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Improving carotenoids production in yeast via adaptive laboratory evolution

Luis H. Reyes, Jose M. Gomez, Katy C. Kao *

Department of Chemical Engineering, Texas A&M University, College Station, United States

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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

* Correspondence to: 3122 TAMU, College Station, TX 77843-3122, United States. Fax: +1 979 845 6446.
E-mail address: kao.katy@mail.che.tamu.edu (K.C. Kao).

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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