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Improving carotenoids production in yeast via adaptive laboratory evolution

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ABSTRACT

Adaptive laboratory evolution is an important tool for the engineering of strains for industrially relevant phenotypes. Traditionally, adaptive laboratory evolution has been implemented to improve robustness of industrial strains under diverse operational conditions; however due to the required coupling between growth and survival, its application for increased production of secondary metabolites generally results in decreased production due to the metabolic burden imposed by, or toxicity of, the produced compound.

In this study, adaptive laboratory evolution was successfully applied to improve carotenoids production in an engineered *Saccharomyces cerevisiae* producer strain by exploiting the antioxidant properties of carotenoids. Short-term evolution experiment using periodic hydrogen peroxide shocking schemes resulted in a 3-fold increase in carotenoids production (from 6 mg/g dry cell weight to up to 18 mg/g dry cell weight). Subsequent transcriptome analysis was used to elucidate the molecular mechanisms for increased carotenoids production. Upregulation of genes related with lipid biosynthesis and mevalonate biosynthesis pathways were commonly observed in the carotenoids hyper-producers analyzed.

1. Introduction

Carotenoids are an attractive class of tetraterpenoid pigmented compounds naturally produced by diverse organisms including plants and numerous fungi and bacteria (Armstrong and Hearst, 1996; Huang et al., 2013). These compounds are known antioxidants (Edge et al., 1997) that have potential positive impact on human health (playing an important role as pro-vitamin A compounds), and are used in the nutraceutical industry as supplements, as additives in fortified foods and in the cosmetics industry, with a total market value estimated in $1.2 billions in 2010 (BCC research published Sept 2011). The biological pathways of all carotenoids use isopentenyl diphosphate (IPP) as precursor. Biosynthesis of IPP falls in two different pathways: the mevalonate (MVA) pathway and the mevalonate-independent methyl erythritol 4-phosphate (MEP) pathway. In the MVA pathway, acetyl-CoA is the precursor for mevalonate. In the MEP pathway, MEP is produced from 1-deoxy-D-xylulose-5-phosphate (DXP), which is synthesized from pyruvate and glyceraldehyde-3-phosphate (G3P). The MVA pathway is the one naturally used by yeast for the production of IPP (Armstrong and Hearst, 1996).

Currently, most industrially produced carotenoids are chemically synthesized through multistep chemical synthesis or by solvent-based chemical extraction from non-microbial sources. Chemical synthesis is not a viable option to produce most carotenoids due to their structural complexity (Vachali et al., 2012). Advances in metabolic engineering and synthetic biology have enabled the engineering of microorganisms for the heterologous production of carotenoids as a potentially more sustainable route of production (Asadollahi et al., 2009; Farhi et al., 2011; Farmer and Liao, 2001; Harada et al., 2009; Lee et al., 2004; Nishizaki et al., 2007; Peralta-Yahya et al., 2011; Verdoes et al., 2003; Verwaal et al., 2007; Zhao et al., 2013). These efforts have thus far involved the optimizations of native pathways, introduction of foreign genes to enhance metabolic flux, and through co-factor balancing. One option that has not been explored is the use of adaptive laboratory evolution (or whole genome directed evolution) to improve the production of carotenoids. Adaptive laboratory evolution uses a selective pressure as driving force for the selection of mutants with enhanced phenotypes. Since carotenoids have antioxidant properties (Paiva and Russell, 1999; Ukibe et al., 2009), we hypothesized that oxidative stress can be used as a driving force for the directed evolution of microbial systems for enhanced carotenoids production.

The main objective of this work is to develop a proper selective pressure in order to use directed evolution to improve heterologous carotenoids production in a microbial host. A *Saccharomyces cerevisiae* (*S. cerevisiae*) strain was engineered to produce carotenoids using heterologous genes from the yeast *Xanthophyllomyces dendrorhous* (Verwaal et al., 2007, 2010). Enhancement of carotenoids yield was achieved through adaptive evolution using a periodic hydrogen peroxide shocking strategy to select for higher producers. The

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potential mechanisms involved in the enhanced production of carotenoids in the hyper-producing mutants were assessed using transcriptome analysis. Upregulation of genes involved in lipid biosynthesis was identified as the likely regulatory perturbation that led to increased carotenoids production. The highest β-carotene yield achieved after a short-term evolution experiment was 18 mg/g [dcw], corresponding to a 3-fold increase compared with the ancestral strain (6 mg/g [dcw]).

2. Materials and methods

2.1. Strains, plasmids and growth conditions

S. cerevisiae strain GSY1136 (Kao and Sherlock, 2008), derived from FY2 (*MATα*, *URA3-52*, isogenic to S288C), was chosen as the background strain for this work. All yeast strains used are listed in Table 1. The evolution experiments were carried out in Yeast Extract Peptone Dextrose medium (YPD) to ensure high biomass formation at 30 °C. The shuttle vector YIplac211YB/I/E* (Verwaal et al., 2007) was used to introduce *crtE*, *crtYB* and *crtI* genes into the yeast strain. For the determination of carotenoids yields on glycerol as a carbon source, YPG medium (Yeast Extract Peptone Glycerol) supplied with 4% (v/v) glycerol was used.

2.2. Construction of the carotenoids yeast producer

The *URA3* gene was excised out from GSY1136 using 5-fluoroorotic acid (5-FOA) as counter-selection in order to create the uracil auxotrophic strain YLH0. The transformation of the YIplac211YB/I/E* shuttle vector into YLH0 was carried out using the lithium acetate method (D Gietz, 1992), with *URA3* as selectable marker for integration, generating the strain YLH1. The transformed cells were selected on YNB+2% D-glucose (w/v), incubated at 30 °C for 3 days, and verified using PCR amplification.

2.3. Deletion of CTT1 catalase

The catalase *CTT1* was knocked out from the genotype of interest using homologous recombination with the *NEO* gene. The primers used for *NEO* amplification, including the homologous regions used to delete *CTT1*, were: forward: 5′-TTA AAA AAA TCC TTC TCT TGT CTC ATG CCA ATA AGA TCA ATC AGC TCA GCT TCA CAA ATG CGG ATC CCC GGG TTA ATT AA-3′ and reverse: 5′-TAT AAT TAC GAA TAA TTA TGA ATA AAT AGT GCT GCC TTA ATT GGC ACT TGC AAT GGA CCA GAA TTC GAG CTC GTT TAA AC-3′. The plasmid pFA6a-kanMX6 (Bähler et al., 1998) was used as template for the *NEO* cassette. Transformation of the integration cassette was carried out using lithium acetate procedure. The integrants were selected on YPD+geneticin (G418) plates (200 μg/ml), incubated at 30 °C for 2 days, and verified using PCR amplification. The primers used for verification were: forward 5′-ATT CGA CGT AGC CTG GAC AC-3′ and reverse 5′-TAA TCG TTG AGT TCA TGC CG-3′. No significant differences in growth kinetics were observed between any of the strains used in this study in either YNB+glucose or YPD+glucose at 30 °C (see Supplementary Fig. 1).

Table 1
List of strains used in this work.

Name	Relevant genotype	Source
GSY1136	Matα, *ura3-52, gal+* in S288c background, *YBR209W*::Act1p-GFP-Act1t-*URA3*	Kao and Sherlock (2008)
YLH0	GSY1136 Δ*URA3*	This work
YLH1	GSY1136 YIplac211YB/I/E*	This work
YLH2	GSY1136 YIplac211YB/I/E* Δ*CTT1*	This work
YLH3	GSY1136 Δ*CTT1*	This work

2.4. Carotenoids quantification

For the carotenoids quantification of the evolving populations, 3 mL of YPD was inoculated with cells from frozen stocks, and incubated at 30 °C for 72 h. Cell density was determined using spectrophotometry (OD_{600}). 250 μL of culture were transferred to a 2 mL collection tube and the cells were collected by centrifugation at 12,000 rpm for 2 min. Supernatant was vacuum aspirated. The pelleted cells were disrupted using approximately 250 μL of 425–600 μm acid-washed glass beads (Sigma) and dodecane (1 mL) to extract the carotenoids. Yeast cells were lysed using an analog Disruptor Genie Cell Disruptor (Scientific Industries). Samples were treated twice for 6 min each to ensure maximum cell disruption and carotenoids recovery. Cell debris and glass beads were separated from the supernatant by centrifugation at 15,000 rpm for 2 min. 200 μL of the supernatant was transferred to a Corning® 96 well black-wall clear-bottom plate for quantification. Total carotenoids were quantified using a survey scan from OD_{350} to OD_{550} to determine any shifts in the spectrum using a microplate reader (TECAN Infinite® M200). The relative total carotenoids production was determined by calculating the area under the curve of the survey scan, using the ancestral strain YLH2 (see Table 1 for genotype information) as reference. β-carotene quantification was determined by the absorption at OD_{454} (Verwaal et al., 2007). A standard curve for β-carotene quantification was generated using commercially available β-carotene (Enzo Life Sciences) at OD_{454}.

2.5. Isolation of hyper-carotenoids producing mutants

Selected populations were revived from frozen stocks and plated on YPD agar plates for isolation of single colonies. Plates were incubated for 72 h at 30 °C and several colonies were chosen based on their apparent red color and normal colony size (compared with the ancestral strain YLH2). The colonies were cultured in 3 mL of YPD media and incubated for 72 h for total carotenoids quantification. The highest producer from each population was selected for further analysis.

2.6. Hydrogen peroxide shock experiments

Each isolated mutant was inoculated in 3 mL of YPD media and incubated for 72 h. Samples were normalized to an OD_{600}~2.0. 500 μL of the normalized culture was transferred to a microcentrifuge tube and shocked with 1.05 M hydrogen peroxide for 30 min. Samples were diluted in YPD, plated on YPD plates and incubated at 30 °C for 48 h for colony counting. A pre-shocked sample was plated on YPD plates to ensure proper normalization of the cell density and dilution.

2.7. RNA extraction

The isolated mutants were inoculated in 25 mL of YNB media (20 g/L D-glucose) in 125 mL flask at an initial OD_{600}~0.05 and cultured at 30 °C. The cells were harvested in late-exponential phase (OD~4.0) by filtration using NALGENE analytical test filter funnels, immediately resuspended in 10 mL of RNA*later* (Sigma) and stored at −80 °C for future analysis. For the extraction of total RNA, the ZR Fungal/Bacterial RNA MiniPrep™ (Zymo Research) kit was used following manufacturers' instructions using 3 mL of each stored sample in RNA*later*. The extracted RNA was quantified using the NanoDrop™ 1000® (Thermo Scientific).

2.8. Labeled cDNA generation, microarray hybridization and data analysis

The reverse transcription reaction was prepared by mixing 10 μg of isolated total RNA, 1U SuperScript® III reverse transcriptase

(Life Technologies), nucleotides (dATP-5 mM, dGTP-5 mM, dCTP-5 mM, dTTP-2 mM and amino-allyl dUTP-3 mM) and Oligo(dT)$_{20}$ primers (Life Technologies) to synthesize the cDNA. cDNA was recovered with ice-cold ethanol precipitation. Cy3- and Cy5- mono-Reactive Dye Pack (GE Healthcare) were used to label cDNA samples. The labeled cDNA was hybridized to the *S. cerevisiae* G4813A Gene Expression Microarray (Agilent Technologies). The arrays were scanned using the GenePix 4100A Microarray Scanner and GenePix Pro 6.0 software (Molecular Devices) for image analysis. The Microarray Data Analysis System (MIDAS) software was used to normalize the data using LOWESS based normalization algorithm (Quackenbush, 2002). Differentially expressed genes were identified using the rank product method with a critical p-value <0.05. The MeV (TM4) (Saeed et al., 2006) microarray analysis software was used for clustering and other expression profile analysis. The *Saccharomyces* genome database (SGD) (Cherry et al., 2011) was used to analyze the data based on gene ontology. Further gene ontology analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al., 2009a, 2009b), in order to identify any enriched biological functions.

2.9. Real-time PCR

The CFX384 Real-Time PCR Detection System (Bio-Rad Laboratories, CA) was used to carry out the quantitative reverse-transcription real-time PCR (qRT-PCR) experiments. RNA extraction and reverse transcription of each sample were performed as described in the previous section. GoTaq® qPCR Master Mix (Promega) was used for all the qRT-PCR experiments, using 20 ng of cDNA and 0.5 μM of each primer. The primers used for the qRT-PCR experiments are: Control gene (*COQ5*): GAC TTC AAT ACA GTC TTC GAA CCA AA (forward) and TCC TTA TAC AGC TGC TGT TAC AAT T (reverse). *crtYB* gene: TGC CAC AAT TGA CAT GGT CT (forward) and AGG CGA AAT GGT ATT GAA CG (reverse). *crtI* gene: GAA GTC GAG CGT TTT GAA GG (forward) and AGG ATT TGG CCA ATG AAC TG (reverse). *crtE* gene: GGG ATT CCG CAG ACA ATA AA (forward) and CTT TCG AGA ACG GAA TCT GC (reverse). The thermocycler protocol used was: 95 °C for 3 min followed by 39 cycles of 95 °C for 10 s followed by 55 °C for 30 s.

2.10. Bioreactor studies

YNB media supplemented with 20 g/L D-glucose was used for bioreactor studies. The seed cultures for fermentation were prepared as follows: Samples from single colonies were used to inoculate 3 mL YNB culture (20 g/L glucose) and incubated at 30 °C for 24 h with constant agitation at 250 rpm. The content of the culture was used to inoculate 50 mL of fresh YNB media (20 g/L glucose) and incubated in the same conditions for 24 h. The 50 mL culture was used to inoculate a batch bioreactor with a total volume of 3 L.

The bioreactor studies were carried out in a 7 L glass autoclavable bioreactor (Applikon®). The pH was maintained at 5.5 by addition of 2 N HCl or 2 N NaOH as needed. The temperature was set at 30 °C. All the bioreactor experiments were performed in batch mode and terminated when the ethanol produced was consumed.

3. Results and discussion

3.1. Directed evolution experiments for enhanced carotenoids productivity

Since the heterologous production of carotenoids by *S. cerevisiae* is not growth-coupled and its production poses a metabolic burden for yeast cells (Verwaal et al., 2010), an appropriate selective pressure is needed to allow the higher producers a growth advantage. Based on the known antioxidant properties of carotenoids (Paiva and Russell, 1999), we hypothesized that yeast strain producing carotenoids will have a fitness advantage compared with a non-producer in the presence of oxidative stress, specifically in the presence of hydrogen peroxide. Since yeast produces catalases for hydrogen peroxide detoxification, we first deleted the cytosolic catalase, encoded by *CTT1*, from a heterologous carotenoids producer (see Materials and Methods for construction of the YLH2 strain). The *CTT1* deletion resulted in an appreciable decrease in carotenoids production (~43% decrease). However, carotenoids production led to an increase in cell viability upon short-term exposure to high concentration of hydrogen peroxide in the absence of *CTT1* (carotenogenic strain YHL2 compared with non-carotenogenic strain YLH3) in late-exponential/stationary phase (see Fig. 1). These protective effects of carotenoids on yeast cells have been observed previously (Ukibe et al., 2009; Yan et al., 2011).

To ensure that higher carotenoids production is not selected for in the absence of oxidative stress, the strain YLH2 (YLH1 Δ*CTT1*) was serially transferred (daily) in the absence of hydrogen peroxide for 6 days. As shown in Fig. 2C, the carotenoids yields during this short-term experiment actually decreased by approximately 30% compared with the parental strain, demonstrating that enhanced carotenoids productivity does not confer a fitness advantage in the absence of oxidative stress.

Two parallel populations (P1 and P2) were initiated from two independent colonies of YLH2 (ancestral strain) for the directed evolution experiments in 3 mL cultures. Approximately 7% (v/v) of each culture was serial transferred daily. Population samples were collected daily and stored in glycerol at −80 °C. Each population was shocked periodically with hydrogen peroxide. Two different phases (Phase I and Phase II) of hydrogen peroxide shock treatments were used as selective pressure to increase carotenoids production (details are given in Table 2). Each “shock treatment” was performed during the late exponential or early stationary phase of growth. 500 μL of culture were transferred to a sterile microcentrifuge tube to be shocked with hydrogen peroxide for 30 min. Concentrations of hydrogen peroxide used for the shocking experiments are shown in Fig. 2A and B (red markers). 200 μL of the shocked culture were inoculated into 3 mL of fresh YPD media and incubated at 30 °C for 24 h for recovery (recovery phase). This shocking treatment was performed on a daily basis during Phase I (see Table 2). Phase I lasted for 7 serial transfers in population P1, and 14 serial transfers in population P2 (Table 2). We observed a gradual decrease in growth rate in both populations as the selection progressed in Phase I. This is likely due to the accumulation of H_2O_2 in the media during each serial transfer after

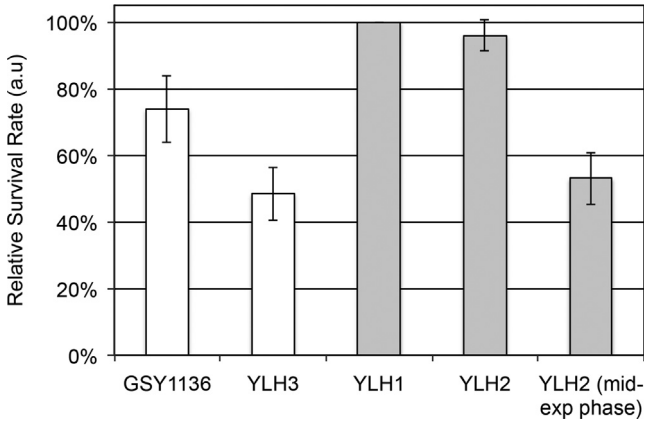


Fig. 1. Hydrogen peroxide shock experiments using 690 mM of hydrogen peroxide. Cells were shocked during late exponential phase unless otherwise specified. Solid gray bars: strains with carotenoids biosynthetic cassette. Open bars: non-carotenoids producing strains.

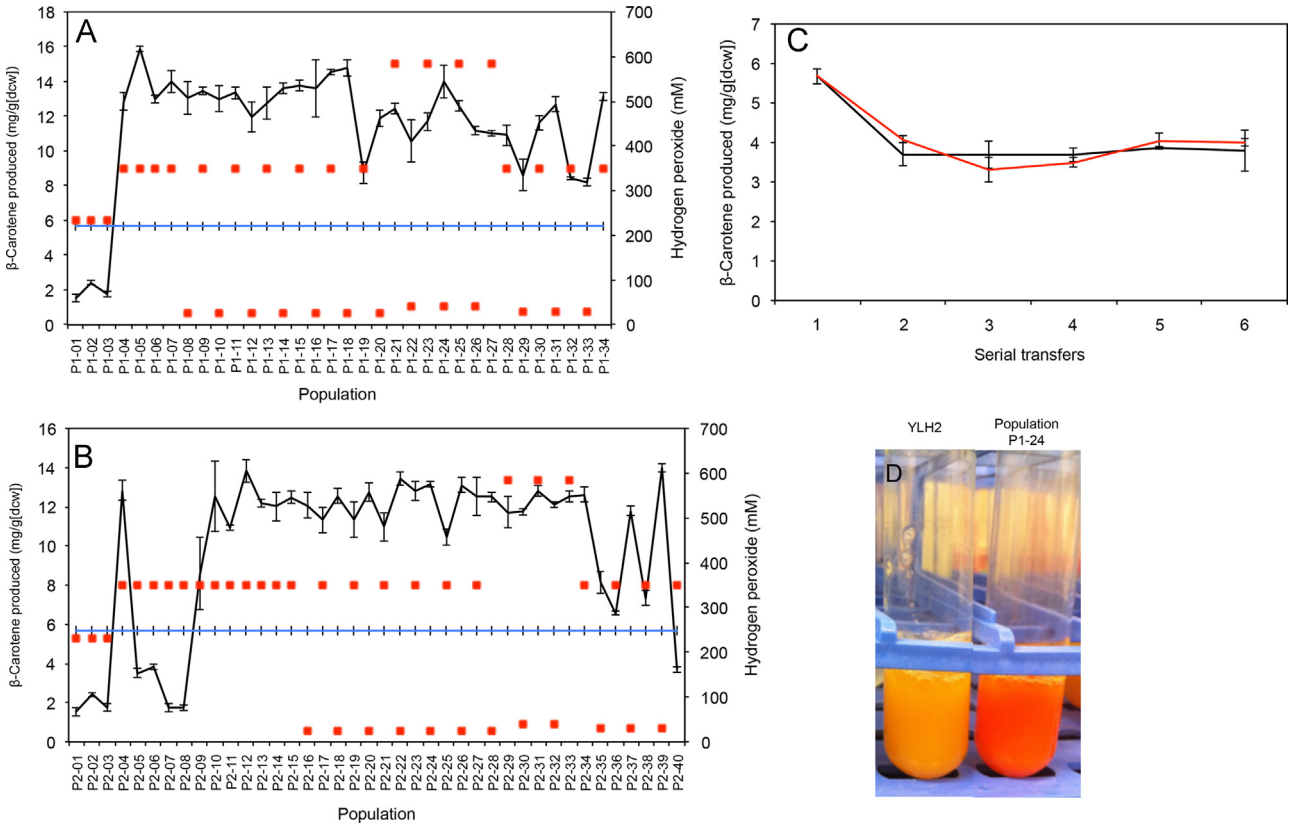


Fig. 2. The observed improvements in carotenoids production during hydrogen peroxide challenged adaptive evolution. Average β-carotene produced (black lines) and the hydrogen peroxide concentration used (red markers) in two independent populations: A. P1 and B. P2. C. Changes in β-carotene concentration in two independent cultures (black and red lines) of YLH2 serially propagated in the absence of hydrogen peroxide. D. Visual comparison of the amount of carotenoids produced between the ancestral strain and the population P1-24. Blue line: the level of β-carotene produced for the ancestor strain. Error bars represent the standard deviation from a minimum of 3 biological measurements. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Hydrogen peroxide shock scheme used in the directed evolution experiment. Each cycle consists of two 24-h periods.

Selective pressure scheme	Populations	First 24 h period	Second 24 h period
Phase 1:	P1: P1-01 to P1-07 P2: P2-01 to P2-15	Shock treatment for 30 min followed by recovery phase	Shock treatment for 30 min followed by recovery phase
Phase 2:	P1: P1-08 to P1-34 P2: P2-16 to P2-40	Shock treatment for 30 min followed by recovery phase	Recovery phase

the "shock treatments". Consequently, Phase II was initiated by performing the "shock treatments" every other serial transfer to increase the recovery period (see Table 2 for details). This increased recovery phase helped to ameliorate the increased toxicity due to H_2O_2 accumulation, as we no longer observed a decrease in cell density.

During the directed evolution experiment with the periodic hydrogen peroxide treatments, the total amount of β-carotene produced (and the total carotenoids content in general) within each population increased shortly after the start of the evolution experiment, reaching more than 12 mg/g dry cell weight [dcw] and resulting in observably redder cultures as shown in Fig. 2D. This enhanced productivity corresponds to greater than 100% increase in the carotenoids yield compared to the ancestral population (originally producing 6 ± 1 mg/g[dcw]). After several days under the Phase II of the selection strategy, an increase in hydrogen peroxide concentration (~600 mM) resulted in an observed decrease in cell mass, likely a result of too high of a selective pressure. Thus, the hydrogen peroxide concentration was decreased back to 350 mM. Unfortunately, the prolonged evolution with Phase II of the selection strategy did not result in additional increases in the average amount of carotenoids produced by the evolving populations. Although there is indication that the carotenoids are transported outside the cell, the quantified rate of secreted carotenoids using a two-phase culture (ratio 2:1 aqueous phase:organic phase) was very low (on the order of 10^{-4} mg/g [dcw]/h), and it is possible that we have reached a physical limit in the amount of carotenoids that can be accumulated inside the cell.

3.2. Isolation of adaptive mutants

Upon the conclusion of the adaptive evolution experiment, mutants were isolated from frozen stocks at different time-points throughout the evolution. The populations to be further analyzed were chosen based on the observed peaks in the average amount of carotenoids produced (see Fig. 2A and B). Mutants were isolated from each of the chosen populations as described in the Materials

and Methods section. Details about each isolated mutant can be found in Fig. 3.

The amount of β-carotene produced in the isolated mutants were measured and compared with the populations from which they were isolated (the results are summarized in Fig. 3). In general, the amount of β-carotene produced by the isolated mutants was significantly higher than the average amount produced by the population they were isolated from. Mutant SM14 was amongst the highest producers, producing approximately 300% more β-carotene (18 ± 1 mg/g [dcw]) compared to the parental YLH2 strain (6 ± 1 mg/g [dcw]).

As we hypothesized that increased carotenoids yield would result in increased resistance to oxidative stress as the basis for this work, we tested the tolerance of each isolated mutant to hydrogen peroxide challenge using a viability assay. A hydrogen peroxide shock experiment using 1.05 M H_2O_2 was performed, and increased cell viability was observed in all of the isolated mutants tested (see Fig. 4).

3.3. Transcriptome analysis of adaptive mutants

The four highest carotenoids producers (SM12, SM13, SM14, and SM22) were chosen for further molecular analysis to identify potential mechanisms for increased carotenoids production in these strains. Since increases in the gene expressions of the carotenoids biosynthetic genes (either due to copy number amplification or mutations in the promoter regions) is a likely cause for increased carotenoids production, we used qRT-PCR to compare the expression levels of the genes *crtI*, *crtE* and *crtYB* (heterologous carotenogenic genes) and the *COQ5* gene, whose expression was found to be unaltered in the mutants based on our transcriptome data (see below), as control. The results showed no expression differences in the carotenogenic genes between the hyper-producing strains and YLH2. In addition, no increase in gene copy number was found between the hyper-producing strains and YLH2. Thus, these results suggest a different route for enhanced carotenoids production in these strains.

Using DNA microarrays, the transcriptome of every isolated mutant was compared to YLH2 as reference. All the strains were grown in YNB media supplemented with 20 g/L glucose at 30 °C until late-exponential phase. Three biological replicates were performed per strain. The genes significantly (p-value < 0.05) differentially expressed were analyzed further to identify potential mechanisms for increased carotenoids production (on average, 107 genes were upregulated and 97 downregulated).

The gene expression profiles between the 4 mutants analyzed (SM12, SM13, SM14 and SM22) were similar, with approximately 29% of the perturbed genes being commonly perturbed (59 genes) between all the mutants, suggesting that the mechanisms of enhanced production are similar between these isolates. Analysis of the set of perturbed genes (significantly differentially expressed in at least one mutant) suggests an increased biosynthesis of HMG-CoA, which is the substrate for the rate-limiting step in IPP production (Britton et al., 1998). Some of the key perturbations are described below. Supplementary Table 1 lists all genes differentially expressed in at least one mutant.

3.3.1. Genes involved in lipid biosynthesis

Several genes involved in the production of lipids were differentially perturbed. The key genes upregulated in the lipid biosynthesis pathway include *CAB1*, *NSG1*, *ERG13*, and *ERG27* (in strains SM12, SM13, SM14 and SM22), and *CYB5* (in SM14 and SM22). *CAB1*, a gene encoding the pantothenate kinase, catalyzes the first step in the synthesis of coenzyme A (required for the formation of HMG-CoA), is regulated by sterol response element (Olzhausen et al., 2009). *NSG1* and *CYB5* are involved in the regulation of the sterol and lipid biosynthesis (Flury et al., 2005; Lamb et al., 1999). Upregulation of *NSG1* has been identified to play a key role in reducing Hmg2p degradation (Flury et al., 2005), which is one of the two HMG-CoA coenzymes. Increased production of HMG-CoA has been reported to improve production of β-carotene in *S. cerevisiae* (Yan et al., 2012).

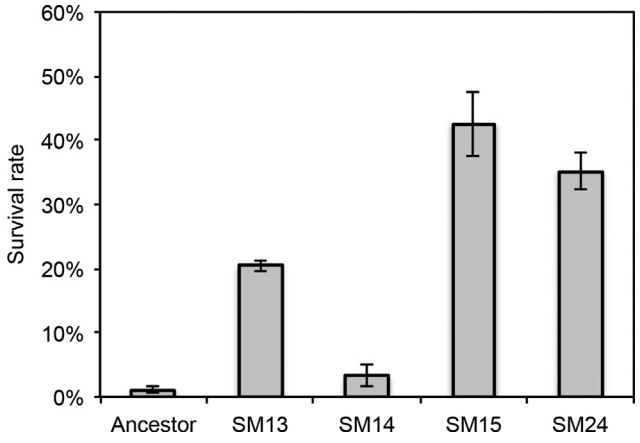
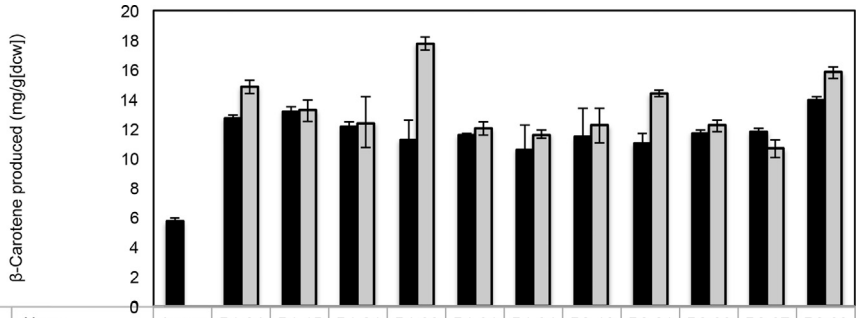


Fig. 4. Hydrogen peroxide shock experiment with selected isolated mutants. All 4 mutants shown here have viabilities that are statistically significantly different from the ancestral strain (p-value < 0.05).



■Population	Name	Ances	P1-04	P1-15	P1-24	P1-30	P1-31	P1-34	P2-10	P2-21	P2-30	P2-37	P2-39
	Yield (mg/g dcw)	6	13	13	12	11	12	11	11	11	12	12	14
■Isolated Mutant	Name		SM11	SM12	SM13	SM14	SM15	SM16	SM21	SM22	SM23		SM24
	Yield (mg/g dcw)		15	13	12	18	12	12	12	14	12	11	16

Fig. 3. Comparison of the amount of β-carotene produced between the evolved populations (solid black bars) and single mutants isolated from each respective population (solid gray bars).

The cytochrome b5, encoded by the gene *CYB5*, acts as an electron donor to support the sterol C-5(6) desaturation (Rogers et al., 2004). This process is carried out through the C-5 desaturase, encoded by *ERG3* (Supplementary Fig. 2). *ERG13* is a 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase, catalyzing the formation of HMG-CoA from acetyl-CoA and acetoacetyl-CoA and is involved in the second step in mevalonate biosynthesis (Parks et al., 1995). Upregulation of *ERG13* potentially suggests an increased biosynthesis of HMG-CoA (Supplementary Fig. 2). The upregulation of *ERG27*, a 3-keto sterol reductase, catalyzing the last of three steps in ergosterol biosynthesis (Parks et al., 1995), is a competing branch to the carotenoids pathway. The upregulation of *ERG27* and *CYB5* may be a potential compensatory mechanism for increased flux towards the competing carotenoids biosynthesis pathway in the hyper-producing mutants. On the other hand, overexpression of *ERG13* and *NSG1* can potentially increase the biosynthesis of the carotenoids precursor, IPP. Downregulation of the ergosterol biosynthetic pathway via deletion of the *ERG24* gene, as well as downregulation of lipid and amino acids biosynthesis, seemed to affect carotenoids production (Ozaydın et al., 2013) in a prior study.

Other lipid biosynthesis-related genes that were differentially expressed are summarized in Fig. 5.

3.3.2. *Change in expression of genes involved in stress response*

We found significant expression changes in genes involved in stress response in all the mutants analyzed. The genes *AAD6* (in SM12, SM13 and SM22), *MXR2* (in SM12, SM13, SM14 and SM22), *UGA1* (in SM22) and *ROX1* (in SM12), involved in oxidative stress response, were downregulated in the isolated mutants. On the other hand, the genes *GND1* (in SM14), *SKO1* (in SM12) and *HOR2* (in SM14 and SM22), whose expressions have been shown to decrease under oxidative stress (Izawa et al., 1998; Pahlman et al., 2001; Rep et al., 2001), were upregulated in our mutants. These results suggest that the increased carotenoids production in the evolved mutants partially alleviates oxidative stress in the cell.

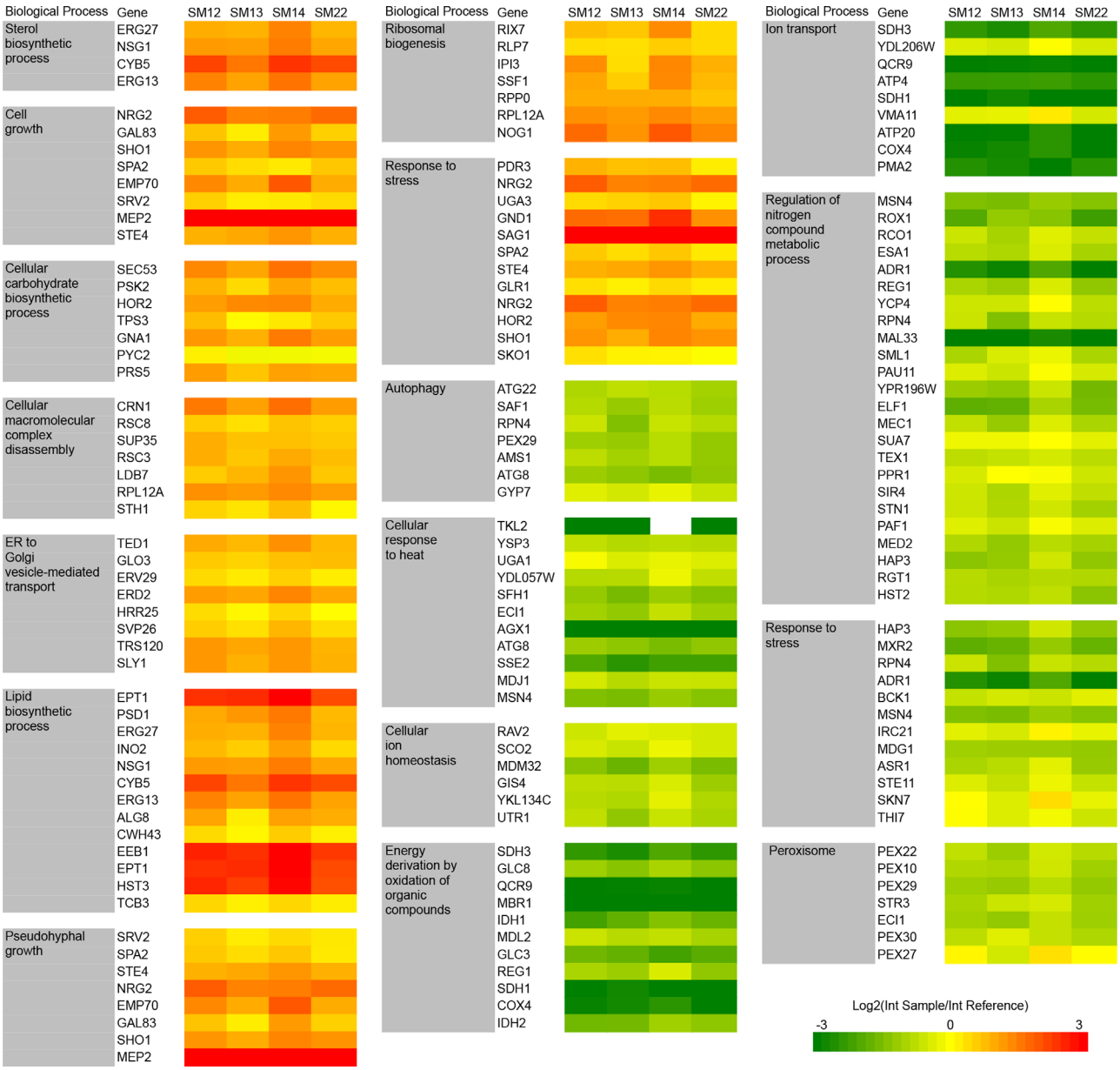


Fig. 5. Selected differentially regulated genes grouped by their biological functions based on gene ontology terms.

The gene encoding a transcriptional activator of the pleiotropic drug resistance network, *PDR3*, was upregulated in the mutants SM12 and SM13. Upregulation of PDR genes, encoding ABC-type and major facilitator transporters, have been reported to be involved in secretion of toxic compounds out of cells and have previously been observed to be upregulated in transcriptome analyses in carotenoids producing cells, possibly due to an inhibitory effect of the carotenoids (Verwaal et al., 2010). Thus we attempted to quantify the amount of carotenoids exported to the media using two-phase (YPD/dodecane) cultures. The average secretion rate quantified during the 72 h of incubation in a two-phases culture, using a ratio of 2:1 aqueous phase/organic phase, were on the order of 10^{-4} mg/g [dcw]/h for the strains tested.

Numerous genes involved in response to hyperosmotic and chemical stimulus were differentially expressed in the mutants (see Fig. 5). The intracellular accumulation of the hydrophobic carotenoids could trigger different mechanisms of stress response, particularly response to osmotic stress. Several genes involved in the biogenesis and regulation of peroxisomes were downregulated in the different isolated mutants as shown in Fig. 5. Since different hydrogen peroxide oxidases and catalases are compartmentalized in peroxisomes in yeast (Purdue and Lazarow, 2001), it is currently not clear if and how downregulation of peroxisome biogenesis genes contribute to enhanced carotenoids production. Although recent work found downregulation of peroxisome biogenesis to be a requirement for increased lipid-related product formation in *Yarrowia lipolytica* (Xue et al., 2013).

3.3.3. Other transcriptional changes

When all differentially expressed genes were categorized according to their cellular component ontology, we found numerous genes involved in mitochondrial proteins (~11% of the perturbed genes) to be perturbed. Most of the perturbed genes were downregulated, and their functions are involved in electron transport and cellular respiration related functions (*MIC14* and *SDH1* in strains SM14 and SM22, *COX4*, *QCR9* and *SDH3* in SM12, SM13, SM14 and SM22, *MBR1* in SM12, SM13 and SM14, and *IDH1* and *IDH2* in mutant SM12).

The genes *GPT2* (glycerol-3-phosphate/dihydroxyacetone phosphate), involved in the lipid biosynthesis via acylation of glycerol-3-phosphate and dihydroxyacetone and *ADR1*, a transcription factor required in the expression of several genes involved in glycerol, ethanol and fatty acid utilization, were downregulated in the mutants SM12 (*GTP2* and *ADR1*), SM13 (*ADR1*) and SM22 (*ADR1*). The downregulation of these two genes suggests a potential defect in glycerol utilization in the evolved mutants. Indeed, we observed a decreased growth rate compared to YLH2 strain (Supplementary Fig. 3) when the evolved cells were grown on glycerol as a carbon source.

3.4. Scale-up studies of carotenoids production

Strain SM14 (the highest carotenoids producer) was chosen for batch bioreactor studies. The seed train was initialized by inoculating 3 ml of YNB media (20 g/L glucose) from a single colony and incubated for 24 h at 30 °C. The culture was transferred to 50 mL of fresh YNB media and incubated for 24 h at 30 °C with constant shaking. A 7 L bioreactor was inoculated with the entire content of the 50 mL culture, maintained at pH=5, temperature at 30 °C and constant agitation at 400 rpm.

In this initial study, the bioreactor was supplied with constant airflow at 6.0 L/min in order to maintain at least 50% dissolved oxygen throughout the experiment. Within 24 h, the glucose was exhausted (verified using HPLC) and the β-carotene yield at that point was 6 mg/g [dcw]. After 24 h, the accumulated ethanol started being consumed, and the net production of β-carotene increased noticeably (reaching 15 mg/g [dcw]) as shown in Fig. 6. To verify that using ethanol as a carbon source increases β-carotene productivity compared with glucose as a carbon source, two batch culture experiments were carried out with SM14 grown in YNB media supplemented with either 2% glucose (w/v) or 4% ethanol (v/v) until late exponential phase (prior to the diauxic shift in the glucose culture). The resulting yields were 8.8 mg/g [dcw] and 15.2 mg/g [dcw] in glucose and ethanol, respectively, an increase of 73% ± 6% in β-carotene yield when ethanol, relative to glucose, was used as the carbon source.

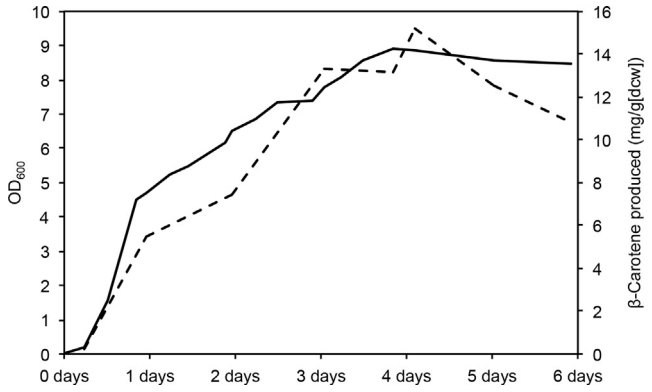


Fig. 6. Batch fermentation of strain SM14 in a bench-top bioreactor. Solid line: OD_{600}. Dashed line: β-carotene productivity.

Due to differences in growth conditions (growth media [YPD vs. YNB], pH and aeration) between the bioreactor and the batch experiments, the obtained productivity in the bioreactor (15 mg/g [dcw]) was lower than the productivity obtained in test tubes (18 ± 1 mg/g [dcw]). The specific bioreactor operational parameters that affect productivity remain to be investigated.

4. Conclusions

In conclusion, adaptive laboratory evolution was successfully applied for the increased production of carotenoids in an engineered *S. cerevisiae* strain. This was accomplished by the rational design of a selective pressure scheme based on the antioxidant properties of carotenoids. Hyper-producers were isolated from the short-term evolution experiment with yields as high as 18 mg/g [dcw], a 3-fold increase in carotenoids production over the parental strain. Transcriptomic analysis revealed increases in expression of several genes involved in lipid biosynthesis, specifically those in the mevalonate biosynthesis pathway, as a potentially key perturbation for the increase in carotenoids production. This work demonstrates a case where the proper selective pressure can be used to improve secondary metabolite production using directed evolution.

Author contributions

LHR and KCK conceived and designed the experiments; LHR and JMG conducted the experiments; LHR and KCK analyzed the data; LHR and KCK wrote the manuscript.

Data availability

All raw microarray data is MIAME compliant and have been deposited in the GEO database with accession number GSE51613.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2013.11.002.

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