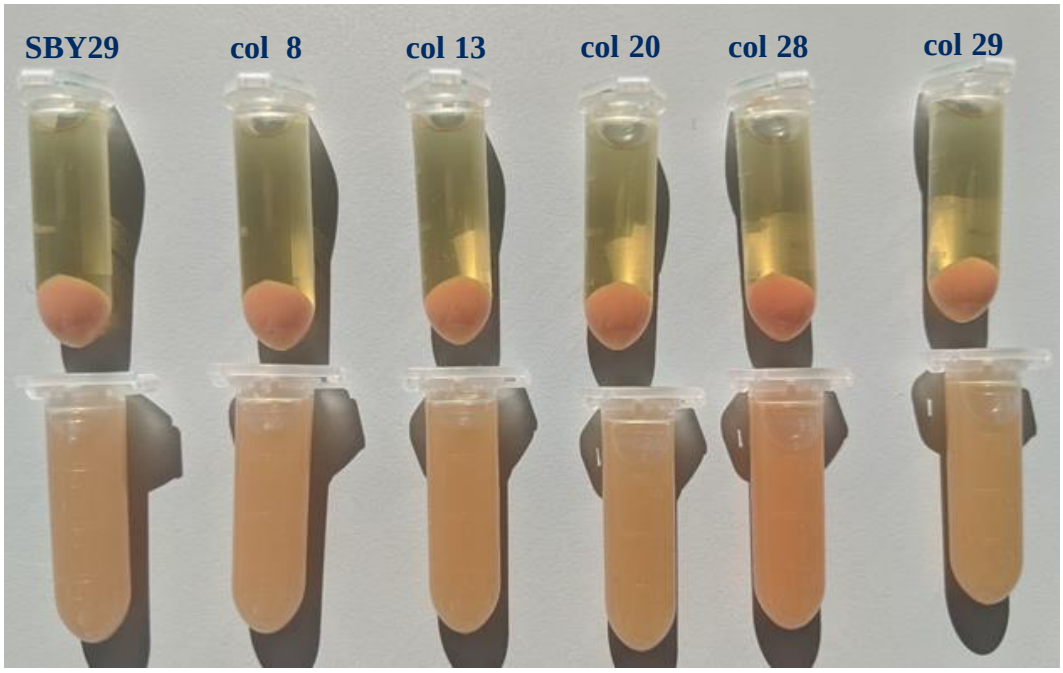

Figure 7. Five colonies and SBY29 grown in YPD. Colonies 8, 13, 20, 28, and 29, along with the parental strain SBY29, were grown in YPD for 48 hours. The difference in intesities for colony 28 can be seen in both pellet and broth.

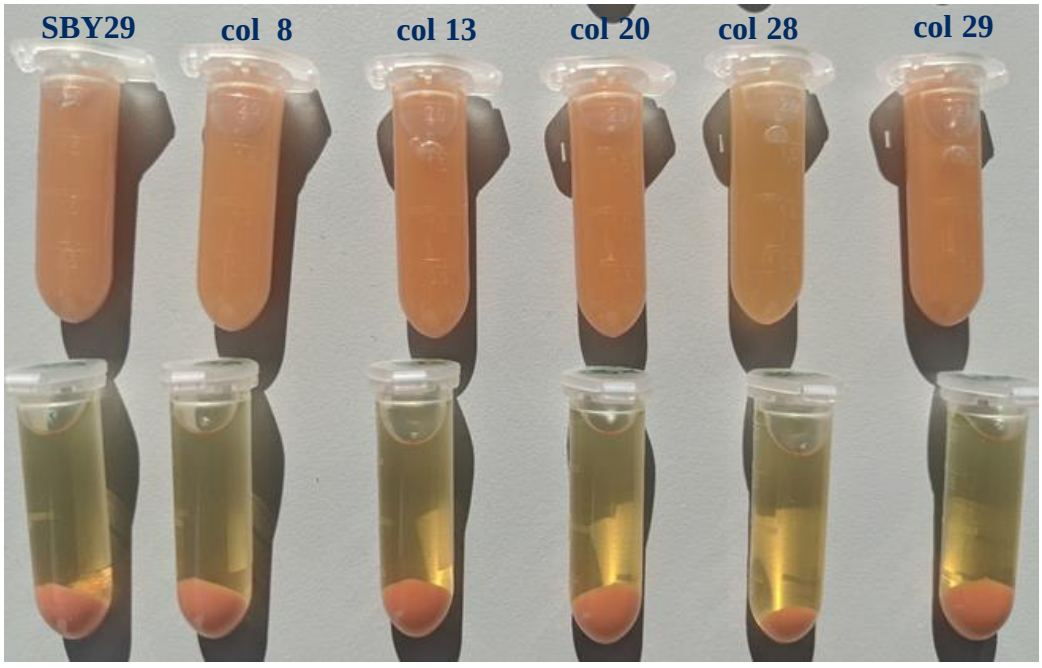

Figure 8. Five colonies and SBY29 grown in YPX. Colonies 8, 13, 20, 28, and 29, along with the parental strain SBY29, were grown in YPX for 48 hours. The difference in intensities for colonies 13, 20, and 29 can be seen in both pellet and broth.

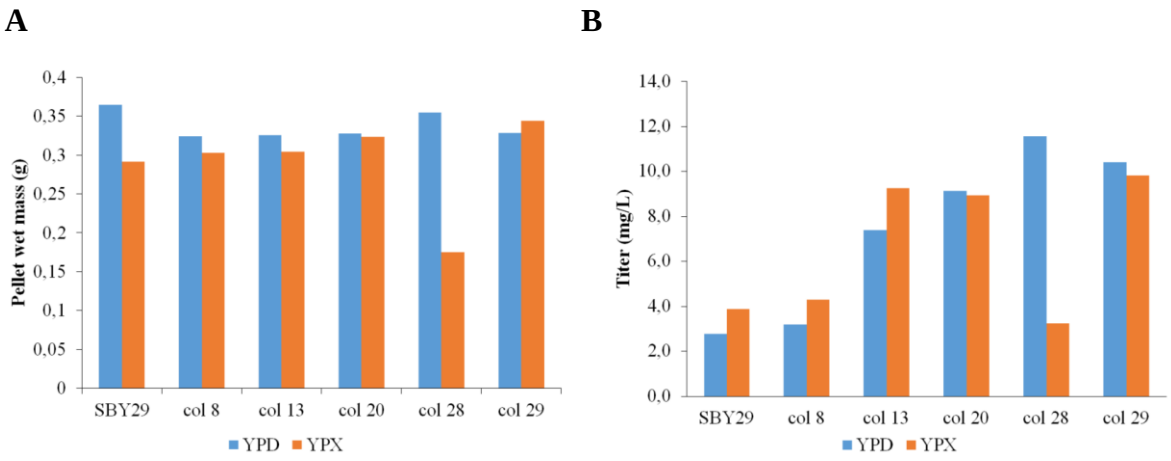

Figure 9. Wet biomass and total carotenoids of five colonies and SBY29. A) Wet biomass of colonies 8, 13, 20, 28, and 29, along with SBY29. B) Total carotenoids of colonies 8, 13, 20, 28, 29, along with SBY29.

3.2.4 Characterization of SBY91 and SBY92

Cultivation of SBY29 and SBY91 in minimal medium containing either glucose or xylose as carbon source for 120 hours revealed decreased biomass and glucose consumption of SBY91 compared to the parental strain (**Fig. 10A, Fig. 11**). A possible reason for this occurrence could be non-homologous recombination during transformation, which would result in the repair fragment integrating at the wrong place in the genome, possibly disrupting the expression of an important gene. Total carotenoids in SBY91 increased by 173% compared to SBY29 (**Fig. 11**). The carotenoids composition of SBY29 and SBY91 were almost identical (**Fig. 12A**).

Conversely, cultivation of SBY29 and SBY92 in minimal medium and xylose as carbon source for 120 hours did not yield any significant differences between the two strains (**Fig. 10B, Fig. 11**), indicating that the increased carotenoids production was only due to redirected flux caused by overexpressing CRTE, CRTI, and CRTYB. Total carotenoids in SBY92 increased by 210% compared to SBY29 (**Fig. 11**). The percentage of torulene produced by SBY92 increased on account of β-carotene decreasing (**Fig. 12B**).

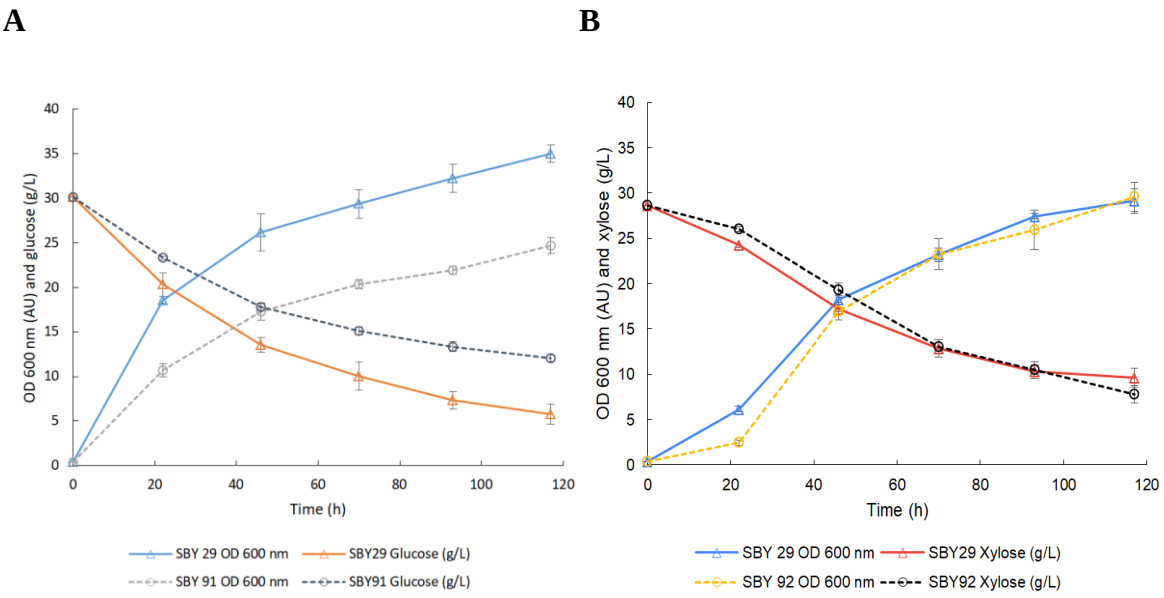

Figure 10. Growth and carbon consumption of SBY91 and SBY92 compared to SBY29. A) SBY29 and SBY91 were grown in YPD for 120 hours. SBY91 showed impaired growth and glucose consumption compared to SBY29. B) SBY29 and SBY92 were grown in YPX for 120 hours; the physiological profiles of both strains were identical.

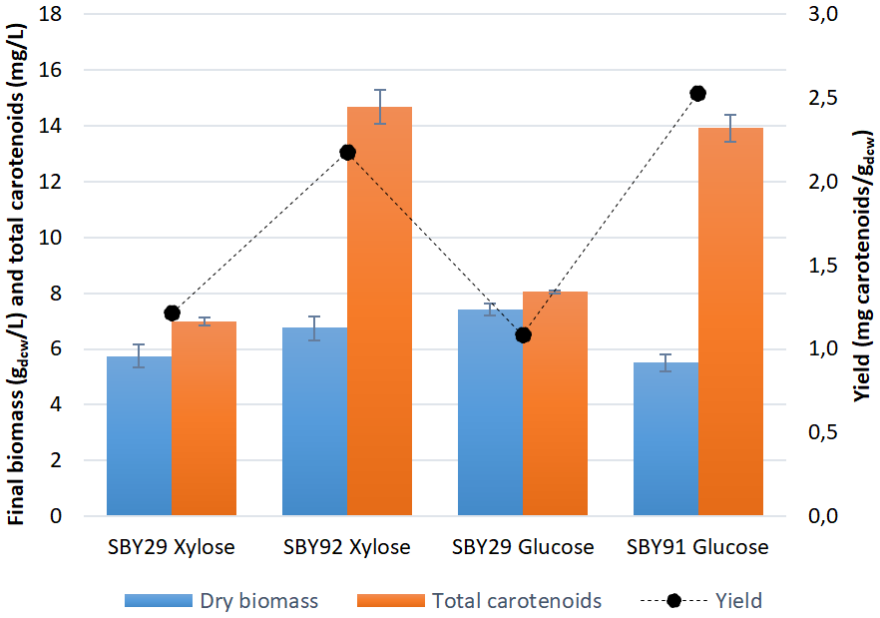

Figure 11. Biomass and carotenoids production by SBY29, SBY91, and SBY92. Final biomass for SBY29 and SBY92, grown on xylose, were almost identical, while SBY91 had impaired growth compared to SBY29 when grown on glucose. Both SBY91 and SBY92 showed ~2-fold increase from SBY29 in total carotenoids produced, grown on glucose and xylose, respectively.

A **B**

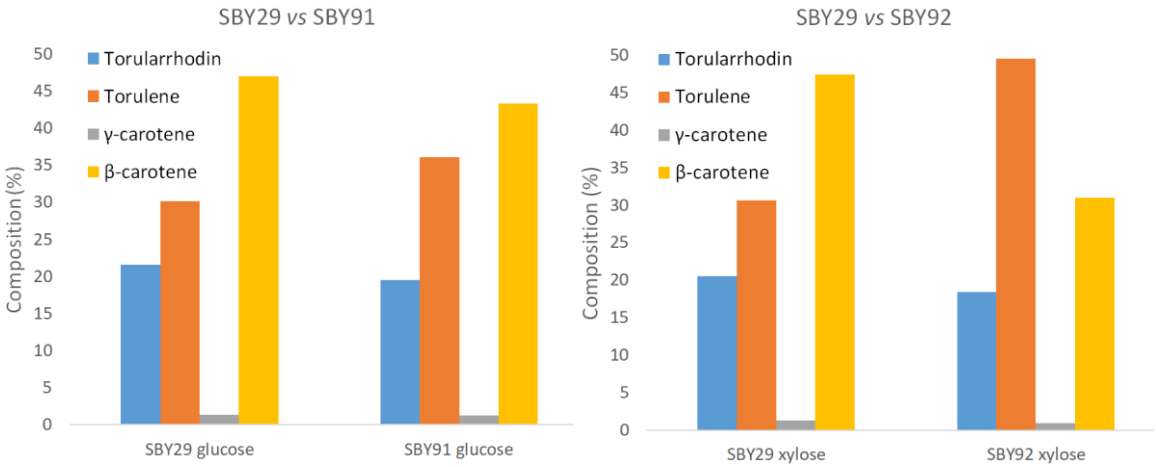

Figure 12. Carotenoids composition of SBY29, SBY91, and SBY92. A) SBY29 and SBY92 have almost identical composition in glucose media. B) SBY92 has higher torulene percentage compared to SBY29, where β-carotene has a larger proportion, suggesting a stronger activity of pGPD.

3.3 DISCUSSION

The foundation of synthetic biology lies in standardized biological parts that allow us to build new biological systems in a more efficient manner and offer better design for metabolic engineering approaches. The Yeast Toolkit, a diverse collection of parts built several years ago, showcases how crucial standardization is for synthetic biology. The YTK has a wide range of regulatory elements and selection markers, which makes engineering the genome and fine-tuning gene expression a much less laborious affair. It is clear to see that turning alternative yeasts into a viable option for wider use in biotechnology requires similar kits to make swift metabolic engineering possible. Maintaining similar standards for assemblies is also helpful for exchanging parts between different yeasts.

With this goal in mind, we have developed a dedicated Golden Gate assembly platform for *Rhodotorula toruloides*, based on the YTK standard and various parts collections developed for other yeasts (Celińska et al., 2017; Prielhofer et al., 2017; Rajkumar et al., 2019). Due to large differences in the genus of *R. toruloides* and other yeasts with dedicated GGA platforms, it was necessary to extensively optimize almost all steps of the assembly. *R. toruloides* has a very GC-rich genome, leading to an outcrop of problems, from extremely high melting temperatures during primer design to the formation of secondary structures. One of the key changes that resulted in a successful assembly of the final construct was switching from linearized DNA parts to plasmids, which allows the restriction enzyme to anchor to the DNA more efficiently. Even while using the TOPO™ Cloning technology that had been developed for the purpose of easy cloning, it was required to optimize the established method to accommodate the GC-rich genome of *R. toruloides*.

The efficiency of the developed GGA was validated by overexpressing the three most important genes in the carotenoids production pathway - geranylgeranyl diphosphate synthase (*CRTE*), which catalyzes the formation of an important precursor of carotenoids; phytoene dehydrogenase (*CRTI*) and phytoene synthase (*CRTYB*), both of which are involved in the production of γ-carotene and downstream formation of β-carotene (CRTYB) and torulene (CRTI) (**Fig. 4**). Regulating the expression of these genes by the promoters of xylose reductase (pXYL), glyceraldehyde 3-phosphate dehydrogenase (pGPD), and alcohol dehydrogenase 2 (pADH2) ensured their overexpression, as pGPD

and pADH2 are high constitutive promoters. pXYL is also a strong constitutive promoter with even higher activity when xylose is present.

The final two strains, chosen based on their carotenoids production compared to the parental strain SBY29, showed different physiological profiles. SBY91 had impaired growth and showed decreased growth rate and glucose consumption when compared to SBY29 (parental strain). This could be explained by non-homologous recombination happening during transformation, leading to the integration of the construct at a detrimental place in the genome. Although there were differences in physiology, this strain showed an increase of 178% in carotenoids production. SBY92, on the other hand, had nearly identical physiological profile when compared to SBY29, showing that the 2-fold increase in the total carotenoids yield was the result of redirected flux towards carotenoid biosynthesis. The carotenoids composition of SBY92 was different from SBY29, the percentage of torulene increasing on the expense of β-carotene, suggesting that pGPD, regulating CRTI, was unexpectedly a stronger promoter than pADH2, which was regulating CRTYB.

SBY91, grown on glucose, showed 13,9 mg/L total carotenoids and 2,53 mg carotenoids/gDCW, while SBY92, grown on xylose, had 14,7 mg/L total carotenoids and 2,18 mg carotenoids/gDCW. For *R. glutinis* cultivated in brewery effluent, the literature reports 1,20 mg/L total carotenoids (Schneider et al., 2013), while culturing the same yeast in crude glycerol resulted in 135,25 mg/L produced (Saenge et al., 2011). The concentrations for carotenoids produced by *R. toruloides* grown on glucose range from 14,0 to 33,4 mg/L (Dias et al., 2015). During cultivation of *R. glutinis* in a photobioreactor, production of only 0.048 mg/g carotenoids was observed (Zhang et al., 2014). On the other hand, by optimizing the fermentation conditions, changing C/N ratios, and doing several rounds of metabolic engineering, Gao et al. (2017) was able to to produce 33 mg/gDCW of carotenoids in *Y. lipolytica*. Using optimum promoter-gene pairs for heterologous carotenoid production pathway, Larroude et al. (2017) was able to engineer a strain of *Y. lipolytica* with a maximum yield of 90 mg/gDCW of β-carotene.

As such, the titers of total carotenoids achieved in this work are superior or within the expected margin of concentrations attained in non-optimized fermentation conditions. However, to transform *R. toruloides* into a competitive biotechnological producer of

carotenoids, it is necessary to further fine-tune the expression of carotenoids synthesis pathway, as well as optimize the fermentation conditions.

In conclusion, the dedicated GGA platform developed in this work has been validated by improved carotenoid production and it is expected to be crucial for further design and modifications required to establish this nonconventional yeast as a biotechnology workhorse.

SUMMARY

Establishment of Golden Gate assembly platform for *Rhodotorula toruloides* fills a gap of advanced genome engineering tools for this nonconventional yeast that has engineering potential for industrial biotechnology purposes. In this work, the existing collection of YTK, which already exists for different yeasts, was adapted to *R. toruloides*, requiring optimization of almost all steps of the assembly.

The efficiency of the platform was first tested by the construction of a deletion cassette designed to knock out the *KU70* gene, coding for a key part of the NHEJ repair complex. The ΔKU70 strain had 6-fold increase in the frequency of successful genomic integration compared to the parental one.

The GGA platform was further validated by the assembly of a construct consisting of three genes, a selection marker, and insertional units. The three genes, coding for essential proteins in the carotenoids biosynthesis pathway, were overexpressed and led to increased carotenoids production visible by changes in the color intensity. Caroten quantification analysis of selected strains revealed 210% and 173% increase in the production of carotenoids, on xylose and glucose media, respectively.

Optimization of fermentation conditions to increase carotenoid yield, as well as using the now established GGA platform to help with overexpressing key proteins in lipid production pathway can make *R. toruloides* an industrial yeast capable of producing fine chemicals and biofuels, helping in the transition towards a biosustainable economy.

To summarize, this work has fulfilled all the objectives set in section 2.

ACKNOWLEDGMENTS

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Appendix I – Supplementary Table 1

Supplementary Table 1. A list of oligonucleotides used for Golden Gate assembly. The list includes the name of the oligonucleotide, the number, the sequence, and for which level of assembly it was used. In the 'Sequence' column, the lowercase base pairs are for enzyme anchoring, BsaI recognition site is underlined, and the 4-base pair overhangs are in *italic*.

Number	Sequence	Purpose
181	gcatGGTCTCA*ACGG*TGTCCGTATTCTACATCGACG	Amplification of promoter from XYL1 gene for P1 position in Level I/II constructs
182	atgcGGTCTCA*CATT*CGACATGGCGTGTATTCTG	
183	gcatGGTCTCA*GCTT*TGTCCGTATTCTACATCGACG	Amplification of promoter from XYL1 gene for P2 position in Level I/II constructs
184	atgcGGTCTCA*TTGT*CGACATGGCGTGTATTCTG	
185	gcatGGTCTCA*GTCA*TGTCCGTATTCTACATCGACG	Amplification of promoter from XYL1 gene for P3 position in Level I/II constructs
186	atgcGGTCTCA*GTGG*CGACATGGCGTGTATTCTG	
187	gcatGGTCTCA*ACGG*TGTGACTGATCTGGTGTTGTTCTGA	Amplification of promoter from GPD gene for P1 position in Level I/II constructs
188	atgcGGTCTCA*CATT*GGAGTTCGACGTTCTCCTCGC	
189	gcatGGTCTCA*GCTT*TGTGACTGATCTGGTGTTGTTCTGA	Amplification of promoter from GPD gene for P1 position in Level I/II/III constructs
190	atgcGGTCTCA*TTGT*TGGAGTTCGACGTTCTCCTCGC	Amplification of promoter from GPD gene for P2 position in Level I/II constructs
191	gcatGGTCTCA*GTCA*TGTGACTGATCTGGTGTTGTTCTGA	Amplification of promoter from GPD gene for P3 position in Level I/II constructs
192	atgcGGTCTCA*GTGG*TGGAGTTCGACGTTCTCCTCGC	
193	gcatGGTCTCA*ACGG*CGGCTGA	Amplification of promoter from ADH2

	GGCTTCCCCGACG	gene for P1 position in Level I/II constructs
194	atgcGGTCTCA*CATT*TGTGACTG TCGGAGACGTGGCAGC	
195	gcatGGTCTCA*GCTT*CGGCTGA GGCTTCCCCGACG	Amplification of promoter from ADH2 gene for P2 position in Level I/II constructs
196	atgcGGTCTCA*TTGT*TGTGACTG TCGGAGACGTGGCAGC	
197	gcatGGTCTCA*GTCA*CGGCTGA GGCTTCCCCGACG	Amplification of promoter from ADH2 gene for P3 position in Level I/II constructs
198	atgcGGTCTCA*GTGG*TGTGACT GTCGGAGACGTGGCAGC	
199	gcatGGTCTCA*TCTA*CGTTCAAA CATTTGGCAATAAAGTTTC	Amplification of terminator from Nos gene for T1 position in Level I/II constructs
200	atgcGGTCTCA*AAGC*CCCGATC TAGTAACATAGATGACA	
201	gcatGGTCTCA*GGAT*CGTTCAA ACATTTGGCAATAAAGTTT	Amplification of terminator from Nos gene for T2 position in Level I/II constructs
202	atgcGGTCTCA*TGAC*CCCGATCT AGTAACATAGATGACA	Amplification of terminator from Nos gene for T2 position in Level I/II/III constructs
203	gcatGGTCTCA*GTAT*CGTTCAAA CATTTGGCAATAAAGTTT	Amplification of terminator from Nos gene for T3 position in Level I/II constructs
204	atgcGGTCTCA*ACTC*CCCGATCT AGTAACATAGATGACA	
169	gcatGGTCTCA*ACAA*ATGCGCC CGCTTGCAC	Amplification of CrtI gene for G2 position in Level I/II constructs
170	CGTTAAAGATCTCGTCAAAC AGTCGCGGG	Site-directed mutagenesis for internal BsaI recognition site removal
171	GTTTGACGAGATCTTTAACGA TCTTGGG	
172	atgcGGTCTCA*ATCC*TCAACCG CGCAGGTACATC	Amplification of CrtI gene for G2 position in Level I/II constructs
173	gcatGGTCTCT*GGAT*TACTAGTA GCGGCCGCTG	Amplification of plasmid vector for G2 fragment insertion for Level I construct
174	gcatGGTCTCT*TTGT*CTCTAGAA GCGGCCGCGA	

175	gcatGGTCTCA*CCAC*ATGGGCG GACTGGACTACTGG	Amplification of CrtY gene for G3 position in Level I/II constructs
176	ATGGGAAGACCGACGGCCCA	Site-directed mutagenesis for internal BsaI recognition site removal
177	CCGTCGGTCTTCCCATCCTCC TC	
178	atgcGGTCTCA*ATAC*TCACAGC GCCTGCCACG	Amplification of CrtY gene for G3 position in Level I/II constructs
179	gcatGGTCTCT*GTAT*TACTAGTA GCGGCCGCTGC	Amplification of plasmid vector for G3 fragment insertion for Level I constructs
180	gcatGGTCTCT*GTGG*CTCTAGA AGCGGCCGCGA	
213	gcatGGTCTCA*AGGT*TGTCCGT ATTCTACATCGACG	Amplification of Marker for Level II constructs
214	atgcGGTCTCA*GTGG*CGACATG GCGTGTATTCTG	
215	gcatGGTCTCA*CCAC*ATGGGCA AGGAGAAGACC	Amplification of G418 gene for Level I/II constructs
216	AGGAAGACGGCGAGGGCGTC GACGATGTTC	Combination of two G418 gene parts by overlapping PCR
217	TCGCCGTCTTCCTCCGCCGCC T	
218	atgcGGTCTCA*ATAC*CTAGAAG AACTCGTCGAGCATGAGGT	Amplification of G418 gene for Level I/II constructs
219	gcatGGTCTCA*GTAT*CGTTCAAA CATTTGGCAATAAAGTTT	Amplification of terminator from Nos gene for Marker in Level I/II constructs
220	atgcGGTCTCA*CCGT*CCCGATC TAGTAACATAGATGACA	
221	acggGGTCTCT*ACGG*TACTAGT AGCGGCCGCTG	Amplification of plasmid vector for Marker insertion for Level I construct
222	acggGGTCTCT*ACCT*CTCTAGA AGCGGCCGCGA	
223	gcatGGTCTCT*AATG*TACTAGTA GCGGCCGCTG	Amplification of plasmid vector for P1 fragment insertion for Level I construct
224	gcatGGTCTCT*CCGT*CTCTAGA AGCGGCCGCGA	
225	gcatGGTCTCT*ACAA*TACTAGTA GCGGCCGCTG	Amplification of plasmid vector for P2 fragment insertion for Level I construct

226	gcatGGTCTCT*AAGC*CTCTAGA AGCGGCCGCGA	
227	gcatGGTCTCT*CCAC*TACTAGTA GCGGCCGCTG	Amplification of plasmid vector for P3 fragment insertion for Level I construct
228	gcatGGTCTCT*TGAC*CTCTAGAA GCGGCCGCGA	
229	gcatGGTCTCT*GCTT*TACTAGTA GCGGCCGCTG	Amplification of plasmid vector for T1 fragment insertion for Level I construct
230	gcatGGTCTCT*TAGA*CTCTAGAA GCGGCCGCGA	
231	gcatGGTCTCT*GTCA*TACTAGTA GCGGCCGCTG	Amplification of plasmid vector for T2 fragment insertion for Level I construct
232	gcatGGTCTCT*ATCC*CTCTAGAA GCGGCCGCGA	
233	gcatGGTCTCT*GAGT*TACTAGTA GCGGCCGCTG	Amplification of plasmid vector for T3 fragment insertion for Level I construct
234	gcatGGTCTCT*ATAC*CTCTAGAA GCGGCCGCGA	
439	atgcGGTCTCA*TAGA*TCAGACTT TGGGAAGCTCGTGC	Amplification of CrtE gene for G1 position in Level II constructs
453	gcatGGTCTCA*AATG*TCGCTGG ACTGGTACGACAAC	
440	gcatGGTCTCA*GGAG*CCGCCTC CTCCACCTCAGCAAC	Amplification of upstream insertional sequence for Level II/III constructs
508	atgcGGTCTCA*ACCT*TCATCGTC GGCGATGAGGAGGAC	Amplification of upstream insertional sequence for Level III constructs
507	agagcaGGTCTCC*TCGG*GATGTG GCGACCGGGCGCG	Amplification of downstream insertional sequence for Level III constructs
444	atgcGGTCTCA*ATGG*GTAGACC GTTTCGGGCGCGAC	Amplification of downstream insertional sequence for Level II/III constructs
501	agagcaGGTCTCC*AGGT*TGTCCG TATTCTACATCG	Amplification of Marker for Level III constructs
502	agagcaGGTCTCC*ACAC*CCGATC TAGTAACATAGA	
503	agagcaGGTCTCC*GTGT*CCGTAT TCTACATCG	Amplification of TU1 for Level III constructs
504	agagcaGGTCTCC*AAGC*CCCGAT	

	CTAGTAACATAGA	
505	agagcaGGTCTCC*GTCA*CGGCTGAGGCTTCCCCGA	Amplification of TU3 for Level III constructs
506	agagcaGGTCTCC*CCGA*TCTAGTAACATAGATGACACCGCGC	
497	ACTAGTAGCGGCCGCTGCAGGGTCTCACCATTCCTGTAGTCT	Amplification of pGGA for Gibson assembly of pGGA_RFP
498	CTAGAAGCGGCCGCGAATTCGGTCTCGCTCCGTACCAAGT	
499	ACTTGGTACGGAGCGAGACCGAATTCGCGGCCGCTTCTAG	Amplification of RFP for Gibson assembly of pGGA_ RFP
500	ACTACAGGAATGGTGAGACCCTGCAGCGGCCGCTACTAGT	

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