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Engineering of *Yarrowia lipolytica* for production of astaxanthin

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ABSTRACT

Astaxanthin is a red-colored carotenoid, used as food and feed additive. Astaxanthin is mainly produced by chemical synthesis, however, the process is expensive and synthetic astaxanthin is not approved for human consumption. In this study, we engineered the oleaginous yeast *Yarrowia lipolytica* for *de novo* production of astaxanthin by fermentation.

First, we screened 12 different *Y. lipolytica* isolates for β-carotene production by introducing two genes for β-carotene biosynthesis: bi-functional phytoene synthase/lycopene cyclase (*crtYB*) and phytoene desaturase (*crtI*) from the red yeast *Xanthophyllomyces dendrorhous*. The best strain produced 31.1 ± 0.5 mg/L β-carotene. Next, we optimized the activities of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*HMG1*) and geranylgeranyl diphosphate synthase (*GGS1*/*crtE*) in the best producing strain and obtained 453.9 ± 20.2 mg/L β-carotene. Additional downregulation of the competing squalene synthase *SQS1* increased the β-carotene titer to 797.1 ± 57.2 mg/L. Then we introduced β-carotene ketolase (*crtW*) from *Paracoccus* sp. N81106 and hydroxylase (*crtZ*) from *Pantoea ananatis* to convert β-carotene into astaxanthin. The constructed strain accumulated 10.4 ± 0.5 mg/L of astaxanthin but also accumulated astaxanthin biosynthesis intermediates, 5.7 ± 0.5 mg/L canthaxanthin, and 35.3 ± 1.8 mg/L echinenone. Finally, we optimized the copy numbers of *crtZ* and *crtW* to obtain 3.5 mg/g DCW (54.6 mg/L) of astaxanthin in a microtiter plate cultivation.

Our study for the first time reports engineering of *Y. lipolytica* for the production of astaxanthin. The high astaxanthin content and titer obtained even in a small-scale cultivation demonstrates a strong potential for *Y. lipolytica*-based fermentation process for astaxanthin production.

1. Introduction

Astaxanthin is a red-colored carotenoid with a global annual market of 250 tonnes worth $447 million [1]. Astaxanthin is used to improve the color of farmed fish, to increase the pigmentation of egg yolks, and for other feed applications. There is also a growing interest in using astaxanthin in food and cosmetics due to its powerful antioxidant activity [2]. The main source of astaxanthin is currently the chemical synthesis from petrochemical sources. The disadvantages of the chemical process are the high cost of the precursors, side reactions, and the fact that chemical astaxanthin is not approved for human consumption due to the presence of by-products. Several biotechnological processes have been developed, but remain too expensive to compete with chemical synthesis. A biological process based on the microalgae *Haematococcus pluvialis* is challenged with low cell densities, even though *H. pluvialis* produces the highest level of astaxanthin (1.5–3.0% dry weight) compared to other astaxanthin producers [1]. Another process, employing the native red yeast *Xanthophyllomyces dendrorhous* [3,4], suffers from the low cellular content of astaxanthin. Multiple studies on the engineering of the red yeast in order to improve astaxanthin accumulation have been published.

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Breitenbach et al. overexpressed geranylgeranyl pyrophosphate (GGPP) synthase, resulting in an 8-fold increase of astaxanthin content and reaching 0.45 mg/g dry cell weight (DCW) [5]. In more recent studies, a combination of mutagenesis and metabolic pathway engineering resulted in *X. dendrorhous* astaxanthin content of up to 9–9.7 mg/g DCW [6,7]. *Saccharomyces cerevisiae* has also been engineered for astaxanthin production by expression of genes encoding astaxanthin synthase (*crtS*) and cytochrome P450 reductase (*crtR*) from *X. dendrorhous* or by expression of β-carotene ketolase (*crtW*) from bacteria *Paracoccus* sp. and β-carotene hydrolase (*crtZ*) from *Pantoea ananatis* [8]. The transformants that co-expressed *crtW* and *crtZ* accumulated more astaxanthin (0.03 mg/g DCW) than the strain co-expressing *crtS* and *crtR*.

In this study, we aimed to engineer oleaginous yeast *Y. lipolytica* for high-level production of astaxanthin. This yeast species is an attractive host for the production of carotenoids because of its naturally high supply of carotenoids precursor, cytosolic acetyl-CoA, and redox co-factor NADPH [9–12]. *Y. lipolytica* has a GRAS status and is genetically more accessible than *X. dendrorhous* [13].

2. Materials and methods

2.1. Strains, culture conditions and chemicals

Escherichia coli DH5α was used for DNA manipulation in this study. *E. coli* was grown at 37 °C and 300 rpm in Lysogeny Broth (LB) liquid medium and at 37 °C on LB solid medium plates supplemented with 20 g/L agar. Ampicillin was supplemented when required at a concentration of 100 mg/L.

Y. lipolytica strain GB20 (*mus*51Δ, *nugm*-Htg2, *ndh*2i, *lys*11^{-}, *leu*2^{-}, *ura*3^{-}, MatB) was obtained from Volker Zickermann (Goethe University Medical School, Institute of Biochemistry II, Germany). Other strains were obtained from ARS Culture Collection (NRRL) collection. All strains used in this study are listed in Supplementary Tables 1 and 2. *Y. lipolytica* was grown at 30 °C on yeast extract peptone dextrose (YPD) or synthetic complete (SC) media supplemented with 20 g/L agar for preparation of solid media. Synthetic drop out media was used for selection of strains expressing auxotrophic markers. Supplementation of antibiotics was done when necessary at the following concentrations: hygromycin B at 50 mg/L and nourseothricin at 250 mg/L. Cultivation of recombinant strains for carotenoids production was performed in yeast extract peptone medium containing 80 g/L glucose instead of 20 g/L glucose (YP+8% glucose). The chemicals were obtained, if not indicated otherwise, from Sigma-Aldrich. Nourseothricin was purchased from Jena Bioscience GmbH (Germany).

2.2. Plasmid construction

The genes encoding phytoene synthase/lycopene cyclase (*crtYB*), phytoene desaturase (*crtI*) and geranylgeranyl diphosphate synthase (*crtE*) from *X. dendrorhous* were obtained from Addgene [14]. Genes encoding *X. dendrorhous* astaxanthin synthase *crtS* (GenBank accession number AX034665) and cytochrome P450 reductase *crtR* (GeneBank accession number EU884134), *Paracoccus* sp. N81106 β-carotene ketolase *crtW* (GenBank accession number AB206672) and *P. ananatis* β-carotene hydrolase *crtZ* (GenBank accession number D90087) were codon-optimized for *Y. lipolytica* and synthesized as GeneArt String DNA fragments by Thermo Fisher Scientific.

The plasmids, BioBricks, and primers used in this study are listed in Supplementary Table 3, 4, and 5, respectively. BioBricks were amplified by PCR using Phusion U polymerase (Thermo Fisher Scientific) under the following conditions: 98 °C for 30 s; 6 cycles of 98 °C for 10 s, 51 °C for 20 s and 72 °C for 30 s/kb; 26 cycles of 98 °C for 10 s, 58 °C for 20 s and 72 °C for 30 s/kb, and 72 °C for 5 min. BioBricks were purified from agarose gels using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). BioBricks were assembled by into EasyCloneYALI vectors using USER cloning [15].

BioBricks were incubated in CutSmart® buffer (New England Biolabs) with USER enzyme and the parental vector for 25 min at 37 °C, followed by 10 min at 25 °C, 10 min at 20 °C and 10 min at 15 °C. Prior to the USER reaction, the parental vectors were digested with FastDigest *Asi*SI (Thermo Fisher Scientific) and nicked with Nb. *Bsm*I (New England Biolabs). The USER reactions were transformed into chemically competent *E. coli* DH5α. Correct assembly was verified by sequencing.

2.3. Construction of Y. lipolytica strains

The yeast vectors were integrated into different previously characterized intergenic loci in *Y. lipolytica* genome as described in Holkenbrink et al. [15]. Prior to the transformation, the integrative vectors were linearized with FastDigest *Not*I (Thermo Fisher Scientific). The digestion reaction was transformed into *Y. lipolytica* using a lithium-acetate protocol [16]. Transformants were selected on YPD + Hygromycin/Nourseothricin or SC (-ura) plates. Transformants carrying the correct integration of the DNA construct into the *Y. lipolytica* genome were verified by colony PCR. Marker loop-out was performed by transformation of the strains with a Cre-recombinase episomal vector pCfB4158. Obtained colonies were cultivated in liquid SC (-leu) medium for 24 h for induction of the Cre recombinase and plated on SC (-leu) plates to obtain single colonies.

The strains with downregulated squalene synthase were constructed by transformation of β-carotene producing strains with BioBricks as detailed in Supplementary Table 4. Obtained colonies were selected on SC (-ura) plates and the correct transformants were confirmed by colony PCR using primers listed in Supplementary Table 5.

2.4. Cultivation of Y. lipolytica

For pre-culture preparation, single colonies were inoculated from fresh plates in 3 mL YPD in 24-well plates with air-penetrable lid (EnzyScreen, NL) and grown for 18 h at 30 °C and 300 rpm agitation at 5 cm orbit cast.

The required volume of the inoculum was transferred to 3 mL YP+8% glucose for an initial optical density at 600 nm (OD_{600}) of 0.1 into new 24-well plates. The plates were incubated for 72 h at 30 °C with 300 rpm agitation.

To screen the astaxanthin producing strains, generated by integration of astaxanthin genes into rDNA loci, we picked 10 clones for each transformation and streaked them on SC (-ura) plates. The resulting single colonies were inoculated into 500 μL of YPD in 96 deep-well plates with air-penetrable lid (EnzyScreen, NL). The plates were incubated for 18 h at 30 °C with 300 rpm agitation. 30 μL of this culture were used to inoculate three wells with 500 μL YP+8% glucose in a new 96 deep-well plate. The plates were incubated at 30 °C and 300 rpm for 72 h. After cultivation, 400 μL of the cultivation volume was transferred into a 2 mL microtube (Sarstedt) for carotenoids extraction and quantification as described further.

2.5. Isoprenoid extraction and sample preparation

After cultivation, OD_{600} was measured using either a Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader (BioTek) or NanoPhotometer (Implen GmbH, Germany). The dry-weight of a sample was measured by taking 1 or 2 mL of fermentation broth

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