Biotechnol Lett
DOI 10.1007/s10529-017-2318-1

ORIGINAL RESEARCH PAPER

Production of β-carotene by expressing a heterologous multifunctional carotene synthase in *Yarrowia lipolytica*

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Received: 3 January 2017 / Accepted: 7 March 2017

Abstract
Objectives To obtain functional expression of a heterologous multifunctional carotene synthase containing phytoene synthase, phytoene dehydrogenase, and lycopene β-cyclase activities encoded by *carS* from *Schizochytrium* sp. in order to allow *Yarrowia lipolytica* to produce β-carotene.
Results To increase the integration efficiency of a 3.8 kb *carS* under the control of P_{GPD} promoter with a 2 kb selection marker, *ura3*, along with a geranylgeranyl diphosphate synthase (GGS1) expression cassette (~10 kb in total), was inserted into the *Y. lipolytica* chromosome, and the DNA assembler method was combined with double chromosomal deletions of *ku70* and *ku80*. This method resulted in a 13.4-fold increase in integration efficiency compared with the original method, reaching 63% (10/16). The resulting recombinant *Y. lipolytica* produced 0.41 mg β-carotene per g dry cell weight, while the wild type did not produce any indicating the functionality of the multifunctional carotene synthase in *Y. lipolytica*.
Conclusion Expression of GGS1 and a multifunctional carotene synthase from *Schizochytrium* sp. in *Y.*

Electronic supplementary material The online version of this article (doi:10.1007/s10529-017-2318-1) contains supplementary material, which is available to authorized users.

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Published online: 13 March 2017

lipolytica led to β-carotene production. DNA assembler efficiency was greatly increased by the deletion of *ku70* and *ku80*, which resulted in decreased in vivo nonhomologous end-joining (NHEJ) in *Y. lipolytica*.

Keywords β-Carotene · Carotene synthase complex · *carS* · DNA assembler · Multifunctional carotene synthase · *Yarrowia lipolytica*

β-Carotene has been isolated from various sources and is used widely in many fields including the health and food industries (Gul et al. 2015). Genes responsible for the synthesis of β-carotene have been cloned and characterized from various organisms (Misawa 2011; Misawa and Shimada 1998; Moise et al. 2014). The formation of β-carotene from geranylgeranyl diphosphate (GGPP) is catalyzed in three steps (Fig. 1). Two molecules of GGPP are catalyzed by phytoene synthase, encoded by the *crtB* gene, to form phytoene. Then, lycopene is produced by dehydration of phytoene with phytoene dehydrogenase encoded by the *crtI* or *carB* genes, and lycopene converted to β-carotene by lycopene β-cyclase, which is encoded by the *crtY* gene. However, in fungi, there is an intriguing family of enzymes that combine phytoene synthesis activity with lycopene β-cyclization activity. These bifunctional enzymes are encoded by *carRA* in *Phycomyces blakesleeanus* (Sanz et al. 2011) and *Blakeslea trispora* (Rodríguez-Sáiz et al. 2004), *crtYB* in *Xanthophyllomyces dendrorhous* (Verdoes et al. 1999) and *Rhodosporidium diobovatum* (Guo et al. 2014), and *carRP* in *Mucor circinelloides* (Martín et al. 2008). However, a multifunctional enzyme containing all three activities has not been described.

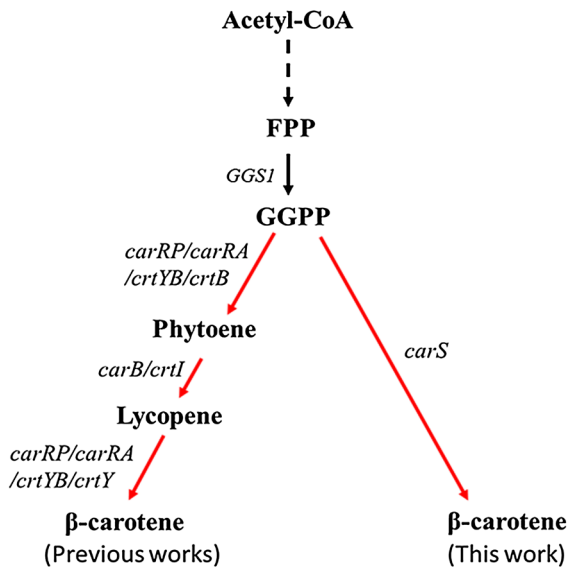

Fig. 1 Graphical depiction of β-carotene biosynthetic pathways. *Black arrows* represent the native pathway in *Y. lipolytica*. *Red arrows* represent the heterologous pathway for biosynthesis of β-carotene from GGPP. *FPP* farnesyl diphosphate, *GGPP* geranylgeranyl diphosphate, *GGS1* GGPP synthase, *carRP or carRA or crtYB* lycopene cyclase/phytoene synthase, *crtB* phytoene synthase, *carB or crtI* phytoene dehydrogenase, *crtY* lycopene cyclase, *carS* multi-functional carotene synthase

As an oleaginous yeast, *Yarrowia lipolytica* is generally regarded as safe (GRAS) and has been engineered to produce many valuable compounds (Cao et al. 2016; Liu et al. 2017; Matthäus et al. 2014; Xu et al. 2016; Ye et al. 2012). Here, we demonstrate that β-carotene can be produced in *Y. lipolytica* by expression of a multifunctional carotene synthase containing phytoene synthase, phytoene dehydrogenase, and lycopene β-cyclase activities encoded by *carS* from *Schizochytrium* sp.

Materials and methods

Strains, medium and culture condition

Strains of all organisms used in this study are shown in Table 1. YPD and SC-ura media were the same as reported previously (Gao et al. 2014), as was SC-leu medium (Gao et al. 2016). SC-Leu + FOA was generated by the addition of 1.2 mg 5-fluoroorotic acid ml^{-1} to SC-Leu medium. YPDS contained 30 g glucose l^{-1}, 10 g peptone l^{-1}, 5 g yeast extract l^{-1} and 15 g sea salts l^{-1}. YPDS was used for cultivation of the strain *Schizochytrium* sp. ATCC20888. YPD60 contained 60 g glucose l^{-1}, 20 g peptone l^{-1} and 10 g yeast extract l^{-1}.

DNA manipulation

rDNAup-P_{TEF}*-GGS1-xpr2t* was amplified with the primers rDNAup-6.14-F and XPR2t-gpd-R. The P_{GPD} promoter was amplified with the primers GPD1p-xpr2t-F and GPD1p-DD253524-R. *lip2t-URA3-rDNAdown* was amplified with the primers rDNAup-6.14-F and XPR2t-gpd-R. CIBTS1776 genomic DNA

Table 1 Strains and plasmids used

Strains or plasmids	Characteristics	Source or reference
Strains		
Y. lipolytica		
ATCC 201249	*MATA ura3-302 leu2-270 lys8-11 PEX17-HA*	ATCC
ATCC MYA-2613	*MATA ura3-302 leu2-270 xpr2-322 axp2-deltaNU49 XPR2::SUC2*	ATCC
CIBTS1776	ATCC201249 *rDNA::TEF1p-GGS1-xpr2t-EXP1p-carB-mig1t-GPDp-carRP-lip2t-URA3*	Gao et al. (2014)
CIBTS1961	ATCC MYA-2613 △*ku70::hisG*△*ku80::URA3*	Gao et al. (2016)
CIBTS1970	ATCC MYA-2613 △*ku70::hisG*△*ku80*	This work
Schizochytrium sp.		
ATCC 20888	*Wild type*	ATCC
Plasmids		
pCen1-Cas9	Only Cas9 expression cassette in pMCSCen1	Gao et al. (2016)
pCAS1yl-trp	*TRP1* guide RNA module and Cas9 expression cassette in pMCSCen1	Gao et al. (2016)
pCAS1yl-ura3	*URA3* guide RNA module and Cas9 expression cassette in pMCSCen1	This work

was used as the template for the three PCR amplifications described above. Then, rDNAup-P_{TEF}-GGS1-xpr2t and P_{GPD} were assembled by overlap extension PCR (OE-PCR) to generate the *rDNAup-P_{TEF}-GGS1-xpr2t-P_{GPD}* cassette. The *carS* gene was amplified from *Schizochytrium* sp. ATCC20888 genomic DNA with the primers DD253524-GPD1p-F and DD253524-lip2t-R.

Three cassettes, *rDNAup-P_{TEF}-GGS1-xpr2t-P_{GPD}*, *carS,* and *lip2t-URA3-rDNAdown,* each at 300 ng, were mixed together and air-dried to 5 µl, followed by transformation into *Y. lipolytica*. Primers used are listed in Supplementary Table 1.

Construction of pCAS1yl-ura3

The ura3 sgRNA cassette was generated by assembly of the TEF fragment amplified with TEF1p-Pme1-F and TEF-ura3.1-HH-R, and the HH-gRNA-HDV-mig1t fragment was amplified with ura3.1-HH-F and Mig1t-Sal1-R. The sgRNA expression cassette and pCen1-Cas9 were ligated after both of them were digested with *Sal*I and *Mss*I. The resulting plasmid was named pCAS1yl-ura3.

Yarrowia lipolytica transformation

The standard method (Claude and Anne-Marie 1987) was used for *Y. lipolytica* transformation, and the procedure used in this work was the same as in our previous report (Gao et al. 2014). For deletion of *URA3*, the transformation mixture was transferred into a tube of SC-leu liquid medium and incubated for four days; then, the culture was diluted and spread onto an SC-Leu + FOA plate. Colonies from the SC-Leu + FOA plate were picked randomly and streaked on SC-ura and SC plates and then incubated at 30 °C for 2 days.

Colony PCR confirmation and sequencing

Colony PCR confirmation was performed with the DNA polymerase KOD FX Neo (Toyobo Co., Ltd), which has a high efficiency and a high success rate. If necessary, the products were sequenced by Sangon Biotech (Shanghai) Co., Ltd. URA3-F/△ku80-yz-up-R primers were used for colony PCR of *Y. lipolytica* transformants to confirm URA3 rescue.

Analysis of β-carotene

Dry cell weights were determined after samples had been dried at 85 °C for 24 h. For each sample, 1 ml cultured cells was harvested by centrifugation and treated with 3 M HCl at 95 °C for 15 min, followed by incubation for 10 min on ice. Then, cells were washed twice with double-distilled water and 0.5 ml acetone was used to extract β-carotene. Extracts were

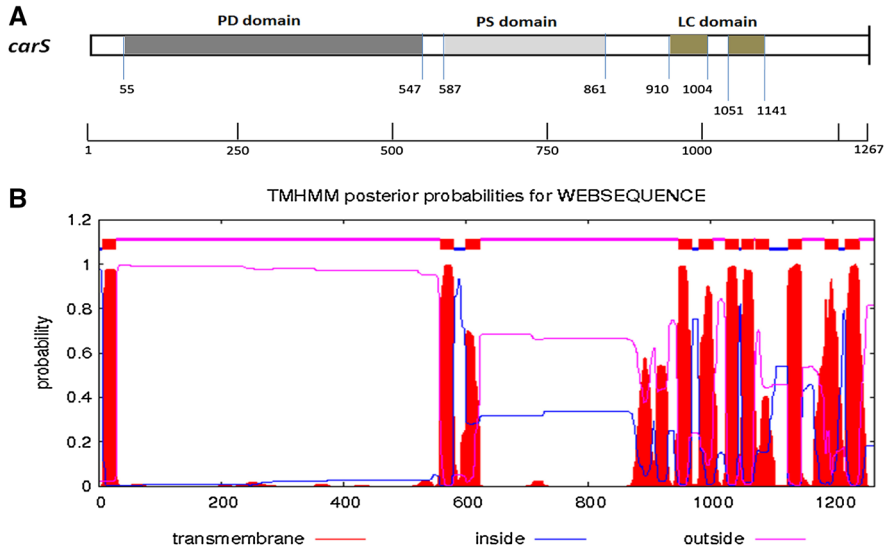

Fig. 2 casS enzyme characteristics. **a** Domains of the carS protein. *PS* phytoene synthase, *PD* phytoene dehydrogenase, *LC* lycopene β-cyclase. **b** Transmembrane prediction analysis of the carS protein. This analysis was performed using the TMHMM tool

centrifuged at ~10,000 rpm for 5 min, and 20 µl of each supernatant was analyzed via HPLC. Cell extracts were analyzed using HPLC with a detector set at 450 nm and an XDB-C18 column (5 µm, 4.6 × 150 mm, Eclipse, USA). Methanol/acetonitrile/dichloromethane (42:42:16, by vol) was used as the mobile phase at 1.5 ml min^{-1} at 30 °C. Samples of β-carotene (98%, Sigma, Aldrich) were dissolved in acetone and used to prepare standard curves.

Results and discussion

Analysis of carotene synthase from *Schizochytrium* sp.

The marine organism *Schizochytrium* sp. can produce several carotenoids, including astaxanthin and canthaxanthin, which are synthesized from a precursor of β-carotene (Aki et al. 2003; Chatdumrong et al. 2007). A carotene synthase containing multiple activities was identified in *Schizochytrium* sp. (Weaver et al. 2007). This candidate gene (GenBank: DD253524.1) consists of 3807 bp and encodes a 1268-amino acid polypeptide. No introns were found in this gene. This polypeptide was analyzed using the BLASTP tool, which indicated that this protein contained at least three conserved functional domains, including phytoene synthase (587–861 aa), phytoene dehydrogenase (55–547 aa), and lycopene β-cyclase (910–1004 and 1051–1141 aa) (Fig. 2a), unlike previously reported enzymes carrying two domains, lycopene β-cyclase and phytoene synthase (Guo et al. 2014; Rodríguez-Sáiz et al. 2004; Sanz et al. 2011; Verdoes et al. 1999). These three domains were responsible for forming β-carotene from GGPP. The results of analysis using the TMHMM tool indicated that this protein also contained multiple transmembrane regions that focused on LC domains (Fig. 2b), a characteristic that can also be found in bifunctional enzymes such as carRP and carRA (Supplementary Figs. 1, 2).

Integration of the carotene synthase expression cassette

A target sequence was amplified from genomic DNA of *Schizochytrium* sp. ATCC20888 with primers designed based on the sequence DD253524.1. Sequencing of PCR products indicated that the sequence was the same as the reference. To efficiently investigate heterologous expression of the *carS* gene in yeast, we used the DNA assembler method to assemble and integrate gene expression cassettes into the chromosome of *Y. lipolytica* (Gao et al. 2014). We designed three DNA cassettes: *rDNAup-*P_{TEF}*-GGS1-xpr2t-*P_{GPD}*, carS* and *lip2t-URA3-rDNAdown.* Overlaps of 60 bp were designed between each pair of successive cassettes. Expression of *carS* began with the P_{GPD} promoter and was terminated by the *lip2t* terminator when these cassettes were assembled successfully (Fig. 3a).

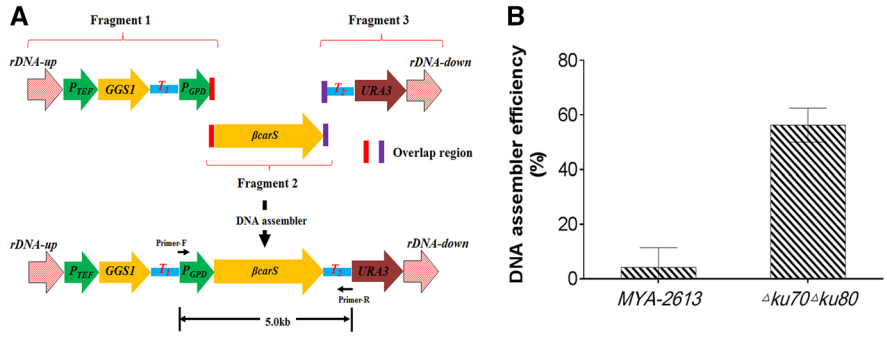

Fig. 3 Integration of the *carS* gene cassette in *Y. lipolytica*. **a** Design of the DNA assembly in this study. **b** Efficiencies of DNA assembly in two hosts. *Error bars* represent standard deviations (n = 3)

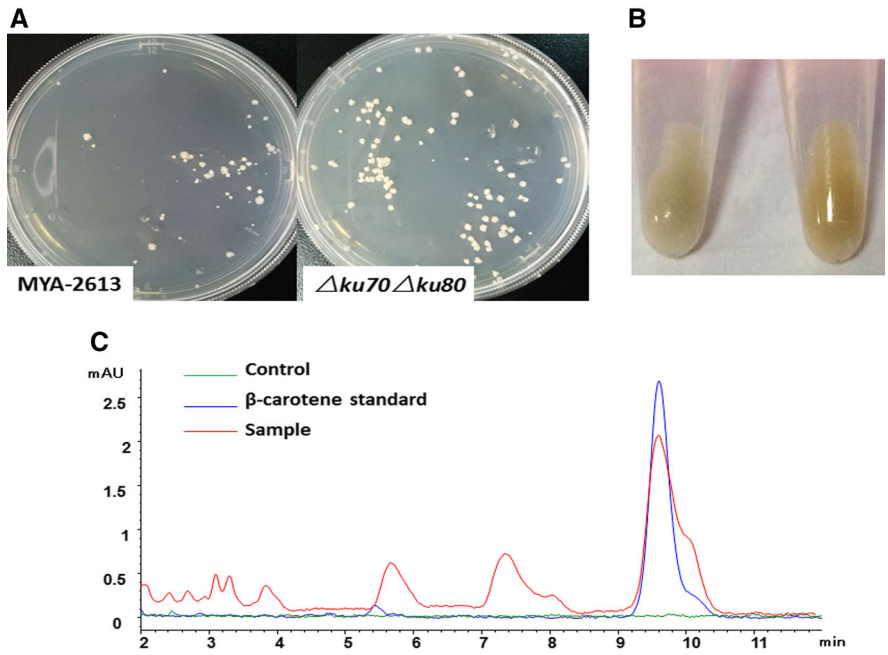

Fig. 4 Functional testing of the multifunctional carotene synthase gene. **a** Transformants of MYA-2613 and △*ku70*△*ku80*. **b** Cells harvested after 7 days of cultivation in YPD60 medium. *Left* control; *right* positive transformants. **c** HPLC analysis of extracted carotenoids. CIBTS1970 was used as the control

Efficiency of DNA assembly is very low in *Y. lipolytica*, perhaps because of its low capacity for homologous recombination. Therefore, NHEJ-deficient *Y. lipolytica* may be a better tool for DNA assembly. The strain CIBTS1970, which lacks the ura3 marker, was constructed through deletion of *URA3* from CIBTS1961 using a CRISPR-Cas9 system and was used to express the *carS* gene (Supplementary Figs. 3, 4). Both the MYA-2613 (*wild type*, WT) and CIBTS1970 (△*ku70*△*ku80*) strains were co-transformed with the three cassettes described above. For these two hosts, the transformation efficiencies were $1 - 2 \times 10^2$ cfu μg^{-1} DNA. High efficiencies of 56.2 ± 6.3% (8/16, 10/16, 9/16) were obtained for △*ku70*△*ku80*, 13.4 times the efficiency that obtained in *WT*, 4.2 ± 7.2% (2/16, 0/16, 0/9) (Fig. 3b). This marked increase in efficiency indicated strongly that down-regulation of NHEJ could significantly improve the efficiency of DNA assembly in *Y. lipolytica*. The highest efficiency using overlaps of only 60 bp was close to that in *Saccharomyces cerevisiae* (Shao et al. 2009).

The highest efficiency was reported to be lower than 23% (7/30) even with 1 kb overlaps between two successive cassettes when engineering *Y. lipolytica* to produce polyunsaturated fatty acids (Liu et al. 2017). NHEJ-deficient *Y. lipolytica* may be a better DNA assembly tool than wild type, and it may be more efficient to construct biosynthetic pathways which lack high-throughput screening method.

Confirmation of the function of carotene synthase

All of the transformants cultured on SC-ura medium plates were white similar to the wild type strain (Fig. 4a) and unlike the mutants of the *carB/carRP* or *crtI/crtYB* pathways (Gao et al. 2014). This difference may be because the multi-functional carotene synthase has lower activity than the other pathway. Five transformants derived from CIBTS1970 and confirmed by colony PCR were cultivated in YPD60 medium for 7 days, and the harvested cells appeared light yellow (Fig. 4b). Carotenoids extracted from the cells were analyzed by HPLC, and a peak appeared at 9.7 min from the correctly assembled strains, consistent with the elution time of authentic β-carotene. No such peak was observed for the cell extracts from the control; therefore, these results were considered to indicate the presence of β-carotene (Fig. 4c). The highest production of β-carotene was 0.41 mg per g dry cell weight.

These results indicated that the novel *carS* gene, which encodes a multi-functional carotene synthase with phytoene synthase, phytoene dehydrogenase and lycopene β-cyclase activities, was successfully expressed in oleaginous yeast and enabled non-carotenogenic yeast to produce β-carotene. This gene is unique in the NCBI database; further, this study presents a novel synthetic biology element for production of β-carotene through metabolic engineering. The production of β-carotene could potentially be enhanced by improving the enzyme activity with a directed evolution strategy (Packer and Liu 2015) or by optimizing the expression of the *carS* gene through a codon optimization strategy (Liu et al. 2012).

Acknowledgements We thank Professor Hal S. Alper for the generous gift of the pMCSCen1 plasmid. This work was financed by the Ministry of Science and Technology of China (973:2014CB745101).

Supporting information Supplementary Table 1—Primers used.

Supplementary Fig. 1—Transmembrane prediction analysis of carB enzymes from (A) *Blakeslea trispora* and (B) *Mucor circinelloides*.

Supplementary Fig. 2—Transmembrane prediction analysis of (A) carRA enzyme from *Blakeslea trispora* and (B) carRP enzyme from *Mucor circinelloides*.

Supplementary Fig. 3—*URA3* disruption of CIBTS1961 via the CRISPR-Cas9 system.

Supplementary Fig. 4—Disruption of URA3 in CIBTS1961, mediated by the CRISPR-Cas9 system.

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