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Competing Interests

The authors declare that they have no competing interests

Running title

Production of β-carotene in *Y. lipolytica*

Abstract

The increasing market demands of β-carotene as colorant, antioxidant and vitamin precursor, requires novel biotechnological production platforms. *Yarrowia lipolytica*, is an industrial organism unable to naturally synthesize carotenoids but with the ability to produce high amounts of the precursor Acetyl-CoA. We first found that a lipid overproducer strain was capable of producing more β-carotene than a wild type after expressing the heterologous pathway. Thereafter, we developed a combinatorial synthetic biology approach base on Golden Gate DNA assembly to screen the optimum promoter-gene pairs for each transcriptional unit expressed. The best strain reached a production titer of 1.5 g/L and a maximum yield of 0.048 g/g of glucose in flask. β-carotene production was further increased in controlled conditions using a fed-batch fermentation. A total production of β-carotene of 6.5 g/L and 90 mg/g DCW with a concomitant production of 42.6 g/L of lipids was achieved. Such high titers suggest that engineered *Y. lipolytica* is a competitive producer organism of β-carotene.

Keywords

Yarrowia lipolytica, β-carotene, metabolic engineering, synthetic biology, golden gate, promoter shuffling.

1. Introduction

β-carotene is an orange pigment, precursor of vitamin A. This compound is a biochemical synthesized terpenoid that belongs to the group of carotenoids, together with lycopene, canthaxanthin, astaxanthin among others. Carotenoids have antioxidants properties which make them very relevant industrial compounds, with an expected market of $1.4 billion in 2018 (Lin et al. 2014; Ye and Bhatia 2012). β-carotene is produced either chemically or biotechnologically using natural producer microorganisms such as *Blakeslea trispora* (Nanou and Roukas 2016), *Xanthophyllomyces dendrorhous* (Contreras et al. 2015) or *Dunaliella salina* (Wichuk et al. 2014) . However, the heterologous production through metabolic engineering is considering a promising way to optimize β-carotene synthesis and face the increasing market demands. Therefore, several strategies to produce this compound have been carried out, mainly in model organisms such as *S. cerevisiae* and *E. coli*. Such strategies include the expression of heterologous genes, the elimination/downregulation of competing pathways, the overexpression of endogenous genes, adaptive evolution, fine control of the metabolic fluxes, etc. (Lin et al. 2014; Ye and Bhatia 2012).

To our knowledge, the best production of β-carotene achieved in engineered baker yeast reached a production of 0.45 g/L and 8.12 mg/g DCW (Xie et al. 2015). An adaptive evolution approach generated the strain with the highest relative production; 18 mg/g DCW (Reyes et al. 2014).

Higher values have been obtained by engineering *E. coli*; 3.2 g/L from glycerol (Yang and Guo 2014) or 2.47 g/L from glucose (Nam et al. 2013). One of the main drawback of using this bacteria for a commercial production of β-carotene is the safety issues associated to the lack of the GRAS status.

Yarrowia lipolytica is an oleaginous yeast widely investigated and modified for the production of biotechnologically relevant compounds (Ledesma-Amaro and Nicaud 2015; Liu et al. 2015; Madzak 2015; Zhu and Jackson 2015). It presents several advantages as industrial host, including a vast repertoire of molecular tools and the ability to grow naturally in low cost substrates such as glycerol

or molasses (Ledesma-Amaro and Nicaud 2016), or once engineered in xylose, raw starch, cellobiose, cellulose or inulin (Guo et al. 2015; Ledesma-Amaro et al. 2015; Ledesma-Amaro et al. 2016b; Wei et al. 2014; Zhao et al. 2010). Moreover, its metabolism, specifically the lipid metabolism, has been widely studied and characterized (Dulermo et al. 2015; Dulermo et al. 2016; Kerkhoven et al. 2017; Kerkhoven et al. 2016; Qiao et al. 2017; Wasylenko et al. 2015). Interestingly, lipid biosynthetic pathway and carotenoid pathway share a common precursor, Acetyl-CoA, which is highly available in *Y. lipolytica*. In addition, the genome of this yeast encodes all the required genes to produce geranylgeranyl diphosphate (GGPP), only two compounds away from β-carotene (Figure 1). Because of this, *Y. lipolytica* has been recently proposed as a potential producer of carotenoids. Both DuPont and Microbia have patented modified strains of *Y. lipolytica* engineered to produce carotenoids (Bailey et al. 2012; Sharpe et al. 2014). Interestingly, the authors proved that modified *Y. lipolytica* was able to produce lycopene (2mg/gDCW), β-carotene (5.7mg/gDCW), canthaxanthin and astaxanthin (Sharpe et al. 2014). Another metabolic engineering approach produced 16 mg/gDCW of lycopene, the direct precursor of β-carotene (Matthaus et al. 2014). Importantly, Grenfell-Lee et al. have demonstrated that the safety profile of the β-carotene produced in *Y. lipolytica* is the same as other commercial products, which would facilitate its commercialization (Grenfell-Lee et al. 2014). Therefore, this oleaginous yeast represents a promising biotechnological chassis for the production of β-carotene.

In addition, during the preparation of this manuscript, Gao et al. (Gao et al. 2017) engineered *Y. lipolytica* to produce up to 4 g/L of β-carotene in fed-batch fermentation; the highest production titer so far described in a heterologous microorganism. The authors engineered one strain after 12 steps where 11 genes were modified and they found that the integration of multiple copies of some of the genes was essential to increase β-carotene production.

In this work we first found that a strain that overproduce lipids is more convenient for the production of β-carotene than a wild type background. We further engineered the lipid

overproducer *Y. lipolytica* strain in order to maximize β-carotene production. For this aim, we used a synthetic biology approach in order to screen the best combination of promoters for each of the studied genes. Finally, we integrated the metabolic engineering of the strain with fermentation condition optimization in order to boost β-carotene production, reaching the best production titer and yield described so far.

2. Material and Methods

2. 1. Strains and media

Escherichia coli strain DH5α was used for cloning and plasmid propagation. Cells were grown at 37 °C with constant shaking on 5 ml LB medium (10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl), and ampicillin (100 μg/ml) or kanamycin (50 μg/ml) were added for plasmid selection.

The *Y. lipolytica* strains used in this study are derived from Po1d (wt), derived from the wild-type *Y. lipolytica* W29 (ATCC20460) strain. All the strains used in this study are listed in Supplementary Figure 1. Media and growth conditions for *Y. lipolytica* have been described elsewhere (Dulermo et al. 2015). Rich media YPD and YPD60 contained 1 or 6% glucose (Sigma) respectively, 1% peptone (BD bioscience) and 1% yeast extract (BD bioscience). Minimal media YNB20, YNB30 and YNB60, contained 2, 3 or 6% glucose respectively (wt/vol; Sigma), 0.17% (wt/vol) Yeast Nitrogen Base (YNBww; Difco), 0.5% (wt/vol) NH_4Cl and 50mM phosphate buffer (pH6.8). YNBgly60 medium was prepared in the same way as the YNB medium except that 6% glycerol was added as sole carbon source. When necessary, the YNB medium was supplemented with uracil (0.1 g/L) and/or leucine (0.1 g/L) or hygromycin (0.2 g/L). Solid media for *E. coli* and *Y. lipolytica* was prepared by adding 15 g/L agar (Invitrogen) to liquid media.

2. 2. Strains construction

All restriction enzymes were purchased from New England Biolabs (NEB). PCR amplifications were performed using Q5 high-fidelity DNA polymerase (NEB) or GoTaq DNA polymerase (Promega). When needed PCR fragment were purified using the QIAquick Gel Extraction Kit (Qiagen). Plasmids from *E. coli* were extracted using the QIAprep Spin Miniprep Kit (Qiagen). All the reactions were performed according to the manufacturer instructions. Transformation of chemically competent *E. coli* cells was performed by thermic shock protocol. Transformation of *Y. lipolytica* was performed using the lithium-acetate method adapted from (Barth and Gaillardin 1996). Transformants were selected on YNBLeu, YNBura, or YNBHygro media, depending on their genotype.

2. 3. DNA constructions

Primer's sequences used in this study can be found in Supplementary Table 1. The carotenoid's (*GGS1-CarB-CarRP*) expression cassette (Supplementary Figure 2) named car-cassette and the t-HMG's expression cassette were kindly provided by Microbia (Bailey et al. 2012) onto plasmids containing the *LEU2* gene and the nourseothricin resistance gene respectively as selection markers. All sequencing processes were done by Eurofins genomics. A complete plasmid list can be found in Supplementary Table 2.

The construction of carTEF-cassette was done using the recently developed Golden Gate (GG) toolbox for *Y. lipolytica* (Celinska et al. 2017) based on (Engler and Marillonnet 2014). Briefly, primers carrying pre-designed 4-nt overhangs and externally located BsaI recognition sites were designed and used to amplify the building blocks which were then cloned in donor vectors (Zero Blunt® TOPO® PCR Cloning Kit, invitrogen). These building blocks and the destination vector carrying a gene encoding for a chromophore, to facilitate selection, which is also flanked with BsaI-site and pre-designed overhangs, are then mixed equimolarly (50 pmoles of ends) in one-pot reaction together with 5 U of BsaI (NEB), 200 U of T4 ligase (NEB), 2 µL of T4 DNA ligase buffer (NEB) and ddH_2O up to 20 µL. The following thermal profile was applied: [37°C for 5 min, 16°C for 2 min]x60, 55°C for 5 min, 80°C for 5 min, 15°C ∞. Subsequently, the reaction mixture was used for *E. coli*

transformation. White colonies were screened for identification of complete Golden Gate Assembly (GGA) through plasmid isolation, restriction digestion and PCR. Complete GGA was subsequently linearized by SfiI restriction enzyme and 10 µL were used for transformation of *Y. lipolytica.*

All the sequences to be used as building blocks of the envisioned GG Assembly were extracted from *Y. lipolytica* W29 genome sequence or from previously constructed vectors of our own collection. After cloned in TOPO vector, the building blocks were screened by restriction digestion and verified by sequencing.

A promoter shuffling was carried out to explore the combination of multiple promoters (Engler and Marillonnet 2013). The assembly of plasmids with three randomized promoters (PGMp-low expression, GAPDHp-medium expression, TEFp-high expression) was split into two parts: pre-assembly construct and then multigene construct assembly. For the pre-assembly, three separate reactions with four Golden Gate parts each (InsertionSiteUp-Marker-Promoter1-Gene1;Terminator1-Promoter2-Gene2-Terminator2;Promoter3-Gene3-Terminator3-InsertionSIteDown) was done. All parts were used in equimolar quantities (50 pmoles), and a mix of all three promoters was made with a final concentration of 50 pmoles. Each reaction has 5 U of BsaI, 200 U of T4 ligase, 1 µL of T4 DNA ligase buffer (NEB) and ddH_2O up to 10 µL and a short thermal profile was applied: [37°C for 3 min, 16°C for 2 min]x30, 55°C for 5 min, 80°C for 5 min, 15°C ∞. Subsequently, for the multigene construct assembly the three previous reactions were mixed together in the same tube and the destination vector (50 pmoles) was added together with 20 U of BsaI, 400 U of T4 ligase, 4 µL of T4 DNA ligase buffer (NEB) and ddH_2O up to 40 µL. The following thermal profile was applied: [37°C for 5 min, 16°C for 5 min]x50, 55°C for 5 min, 80°C for 5 min, 15°C ∞. This Golden Gate reaction was transformed into *E. coli*, and all transformant colonies were mixed together into a single overnight culture. A plasmid library was prepared from this overnight culture, digested with SfiI restriction enzyme and 10 µL were used to transform *Y. lipolytica*. (Figure 4)

Identification of promoters at each site was done by PCR from gDNA of the selected yellow-orange colonies obtained. For promoter in position 1 the primer pair used was Ura3Marker-intern-Fw/GGSI_intern_Rv, for position 2 GGSI_intern_Fw/CarB_intern_Rv, and for position 3 CarB_intern_Fw/CarRP_intern_RV.

2. 4. β-carotene measurement

Intracellular β-carotene content was extracted and quantified by photometric measurement adapted from previous reports (Matthaus et al. 2014). Briefly, 20 ml of medium was inoculated with 0.05 DO pre-culture yeast strain in a 250 ml flask. Cells were cultured during 4 days at 28°C and shaking. Afterwards 200 ul of the culture were harvested in a FastPrep FP120 (Thermo Electron) and 500 ml glass beads (0.75 to 1 mm; Roth) and 1.2 ml extraction solvent (50:50 v/v; hexane-ethyl acetate; 0.01% butyl hydroxyl toluene) were added. The mixture was vortexed three times for 1min30s at maximum speed, alternating with ice incubation. The extract was collected after 5 minutes centrifugation, and the extraction procedure was repeated until the pellet and the supernatant were colorless. The extract was then diluted with extraction solvent and measured photometrically at 448 nm. The concentrations were calculated through a standard curve using β-carotene from Sigma-Aldrich as standard. The OD was correlated with the dry cell weight (DCW) measurement for each corresponding culture. The washed and lyophilized cells coming from a known volume served to measure the DCW. Every sample was cultured in duplicate.

2. 5. Microscopy images

Images were acquired using a Zeiss Axio Imager M2 microscope (Zeiss) with a 100×objective and Zeiss filters 45 and 46 for fluorescent microscopy. Axiovision 4.8 software (Zeiss) was used for image acquisition. Lipid bodies visualization was performed by adding BodiPy ® Lipid Probe (2.5 mg/ml in ethanol; Invitrogen) to the samples and after incubationon at room temperature for 10 min.

189 Microscopic color images were acquired using a Leica DM1000 microscope (Leica) with a 100×
190 objective a moticam 2500 camera. Motic imaging 2.0 software was used for image acquisition.

191 2. 6. Lipid content quantification

192 Lipids were extracted from 15–25 mg aliquots of lyophilized cells and converted into their equivalent
193 methyl esters as previously described (Ledesma-Amaro et al. 2016a). The products were then used
194 in the gas chromatography (GC) analysis, performed using a Varian 3900 gas chromatograph
195 equipped with a flame ionization detector and a Varian FactorFour vf-23 ms column, where the
196 bleed specification at 260 °C was 3 pA (30 m, 0.25 mm, 0.25 µm). FA were identified by comparison
197 to commercial fatty acid methyl ester standards (FAME32, Supelco) and quantified by the internal
198 standard method with the addition of 50 µg of commercial C12:0 (Sigma).

199 The washed and lyophilized cells coming from a known volume served to measure the DCW.

200 2. 7. Biomass, sugar and acid quantification

201 Dry cell weight (DCW) was calculated by weighting the lyophilized cells. The harvested cells were
202 washed twice and centrifuged in order to separate all the mass remaining in the culture media prior
203 to lyophilisation.

204 Sugar and citric acid were quantified by HPLC (UltiMate 3000, Dionex-Thermo Fisher Scientific, UK)
205 using an Aminex HPX 87 H column coupled to UV (210 nm) and RI detectors. The column was eluted
206 with 0.01 N H_2SO_4 at room temperature and a flow rate of 0.6 ml min^{-1}. Identification and
207 quantification were achieved via comparisons to standards. Before being subject to HPLC analysis,
208 samples were filtered on 0.45-µm pore-size membranes.

209 2. 8. Bioreactor procedures

210 Fed-batch cultivations were performed in a 5 L bioreactor (Sartorius Stedim Biotech, Göttingen,
211 Germany) with an initial working volume of 2 L. The initial medium (Y10P20D) contained 10 g/L of

Yeast extract (Becton Dickinson, Franklin Lakes, USA), 20 g/L of peptone (Becton Dickinson, Franklin Lakes, USA) and 5 g/L of glucose (Sigma-Aldrich, St. Louis, USA). The concentrated medium (Y20P40D) contained 20 g/L of Yeast extract, 40 g/L of peptone and 5 g/L of glucose. The temperature was held constant at 28°C, the aeration at 2 VVM, the agitation at 500 - 900 rpm, the pH at 5.5 automatically by injection of 100 g/L H3PO4 or 200 g/L KOH, and the dissolved oxygen was set up at 20 %. Foam was prevented by the addition of antifoam 204 (Sigma-Aldrich, St. Louis, USA). The medium was inoculated with 100 mL from a 24 h preculture performed in a shake flask containing 10 g/L of Yeast extract, 20 g/L of peptone and 20 g/L of glucose. The fed-batch process was initiated after 6 h of cultivation at a rate of 6 g/L (0.2 molcarbon/h) of glucose from a 500 g/L concentrated stock solution.

3. Results and Discussion

3. 1. Lipid overproducer strain synthesizes higher amount of β-carotene

Previous reports have proven that the overexpression of three genes, geranylgeranyl diphosphate synthase (*GGS1* from *Y. lipolytica*), pytoene synthase/lycopene cyclase and phytoene dehydrogenase (*carPR* and *carB* from *Mucor circinelloides*) promote β-carotene production in *Y. lipolytica* (Celinska et al. 2017; Gao et al. 2014). We here used an expression cassette (car-cassette) where the expression of *GGS1* is controlled by the promoter PGMp, of *CarB* by GAPDHp and of *CarRP* by TEF1p. As expected, the sole expression of this cassette in the parental strain (named wt-C) allows it to produce substantial amounts of β-carotene (3.4 mg/gDCW and 18.4 mg/L) (Figure 2). This production level was higher than the 2.2 mg/gDCW previously obtained for a similar approach (Gao et al. 2014). The differences could be caused by the use of different promoters controlling each of the three genes or due to the use of different parental strains.

It is well known that the lipophilic nature of carotenoids promotes their storage in the lipid bodies of the cells. In Supplementary Figure 3 the co-localization of the pigment and the neutral lipids can be seen. Here we hypostatized that lipid overproducer strains could boost both β-carotene production, due to a higher availability of the precursor Acetyl-CoA, and storage, due to an increase of the lipophilic structures inside the cells. In order to verify this, we transformed a lipid overproducer strain (JMY3501, hereafter called 'obese' or 'ob') with the car-cassette. As expected, the generated strain (ob-C) accumulated higher amount of lipids, 3.6 times more than the strain wt-car, and interestingly it also boosted β-carotene synthesis, producing 8.9 mg/gDCW and 35.7 mg/L, 2.61 and 1.93 times more than wt-car (Figure 2). The higher amounts of lipids could be important for the solubilisation of β-carotene, which would reduce the formation of crystals that could impair the cellular homeostasis. These results indicate the importance of selecting the proper parental strain in metabolic engineering approaches. Unexpectedly, the expression of the β-carotene pathway favoured the total production of lipids, which increased 82% and 56% in the wild type and obese background respectively. Importantly, the co-production of the two biotechnologically relevant compounds, β-carotene and lipids, could facilitate the industrial viability of the process.

3. 2. Further metabolic engineering increases carotene content and reveals pathway bottlenecks

The overexpersion of hydroxymethylglutaryl-CoA reductase (*HMG1*) is known to channelized the flux towards carotenoids in engineered microorganisms (Matthaus et al. 2014). Here we overexpressed a truncated version of this gene (YALI0E04807,(Bailey et al. 2012)) under the control of the constitutive TEF promoter in the strain ob-C. The generated strain (ob-CH) increased β-carotene content up to 17.4 mg/gDCW and 121.6 mg/L, 2.0 and 3.4 times more than the parental strain ob-C (Figure 3). Again, this results are higher than the ones recently obtained from a strain overexpressing the same genes but using different parental strain and set of promoters (4.36 mg/gDCW and 64.6 mg/L)(Gao et al. 2017).

In order to analyze if the production of β-carotene is limited by the expression levels of the overexpressed genes we introduced in the genome of ob-CH a second copy of the car-cassette, generating the ob-CHC. Interestingly, we found a further increase in the desired product, which reached 24.0 mg/gDCW and 175.6 mg/L, 1.4 and 1.4 times more than the parental strain ob-CH. This result indicates that the expression of some of the gene encoded in the car-cassette is limiting the production of β-carotene. Similar conclusion was reached by Gao et al. (Gao et al. 2017) in a parallel study where the authors improved the β-carotene content by increasing the copy number of the genes expressed. Here, in order to overcome this limitation we considered a strategy that combines the increase of the copy number and the optimization of the promoter strength for each gene.

3.3. Promoter shuffling using Golden Gate indentifies the best promoter set for the production of β-carotene

We have previously seen (Dulermo et al. 2017) that the increase in the promoter strength, even in strong promoters, can enhance the transcription level more than 6 times, while an extra copy of a gene under the same promoter can typically only duplicate the expression level. We therefore decided to identify the best combination of promoters and genes in order to maximize β-carotene production. The construction of the car-cassette with different promoters via traditional cloning would have been a very long and inefficient process. Thus, we took advantage of the recently developed Golden Gate toolbox for *Y. lipolytica* (Celinska et al. 2017) to perform a promoter shuffling strategy. The strategy, summarized in Figure 4, consists in a digestion-ligation reaction guided by BsaI defined sites where the three promoters can be introduced in each of the three promoter positions in the car-cassette. It has been recently proven by sequencing the Golden Gate reaction products that every possible combination is unbiased generated during combinatorial assemblies (Awan et al. 2017). Then, the pool of cassettes were simultaneously amplified, linearized and used to transform the wild type parental strain (wt), where the difference in colour intensity

could be easily screened (Supplementary Figure 4). After the transformation, 387 colonies with different colour were obtained. Here, we selected the 15 more orange-yellow strains and we checked the set of promoter controlling each gene (Figure 5). Interestingly, the combination of promoters originally present in the car-cassette was not found among the best producer strains and, accordingly, the analyzed strains generated by the shuffling produced higher β-carotene content than the wt-car previously analyzed (Figure 2). The major conclusion of these results is that the cassette with the three genes controlled by TEF1p is the optimum producer. Analyzing the overall combination of promoters found as convenient for overproducing β-carotene, it seems that the presence of a strong TEF promoter is favoured in the second position of the car-cassette (11 out of 15). The best of these strains were able to produce 6.3 times more β-carotene than the wt-car, 16.7 mg/gDCW and 111.8 mg/L.

Based on these results we constructed using Golden Gate a new car-cassette where the three genes, *GGS1*, *CarB* and *CarRP* are under the control of TEF1 promoter. This new cassette was called car^{TEF}-cassette and it was used to further increase the β-carotene content of ob-CH.

3. 4. Construction of a β-caratone overproducer strain

We have previously seen that an extra copy of the car-cassette in the ob-CH increased β-carotene production and, in addition, we have improved the cassette (car^{TEF}-cassette) by an optimized set of promoters. We therefore attempted to further increase total β-carotene content by the expression of car^{TEF}-cassette in the strain ob-CH. The generated strain, ob-CHC^{TEF} , as hypothesized, produced higher β-caratone; 54.4 mg/gDCW and 293.3 mg/L (Figure 6).

Finally, an extra copy of the car^{TEF}-cassette was introduced to construct the strain ob-$CHC^{TEF}C^{TEF}$. This strain was able to produce 61.1 mg/gDCW and 454.36 mg/L of β-carotene (Figure 6), which represent the best production of this compound so far described in flask culture (260 mg/L in *E. coli*

(Yang and Guo 2014) and 353.6 mg/L in *Y. lipolytica* (Gao et al. 2017) and the best yield reported so far (0.048 g/g followed by 0.027 g/g achieved in *E. coli* (Yang and Guo 2014) and 0.018 g/g in *Y. lipolytica* (Gao et al. 2017)). We also tested the stability of this strain, since some reports in *S. cerevisae* showed the appearance of spontaneous white colonies (Beekwilder et al. 2014) but in *Y. lipolytica* all the cells plated after 6 days of culture produced β-carotene (Supplementary Figure 5), which could be expected from the low homologous recombination rate in this yeast. We therefore selected this strain in order to optimize culture conditions to further increase β-carotene production.

3. 5. Media optimization shows a trade-off between production titer and yield

In order to study the effect of the media composition in the production of β-carotene, we tested two different kind of media, rich media (YPD) and synthetic media (YNB). We also tested different concentrations of carbon source keeping constant the nitrogen amount, referred by a number that indicates the concentration in g/L (10, 20, 30 and 60). It is well known that a higher C/N ratio promotes lipid production as well as other carbon based molecules such as carotenoids (Braunwald et al. 2013). We tested glucose as a standard carbon source and glycerol (GLY) as a cheaper carbon sources. The selected media were YPD10, YPD60, YNB20, YNB30, YNB60 and YNBGLY60. Large variations in the β-carotene production were found depending on the culture media (Figure 7). According to the results we can suggest that there is no much influence on the carbon source since glucose and glycerol showed similar titer and yields. In addition, a clear correlation between the increase in the initial glucose content and the production titer was found in both rich and synthetic media. Interestingly, the production yields for all the YNB based-media tested was similar independently of the amount or kind of carbon source. However, this was not the case for rich media where a trade-off between production titer and yield was found. The best β-carotene titer so far, 1.5 g/L, was found in YPD60 while the best yield, 0.048 g/g was produced in YPD10. In any case,

YPD showed higher titers and yields than YNB and therefore we selected rich media for further optimization of the culture conditions in a controlled fermentation in bioreactor.

3.6. Bioreactor controlled conditions boosts β-carotene production by the engineered strain ob-CHC^{TEF}C^{TEF}

In order to improve the production of β-carotene in the strain ob-CHC^{TEF}C^{TEF} we decided to optimize the culture conditions using a 5 L bioreactor. We here performed a fed-batch fermentation using rich media (Y10P20D) where glucose was added after 6 h of cultivation at a rate of 6 g/h. The fermentation was stopped after 143.5 h when the glucose concentration started to rise in the culture media indicating its lack of consumption. The strain reached a production 2.9 g/L β-carotene and a concomitant production of 40 g/L lipids and 31 g/L of citric acid (Supplementary Figure 6).

In order to further optimize bioreactor conditions we designed a culture media with double amounts of yeast extract and peptone, named Y20P40D, where the rate of glucose feeding was used as carbon source in fed-batch. In this experiment, a maximum production of 6.5 g/L of β-carotene was produced after 122 hours, the best titer described so far (Figure 8). In this conditions, the production of citric acid was kept under 1 g/L along the fermentation while the lipid titer reached 40 g/L (Figure 8). Interestingly, the maximum β-carotene content was 89.6 mg/g of DCW, 50 % higher than in flask and one of the highest described in the literature. Such results indicates that herein engineered *Y. lipolytica* using synthetic biology and metabolic engineering is a potential industrial producer of β-carotene.

4. Conclusions

In this work we have combined traditional metabolic engineering strategies with novel synthetic biology tools in order to turn *Y. lipolytica* an industrially competitive producer of β-carotene. We found that increasing lipogensis and gene copy number as well as using the most favourable set of promoters, greatly enhanced β-carotene production. Finally a fed-batch fermentation lead to the highest β-carotene production reported so far.

This work shows the enormous potential of *Y. lipolytica* to produce β-carotene in an economically feasible manner, not only by the high titer achieved but also due to the co-production of high amount of lipids, which can be used as fuels or chemicals (Singh et al. 2016). Moreover, the process can be further improved not only by strain engineering and bioreactor condition optimization but also by the use of low cost carbon sources such as starch or lignocellulosic materials. In addition, this work also highlights the importance of selecting the right microorganism and strain in a synthetic biology or metabolic engineering strategy. The selection of an oleaginous organism has permitted production levels far beyond the so far obtained in other yeasts such as *S. cerevisiae*, while the selection of a lipid overproducer strain further increased β-carotene content. This work is an example of how the rapid development of synthetic biology tools for DNA assembly and genome editing is facilitating the manipulation of non-conventional organisms, expanding the range of biotechnological chassis for metabolic engineering.

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Figures

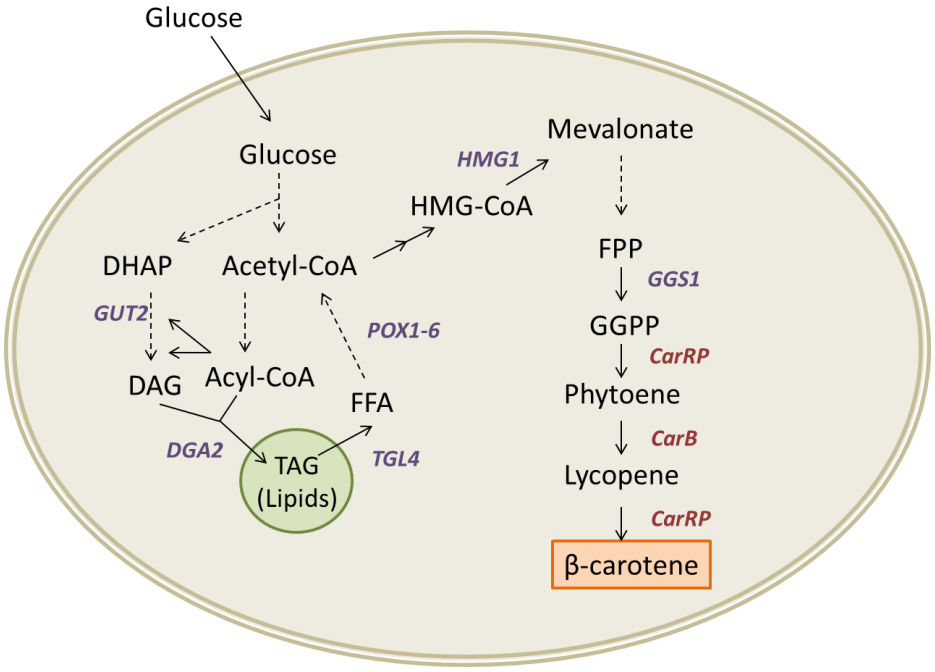
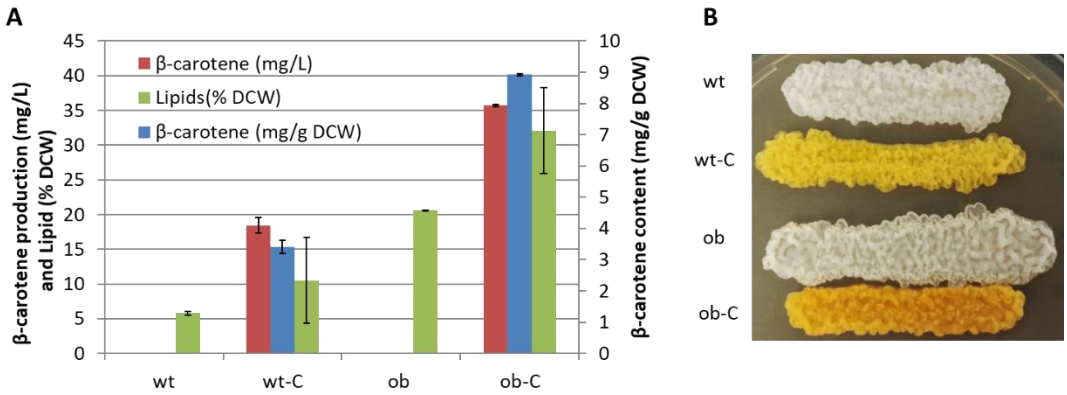


Figure 1: Scheme of the metabolic pathways leading to the production of lipids and β-carotene in *Y. lipolytica.*

Metabolites are shown in black, native enzymes in purple and heterologous enzymes in red. The continuous arrows indicate a metabolic step while the dashed arrows indicate multiple metabolic steps. DHAP, dihydroxyacetone phosphate; DAG, diacylglycerol; TAG, triacylglycerol; FFA, free fatty acids; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate.



492 Figure 2: Lipids and β-carotene production in wt and ob backgrounds.

493 A) β-carotene production (mg/L), content (mg/g DCW) and lipid content (% DCW) in the strains wt,
494 wt-C, ob and ob-C after growing in YPD for 4 days. The average value and the SD from two
495 independent experiments are shown. B) Strains wt, wt-C, ob and ob-C growing on YPD-agar plate
496 where the color provoked by the β-carotene can be seen.

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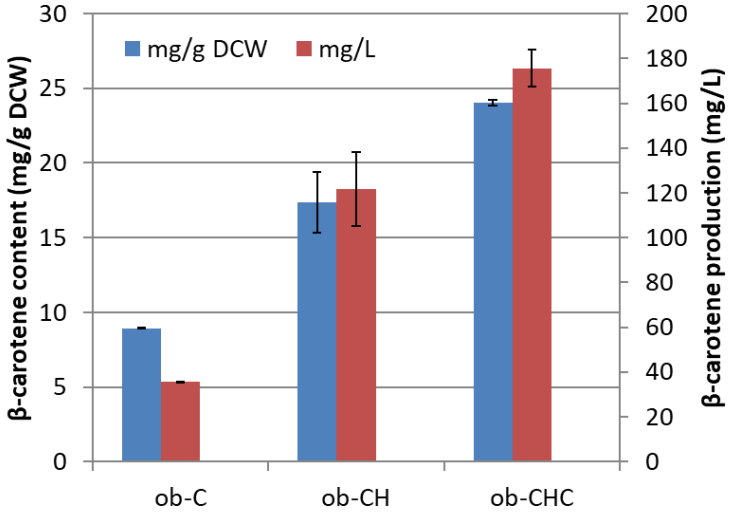


498 Figure 3: β-carotene production in ob-derived background.

499 A) β-carotene production (mg/L) and content (mg/g DCW) in the strains ob-C, ob-CH and ob-CHC
500 after growing in YPD for 4 days. The average value and the SD from two independent experiments
501 are shown.

502

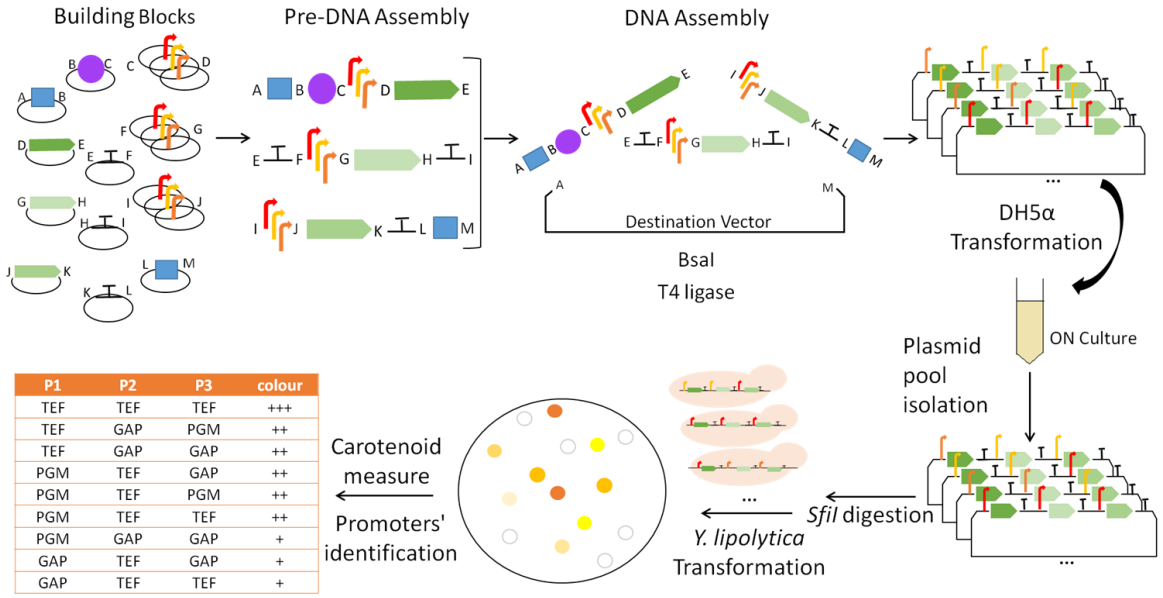


Figure 4: Scheme of the promoter shuffling strategy to optimize β-carotene production in *Y. lipolytica.*

Briefly, Golden Gate cloning system was used to generate a pool of randomized expression cassettes bearing different set of 3 different promoters. The pool of expression cassettes was used to transform Y. lipolytica and the generated clones were screened by colour intensity. The selected clones were further analyzed, each promoter set was determined and the β-carotene content was measured (see Materials and methods for a detailed explanation).Letters A to M represent the designed overhangs enabling the ordered assembly of DNA parts after BsaI digestion. Blue squares represent genomic integration targeting sequences, violet circle represents the selection marker gene, red-orange-yellow arrows represent promoters, green arrows represent genes and T represent terminators.

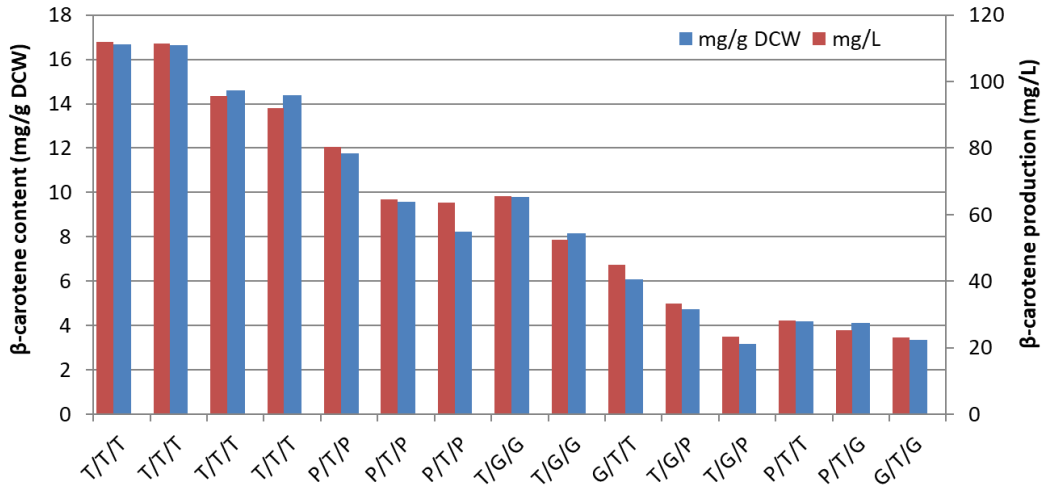


Figure 5: β-carotene production in the strains selected from the promoter shuffling approach.

β-carotene production (mg/L) and content (mg/g DCW) in the strains derived from the promoter shuffling experiment after growing in YPD for 4 days. Each strain is identified by the set of promoters: T, TEFp; P, PGMp; G, GAPDHp in the car-cassette order 1st GGS1, 2nd carB and 3rd carRP. Some of the selected strains presented the same combination of promoters.

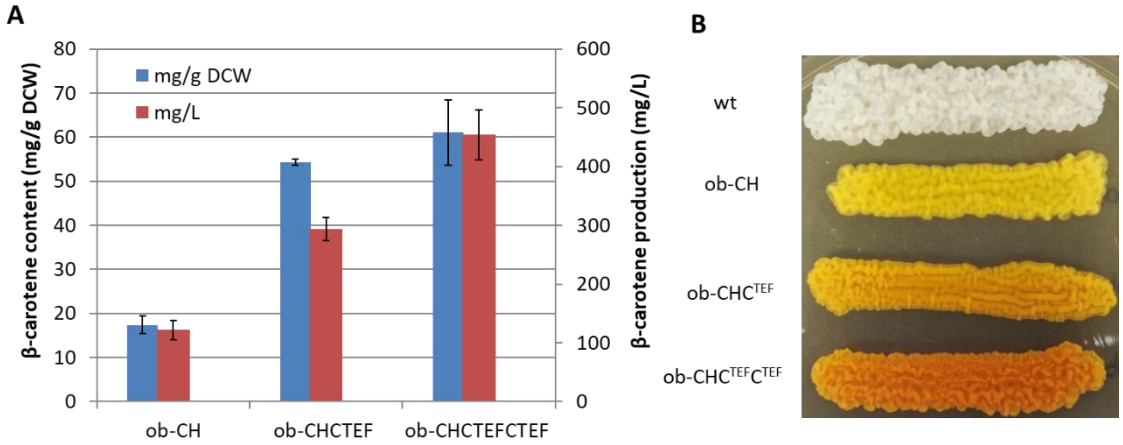


Figure 6: β-carotene production in engineered strains of Y. lipolytica.

A) β-carotene production (mg/L) and content (mg/g DCW) in the strains ob-CH, ob-CHCTEF and ob-CHCTEFCTEF after growing in YPD for 4 days. The average value and the SD from two independent experiments are shown. B) Strains wt, ob-CH, ob-CHCTEF and ob-CHCTEFCTEF growing on YPD-agar plate where the color provoked by the β-carotene can be seen.

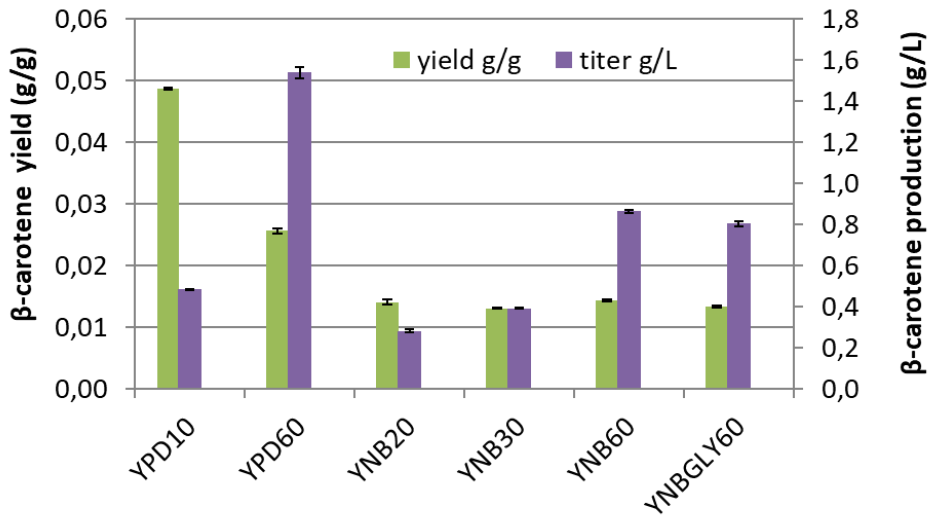


Figure 7: Yield and titer of β-carotene production in different culture media.

A) β-carotene yield (g β-carotene/g substrate) and titer (g β-carotene/L) in the strain ob-CHCTEFCTEF after growing for 4 days in rich media (YPD10, 10g/L glucose or YPD60, 60g/L glucose) or synthetic

media (YNB20, 20g/L glucose, YNB30, 30g/L glucose, YNB 60, 60 g/L glucose or YNBGLY60, 60 g/L glycerol). The average value and the SD from two independent experiments are shown.

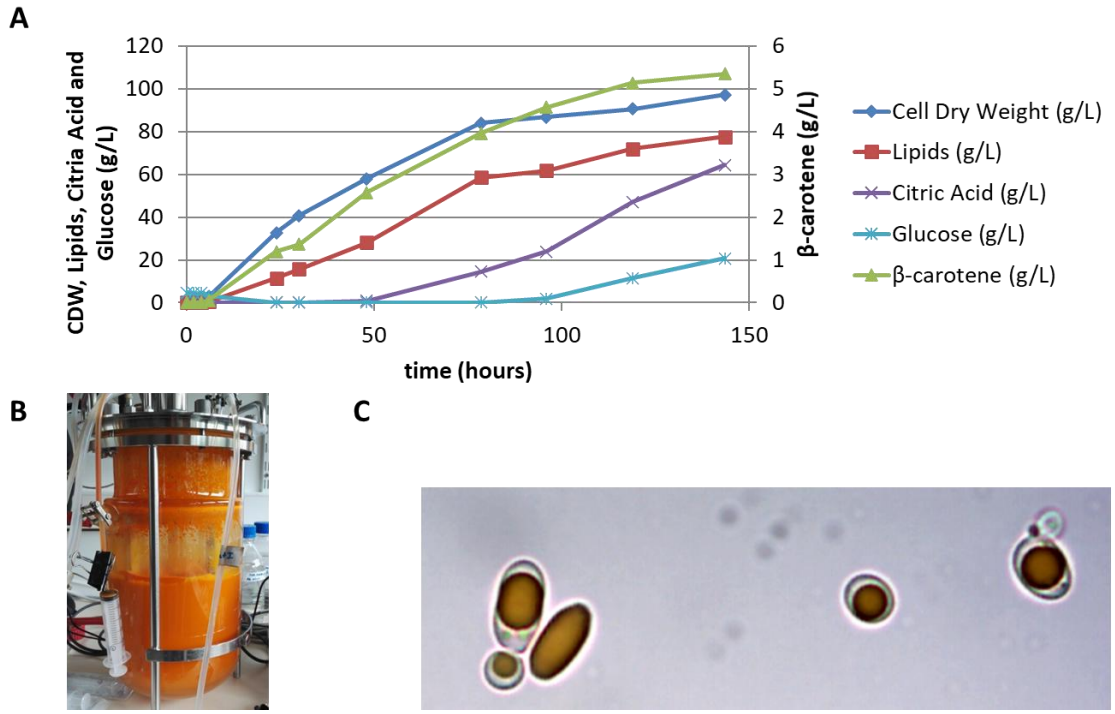
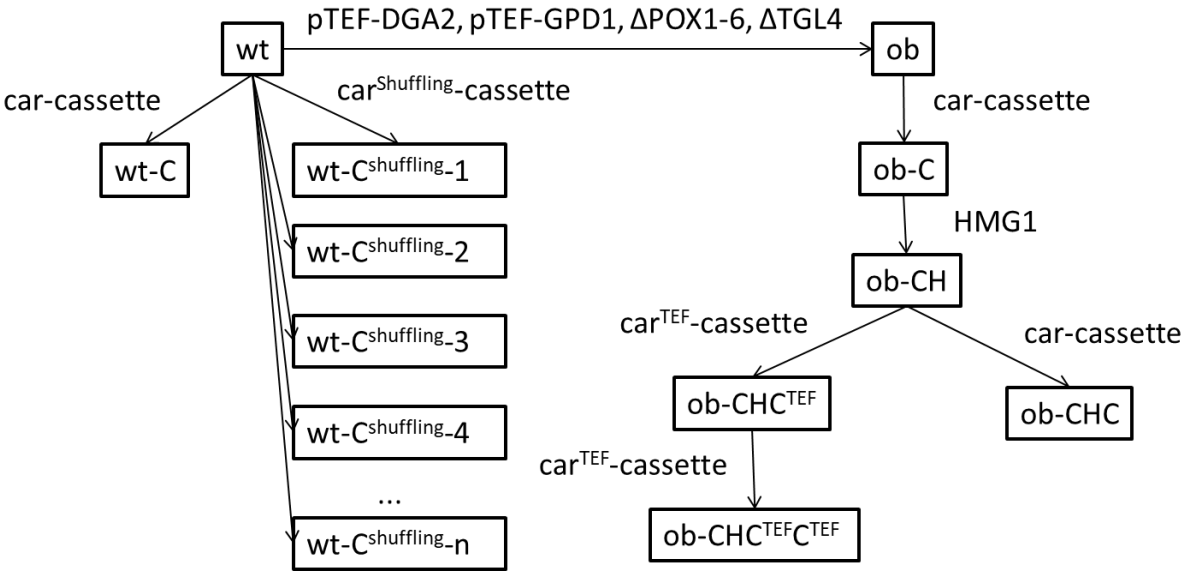
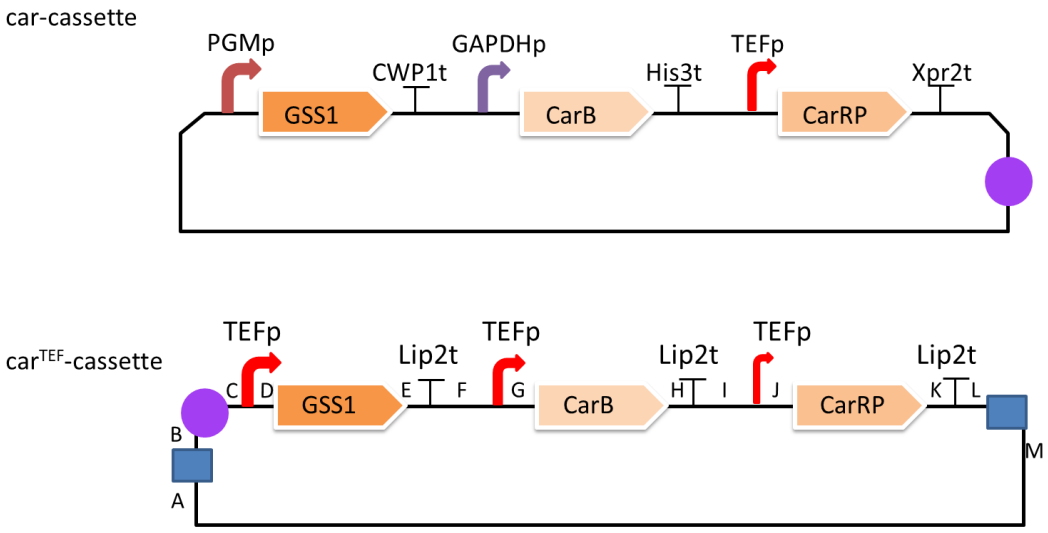


Figure 8. Production of β-carotene by engineered Y. lipolytica in bioreactor in Y20P40D media.

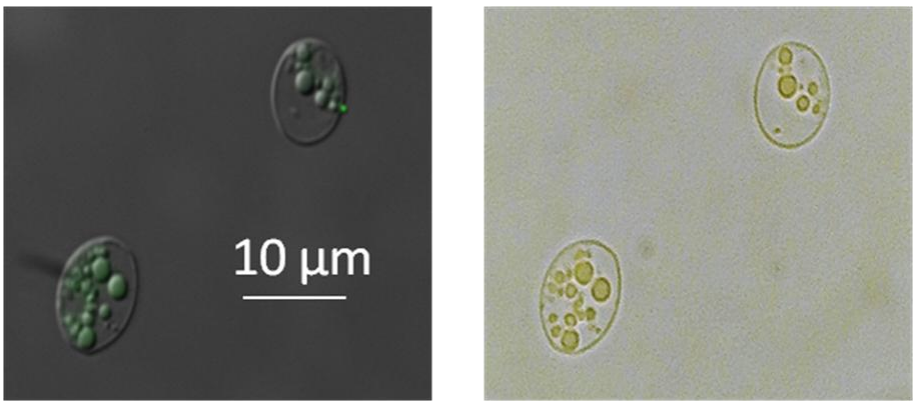
A) Kinetics of the 5-L bioreactor fermentation along 143.5 hours showing Cell Dry Weight (g/L), lipids (g/L), citric acid (g/L), glucose (g/L), β-carotene (g/L) and bioreactor broth volume (L). B) Picture of the bioreactor after 120 hours in Y10P20D. C) microscopic image of the cells grown in Y10P20D where the β-carotene can be seen as the orange colour staining the lipid bodies of the cells.



Supplementary Figure 1. Scheme showing the strains constructed and used in this study.

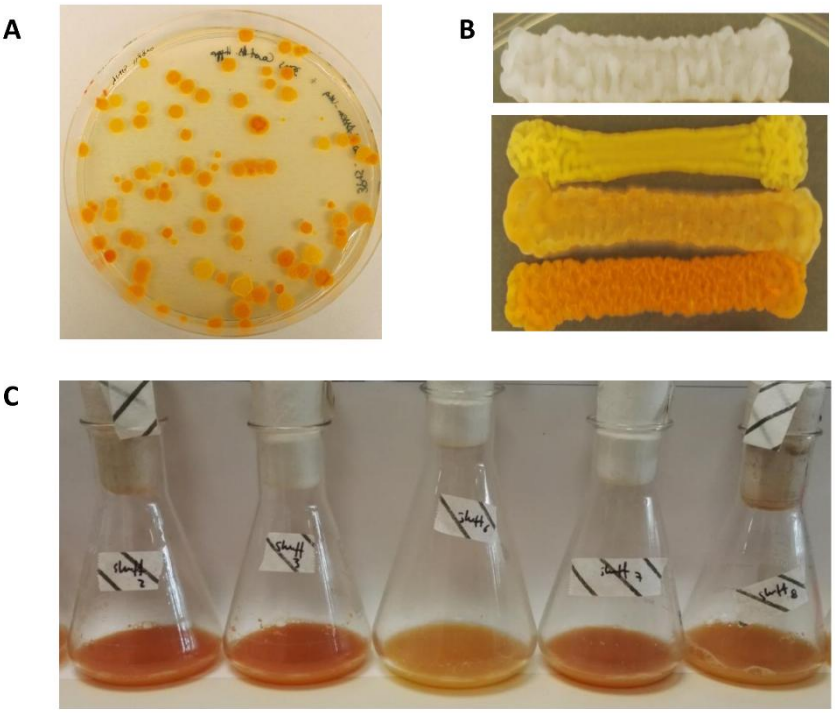


Supplementary Figure 2. Schematic representation of the car-cassettes used in this study.



Supplementary Figure 3: co-localization of lipid bodies (left, fluorescence microscopy stained with bodipy) and β-carotene (right, light microscopy revealing the orange β-carotene).

548



549 Supplementary Figure 4. Example of colour variation in the shuffling promoter experiment after
550 plating the transformation (A) and growing some of the selected strain in YPD solid (B) or liquid (C)
551 for 4 days.

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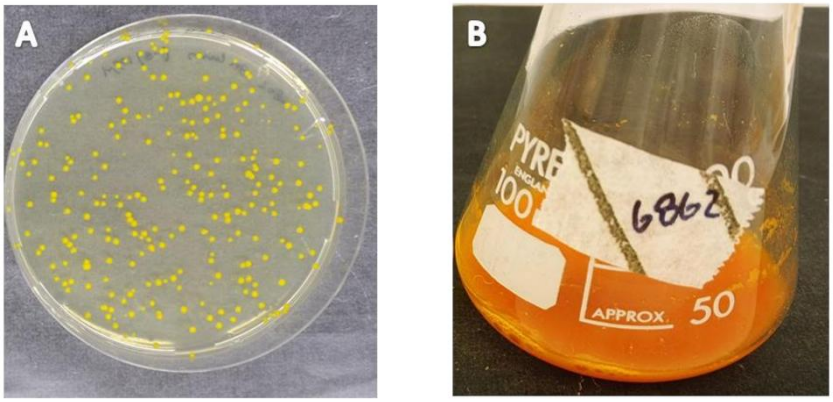
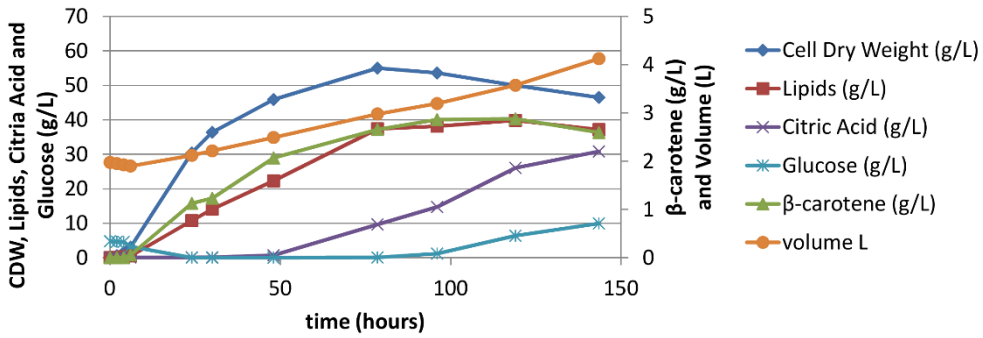


553 Supplementary Figure 5. Stability test. The engineered strain ob-CHCTEFCTEF was plated (A) from a 6
554 days culture in YPD (B).



Supplementary Figure 6. Production of β-carotene by engineered Y. lipolytica in bioreactor in Y10P20D media.

Kinetics of the 5-L bioreactor fermentation along 143.5 hours showing Cell Dry Weight (g/L), lipids (g/L), citric acid (g/L), glucose (g/L), β-carotene (g/L) and bioreactor broth volume (L).

Tables

Supplementary Table 1. Oligonucleotide table. The nucleotides in capital letters show the BsaI recognition site and the 4 nt overhangs for the Golden Gate strategy.

OLIGONUCLEOTIDES	
Name	***Sequence***
Amplification of GG Parts	
GGP_InsertUP_zeta_A_F_SfiI	GGTCTCtGCCTggccacctaggcctgtcgggaaccgc
GGP_InsertUP_zeta_B_R	GGTCTCTACCTtctagcaaagtgctttgtgc
GGP_M_Ura3_B_F	GGTCTCTAGGTataacttcgtatagcatacattatacgaag
GGP_M_Ura3_C_R _new	GGTCTCtCCGTtcgcttcggataactcc
GGP_M_Hygro.2.1_B_F	gggGGTCTCTAGGTgcatacattatacgaagttattctg
GGP_M_Hygro.2.1_C_R _new	ggGGTCTCTCCGTatacgaattctcgactattcctttg
GGP_P1_TEF_C_F_New	GGTCTCtACGGgggttggcggcg
GGP_P1_TEF_D_R	GGTCTCTCATTcttcgggtgtgagttgac
GGP_P1_PGM_C_F_New	GGTCTCtACGGtaccaaccacagattacgac
GGP_P1_PGM_D_R	GGTCTCTCATTttttgtatgtgttttggtgatgtc
GGP_P1_GAPdh_C_F_new	GGTCTCtACGGcggtagtcggaaagagc
GGP_P1_GAPdh_D_R	GGTCTCTCATTtgttgatgtgtgtttaattcaagaatg
GGP_G1_GGS1_D_F	GGTCTCTAATGgattataacagcgcgg
GGP_G1_GGS1_E_R	GGTCTCTTAGAtcactgcgcatcctc
GGP_T1_LIP2inclT_E_F	GGTCTCTTCTAgtgtctgtggtatctaagctatttatcactctttacaac ttctacctcaactatctactttaataaatgaatatcg
GGP_T1_LIP2inclT_F_R	GGTCTCTAAGCtgtcttagaggaacgcatatacagtaatcatagaga ataaacgatattcatttattaaagtagatagttgaggtagaagttg
GGP_P2_TEF_DSM_F_F	GGTCTCTGCTTgacgggttggcgg
GGP_P2_TEF_DSM_G_R	GGTCTCTTTGTtgattcttatactcagaaggaaatgc
GGP_P2_PGM_F_F	GGTCTCTGCTTtaccaaccacagattacgac
GGP_P2_PGM_G_R	GGTCTCTTTGTttttgtatgtgttttggtgatgtc
GGP_P2_GAPdh_F_F	GGTCTCTGCTTcggtagtcggaaagagc
GGP_P2_GAPdh_G_R	GGTCTCTTTGTtgttgatgtgtgtttaattcaagaatg
GGP_G2_carB_G_F	GGTCTCTACAAtgtccaagaaacacattgtcattatc
GGP_G2_carB_H_R	GGTCTCTATCCttaaatgacattagagttatgaacgc
GGP_T2_LIP2inclT_H_F	GGTCTCTGGATgtgtctgtggtatctaagctatttatcactctttacaa cttctacctcaactatctactttaataaatgaatatcg
GGP_T2_LIP2inclT_I_R	GGTCTCTTGACtgtcttagaggaacgcatatacagtaatcatagaga

	ataaacgatattcatttattaaagtagatagttgaggtagaagttg
GGP_P3_TEF_DSM_I_F	GGTCTCTGTCAgacgggttggcgg
GGP_P3_TEF_DSM_J_R	GGTCTCTGTGGtgattcttatactcagaaggaaatgc
GGP_P3_PGM_I_F	GGTCTCTGTCAtaccaaccacagattacgac
GGP_P3_PGM_J_R	GGTCTCTGTGGttttgtatgtgttttggtgatgtc
GGP_P3_GAPdh_I_F	GGTCTCTGTCAcggtagtcggaaagagc
GGP_P3_GAPdh_J_R	GGTCTCTGTGGtgttgatgtgtgtttaattcaagaatg
GGP_G3_carRP_J_F	GGTCTCTCCACAatgctgctcacctacatg
GGP_G3_carRP_K_R	GGTCTCTGTATttaaatggtatttagatttctcatttttccc
GGP_T3_LIP2inclT_K_F_NEW	GGTCTCTGTATgtgtctgtggtatctaagctatttatcactctttacaa cttctacctcaactatctactttaataaatgaatatcg
GGP_T3_LIP2inclT_L_R	GGTCTCTACTCtgtcttagaggaacgcatatacagtaatcatagaga ataaacgatattcatttattaaagtagatagttgaggtagaagttg
GGP_InsertDOWN_zeta_L_F	GGTCTCTGAGTcatgtgtaacactcgctctg
GGP_InsertDOWN_zeta_M_R_SfiI	GGTCTCTCGCAggcctccttggccactgaagggctttgtgag
Elimination of internal BsaI site through assembly PCR	
GGS1_Bsa_del_F	gaaatagtctcgagatcgctcttg
GGS1_Bsa_del_R	gagactatttcgcacatcaccaag
Verification of constructions	
ZetaUp-Intern-Fw	tatcttctgacgcattgaccac
Ura3Marker-intern-Rv	caactaactcgtaactattacc
Ura3Marker-intern-Fw	acatccagagaagcacacagg
GGSI_intern_Rv	cactgcgcatcctcaaagta
GGSI_intern_Fw	tcaaggagatatggggcaag
CarB_intern_Rv	ccattggattgagggaagaa
CarB_intern_Fw	atgccaccaaacaagtgaca
CarRP_intern_RV	tgtgctctgctgggatagtg
CarRP_intern_Fw	ccttctgtttacccgaccaa
ZetaDown-Intern-Rv	ggtaacgccgattctctctg

564

565 Supplementary Table 2: Plasmid table

PLASMIDS		
Name	***Resistance/Marker***	***Function (reporter)***
pCR™-Blunt II-TOPO®	Kanamycin	GG Donor vector
GGV TOPO InsUp-Zeta	Kanamycin	GG building block storage Insertion sequence 5' Zeta
GGV TOPO M-URA	Kanamycin	GG building block storage. Marker URA3
GGV TOPO M-Hygro	Kanamycin	GG building block storage. Marker Hygromycin Resistance
GGV TOPO P1-TEF	Kanamycin	GG building block storage. Promoter 1st position TEF
GGV TOPO P1-PGM	Kanamycin	GG building block storage. Promoter 1st position PGM
GGV TOPO P1-GAPdh	Kanamycin	GG building block storage. Promoter 1st position GAPdh
GGV TOPO G1-GSS1	Kanamycin	GG building block storage. Gene 1st position GSS1
GGV TOPO T1-Lip2	Kanamycin	GG building block storage. Terminator 1st position Lips2
GGV TOPO P2-TEF	Kanamycin	GG building block storage. Promoter 2nd position TEF
GGV TOPO P2-PGM	Kanamycin	GG building block storage. Promoter 2nd position PGM
GGV TOPO P2-GAPdh	Kanamycin	GG building block storage. Promoter 2nd position GAPdh
GGV TOPO G2-CarB	Kanamycin	GG building block storage. Gene 2nd position GSS1
GGV TOPO T2-Lip2	Kanamycin	GG building block storage. Terminator 2nd position Lips2
GGV TOPO P2-TEF	Kanamycin	GG building block storage. Promoter 3rd position TEF
GGV TOPO P2-PGM	Kanamycin	GG building block storage. Promoter 3rd position PGM
GGV TOPO P2-GAPdh	Kanamycin	GG building block storage. Promoter 3rd position GAPdh
GGV TOPO G3-CarRP	Kanamycin	GG building block storage. Gene 3rd position GSS1
GGV TOPO T3-Lip2	Kanamycin	GG building block storage. Terminator 3rd position Lips2
GGV TOPO InsD-Zeta	Kanamycin	GG building block storage Insertion sequence 3' Zeta
pSB1A3-GB3	Ampicilin	GG Destination vector (GB3 – red chromophore)
GGAV 3TEF-Carotenoids-URA	Ampicilin/ URA3	GG Assembly expression cassette 3TEF_GSS1-CarB-CarRP-

		_URA3
GGAV 3TEF-Carotenoids-Hygro	Ampicilin/ Hygromycin	GG Assembly expression cassette 3TEF_GSS1-CarB-CarRP_Hygromycin
pMB6511	Ampicilin / Leu2	Carotenoid expression cassette
pMB6205	Kanamycin/ Hygromycin	HMG expression cassette

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