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Screening of novel β-carotene hydroxylases for the production of β-cryptoxanthin and zeaxanthin and the impact of enzyme localization and crowding on their production in *Yarrowia lipolytica*

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Abstract

Zeaxanthin, a vital dietary carotenoid, is naturally synthesized by plants, microalgae, and certain microorganisms. Large-scale zeaxanthin production can be achieved through plant extraction, chemical synthesis, or microbial fermentation. The environmental and health implications of the first two methods have made microbial fermentation an appealing alternative for natural zeaxanthin production despite the challenges in scaling up the bioprocess. An intermediate between β-carotene and zeaxanthin, β-cryptoxanthin, is found only in specific fruits and vegetables and has several important functions for human health. The low concentration of β-cryptoxanthin in these sources results in low extraction yields, making biotechnological production a promising alternative for achieving higher yields. Currently, there is no industrially relevant microbial fermentation process for β-cryptoxanthin production, primarily due to the lack of identified enzymes that specifically convert β-carotene to β-cryptoxanthin without further conversion to zeaxanthin. In this study, we used genetic engineering to leverage the oleaginous yeast *Yarrowia lipolytica* as a bio-factory for zeaxanthin and β-cryptoxanthin production. We screened 22 β-carotene hydroxylases and identified eight novel enzymes with β-carotene hydroxylating activity: six producing zeaxanthin and two producing only β-cryptoxanthin. By introducing the β-carotene hydroxylase from the bacterium *Chondromyces crocatus* (CcBCH), a β-cryptoxanthin titer of 24 ± 6 mg/L was achieved, representing the highest reported titer of sole β-cryptoxanthin in *Y. lipolytica* to date. By targeting zeaxanthin-producing β-carotene hydroxylase to the endoplasmic reticulum and peroxisomes, we increased the production of zeaxanthin by 54% and 66%, respectively, compared to untargeted enzyme. The highest zeaxanthin titer of 412 ± 34 mg/L was achieved by targeting β-carotene hydroxylases to peroxisomes. In addition, by constructing multienzyme scaffold-free complexes with short peptide tags RIDD and RIAD, we observed a 39% increase in the zeaxanthin titer and a 28% increase in the conversion rate compared to the strain expressing unmodified enzyme. The zeaxanthin titers

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obtained in this study are not the highest reported; however, our goal was to demonstrate that specific approaches can enhance both titer and conversion rate, rather than to achieve the maximum titer. These findings underscore the potential of *Y. lipolytica* as a promising platform for carotenoid production and provide a foundation for future research, where further optimization is required to maximize production.

Keywords Carotenoids, Zeaxanthin, β-cryptoxanthin, *Yarrowia lipolytica*, Metabolic engineering, Subcellular compartments, Modular enzyme assembly, Multienzyme complex

Introduction

Zeaxanthin is a naturally occurring pigment synthesized by photosynthetic organisms (plants and microalgae) and certain non-photosynthetic yeasts and bacteria [1–3]. It is a dihydroxy derivative of β-carotene and belongs to a group of pigments known as oxycarotenoids, which are part of a large group of natural colorants known as xanthophylls [2, 4, 5]. Beyond its role as a pigment, zeaxanthin is recognized for its diverse physiological properties, including antioxidant, anti-inflammatory, anticancer, and neuroprotective effects [2]. It is naturally present in human eyes, where it has a protective role, but it is also present in kidneys, liver, spleen, pancreas, and other organs [1, 2, 4, 6]. Since humans are unable to synthesize zeaxanthin, it must be acquired through dietary sources [7].

Awareness of zeaxanthin's health benefits has led to a higher demand for its production. Three main production methods are possible: plant extraction, chemical synthesis, and microbial fermentation [8]. Plant extraction is the primary method for producing zeaxanthin, but the employment of organic solvents contributes to environmental pollution, poses risks to the health of operators, and incurs high process costs [9–14]. Additionally, the plant biomass used for zeaxanthin extraction may be contaminated with heavy metals, pesticides, herbicides, mycotoxins, environmental pollutants, pathogens, and allergens, which could negatively impact human health [11, 15, 16]. Currently, natural zeaxanthin is primarily sourced from the marigold flower (Tagetes erecta L). However, this source has several drawbacks, including low yield (only 0.3 mg/g), labor intensity, dependence on season and timing, and significant land and water requirements. These factors combine to make year-round zeaxanthin production from marigolds an expensive and resource-intensive process [17–19]. Chemical synthesis of zeaxanthin may lead to toxicity concerns due to the potential presence of residual reagents or by-products from the synthetic process [3, 20]. This is why microbial fermentation is a promising method for producing highquality natural zeaxanthin, although achieving industrial-scale yields has been challenging [8].

Unlike other carotenoids, the intermediate between β-carotene and zeaxanthin, i.e., βcryptoxanthin (BCX), is found only in specific fruits and vegetables, such as Citrus unshiu, Citrus reticulate, Cucurbita maxima, Capsicum annuum, Rubus palmatus and Carica papaya [21, 22]. Its benefits for human health, including cell-to-cell communication, roles in antioxidant defense, anticancer activity, as well as its function as a vitamin A precursor, make BCX an interesting molecule [22, 23]. Biotechnological production of BCX is a promising approach because extraction from natural sources generates low yield due to low concentrations [24, 25]. Few studies have explored the production of BCX using microorganisms. Serrato-Joya et al. (2006) demonstrated that BCX production is feasible using the bacterium Flavobacterium lutescens, achieving a yield of 770 mg per kg of dry cell weight [26]. Lautier et al. (2023) expressed CYP97H1, a P450 β-carotene monohydroxylase from the freshwater mixotrophic protist Euglena gracilis, in E. coli, which resulted in a BCX production of 2.7 mg/L [22]. However, these production levels are insufficient for industrial-scale applications, requiring further research to increase yields. Currently, there is no information available on the exclusive production of BCX through microbial fermentation in Y. lipolytica. It only appears in mixture with zeaxanthin as an intermediate in the process of its biosynthesis from β-carotene. This is due to a lack of the enzymes involved in converting β-carotene solely to BCX without further conversion to zeaxanthin, so finding those enzymes is an important biotechnological challenge.

Yarrowia lipolytica, an oleaginous yeast known for its versatile substrate use, rapid metabolism, and robust biosynthetic abilities, offers a solution for microbial production of carotenoids [27, 28]. It can produce substantial quantities of acetyl coenzyme A (acetyl-CoA), a precursor for fatty acid biosynthesis, which leads to the accumulation of fatty acids in the organism's lipid droplets. These droplets function as energy storage but also as storage for lipophilic molecules, including most carotenoids [14, 29]. The synthesis of acetyl-CoA through the mevalonate pathway facilitates the enhanced generation of geranyl diphosphate, farnesyl diphosphate, and geranylgeranyl diphosphate, which are critical intermediates in the carotenoid biosynthesis (Fig. 1) [28, 30]. GRAS (Generally Regarded As Safe) and QPS (Qualified Presumption of Safety) statuses, granted by the Federal Drug Administration (FDA) and the European Food Safety Authority (EFSA), respectively, designate Y. lipolytica as a suitable microorganism for producing compounds safe for human consumption [31].

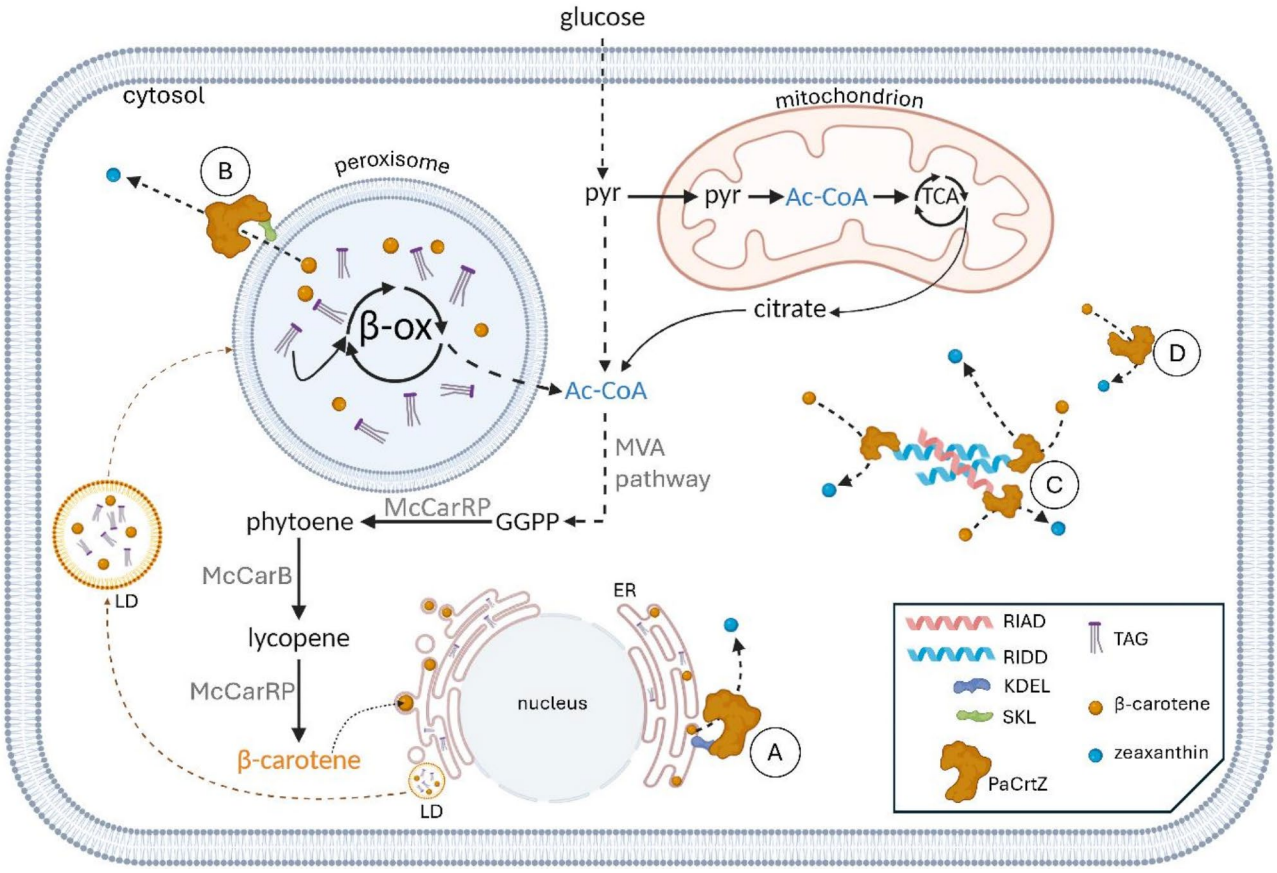

Fig. 1 Scheme of engineered biosynthetic pathway for zeaxanthin production in *Y. lipolytica*. Mevalonate pathway genes (upstream of GGPP) are naturally present in *Y. lipolytica*. Phytoene, lycopene, and β-carotene are synthesized by expression of genes encoding heterologous enzymes McCarRP and McCarB. Synthetized βcarotene is stored in the endoplasmic reticulum (ER) and, along with triacylglycerols (TAG), transported to peroxisomes [32]. (**A**) PaCrtZ_KDEL protein targeting ER; (**B**) PaCrtZ_SKL protein targeting peroxisome; (**C**) Two PaCrtZ_RIDD and one PaCrtZ_RIAD proteins creating PaCrtZ_DAD multienzyme homotrimer complex; (**D**) unmodified cytosolic PaCrtZ protein. Ac-CoA: acetyl coenzyme-A; pyr: pyruvate; MVA pathway: mevalonate pathway; GGPP: geranylgeranyl diphosphate; McCarRP: bifunctional phytoene synthase/lycopene cyclase from *Mucor circinelloides*; McCarB: phytoene dehydrogenase from *M. circinelloides*; LD: lipid droplets (filled with βcarotene); β-ox: β-oxidation; TCA: the citric acid cycle (the Krebs cycle)

As a non-carotenoid-producing organism, Y. lipolytica must be genetically modified to redirect acetyl-CoA flux toward carotenoid biosynthesis. Expression of genes involved in the biosynthesis of carotenoids leads to their production in Y. lipolytica [33–35]. A significant titer has been reached by expressing the bifunctional phytoene synthase/lycopene cyclase and phytoene dehydrogenase from the β-carotene-producing fungus Mucor circinelloides (denoted as McCarRP and McCarB, respectively). Expression of those genes in Y. lipolytica promotes the biosynthesis of β-carotene, the predominant bicyclic carotenoid, achieving concentrations of up to 7.6 g/L through fed-batch fermentation [36].

Overexpression of genes encoding β-carotene hydroxylases (BCHs) in β-carotene-producing strains of Y. lipolytica leads to the biosynthesis of zeaxanthin, with BCX as an intermediate [14, 30]. In their research, Xie et al. (2021) have evaluated several known BCH genes, including BvCrtZ from Brevundimonas vesicularis, HpCrtZ from Haematococcus lacustris and PaCrtZ from P. ananatis (referred to as EuCrtZ in their study). Their findings have revealed that expression of the gene encoding PaCrtZ in Y. lipolytica exhibits BCH activity, which resulted in the production of 21.98 ± 1.80 mg/L of zeaxanthin [30].

Carotenoid metabolism in Y. lipolytica is related to specific cell compartments. The endoplasmic reticulum (ER) and peroxisomes are important for β-carotene biosynthesis and storage [29, 32, 34, 37, 38]. Targeting BCHs to those organelles could increase β-carotene conversion into zeaxanthin. Certain amino-acid sequences are responsible for protein targeting at specific organelles [39–43]. Two of those sequences are KDEL, responsible for protein targeting to the ER (Fig. 1A), and SKL, responsible for protein targeting to the peroxisomes (Fig. 1B) [32, 41, 44–46].

The study by Ma et al. (2021) demonstrates that targeting enzymes responsible for astaxanthin biosynthesis to both the ER (using KDEL amino-acid sequence) and

peroxisomes (using SKL amino-acid sequence) significantly improves astaxanthin production in Y. lipolytica [32]. This improvement is due to the increased availability of β-carotene stored within these organelles, facilitating its conversion by the enzymes involved in astaxanthin biosynthesis. This strategy, while successful for astaxanthin, has not yet been explored for the production of zeaxanthin in Y. lipolytica. Given the common pathways involved in carotenoid biosynthesis, we aimed to test whether applying similar enzyme-targeting techniques for zeaxanthin production in this organism could lead to significant improvements.

Zhu et al. (2022) explored an alternative approach to astaxanthin production by developing a scaffold-free modular enzyme assembly utilizing a peptide pair with exceptionally strong affinity and relatively short lengths. This pair of peptides, named RIDD and RIAD, belonging to the dock-and-lock peptide interacting family, originates from the cAMP-dependent protein kinase (PKA) and the A kinase-anchoring proteins (AKAPs), respectively [47–49]. RIDD refers to a short N- terminal part of the R subunits of PKAs, responsible for docking and dimerization. The RIAD peptide refers to the anchor domain of AKAP, an amphipathic helix that specifically binds to the RIDD dimer. By adding RIDD and RIAD peptides to the C-terminus of the enzymes involved in astaxanthin biosynthesis, they have created a scaffold-free multienzyme complex that had a positive effect on the increase of astaxanthin production in Y. lipolytica PO1f strain [50–52]. We aimed to utilize this approach to create a homo-trimer complex of the zeaxanthin-producing BCH enzyme to evaluate its effect on zeaxanthin production in the YB-392 strain of Y. lipolytica.

Zhang et al. (2023) have reported that expressing the gene encoding PaCrtZ in a βcarotene-producing strain of Y. lipolytica, along with additional genetic modifications, has achieved the highest recorded zeaxanthin titer to date, reaching 767.2 mg/L with a conversion rate of 44.37% [14]. While achieving a high zeaxanthin titer is crucial for industrial production, it is equally important to consider the rate of β-carotene conversion into zeaxanthin, as the downstream processing of specific carotenoids from a carotenoid mixture presents a significant challenge [53]. These challenges are the reason why we explored different approaches to enhance both the titer and the conversion rate of zeaxanthin in Y. lipolytica compared to the expression of the unmodified gene. Although the zeaxanthin conversion rate in our study surpasses those reported by Zhang et al. (2023), the overall zeaxanthin titer is lower. The goal of this study was to investigate different strategies to increase zeaxanthin titer and conversion rate relative to the unmodified enzyme. Future efforts should focus on employing stronger promoters for gene expression, introducing multiple copies of the modified target genes, and optimizing cultivation and media conditions. These strategies hold the potential for significantly increasing zeaxanthin and BCX titers.

In this study, we thus screened 21 homologs of PaCrtZ enzyme to assess their activity in a previously constructed β-carotene-producing strain of Y. lipolytica, CH_931, a descendant of the YB-392 wild-type strain. We then targeted the best-performing zeaxanthin-producing BCH enzymes to the ER and peroxisomes and implemented a modular enzyme assembly system by constructing protein complexes using BCH fusion with short peptide tags, RIDD and RIAD.

Materials and methods

Strains, media and cultivating conditions

The assembled plasmids were amplified using the Escherichia coli DH10β strain. E. coli transformants were grown on solid LB medium [54] with the appropriate antibiotics. The antibiotics (all ordered from Sigma-Aldrich) were added into the sterilized LB media at the following final concentrations: chloramphenicol 20 μg/L, ampicillin 100 μg/L, and spectinomycin 100 μg/L. The transformants were inoculated into 5 mL of liquid LB medium with appropriate antibiotic and cultivated at 37 °C for 24 h.

All the Y. lipolytica strains used for this study originated from the wild-type strain YB-392, which we obtained from the ARS Culture Collection (NRRL). A previously engineered βcarotene-producing strain CH_931 was used as the background strain for the construction of zeaxanthin-producing strains. For the selection of zeaxanthin-producing transformants of Y. lipolytica, we used SC-U agar plates (1.7 g/L of yeast nitrogen base without amino acids and ammonium sulfate (Sigma-Aldrich), 5 g/L of ammonium sulfate (Roth), 5 mL of 1 M MES buffer (Sigma-Aldrich), 8 mL of 60% glucose (Glentham Life Sciences), 10 mL of yeast synthetic drop-out medium without uracil (Sigma-Aldrich) and 20 g/L of agar (Sigma-Aldrich)). All stock solutions were stored at 4 °C.

SC-U plates with transformants were incubated at 28 °C for three days. Grown colonies were plated onto new SC-U plates for one day and used for inoculation into the seed medium. For the seed medium, we used YPD: 20 g/L of glucose (Glentham Life Sciences), 20 g/L of bacteriological peptone (Sigma-Aldrich), and 10 g/L of yeast extract (Sigma-Aldrich). Strains were cultivated in 48-well deep-well plates (Axygen®) in a shaker (28 °C, 220 rpm, 70% humidity) for 24 h. 10% of the seed culture was inoculated into YPM29 production medium, cultivated in a shaker (28 °C, 220 rpm, 70% humidity), and sampled after 72 h into PCR plates for further extraction and analysis. For the YPM29 production medium, 40 g/L of yeast extract (Sigma-Aldrich), 11.73 g/L of KH_2PO_4 (Glentham Life Sciences), 2.405 g/L of K_2HPO_4

(Glentham Life Sciences), 29.58 mg/L of $MgSO_4 \times 7H_2O$ (Glentham Life Sciences), 0.044 mg/L of $CaCl_2 \times 2H_2O$ (Glentham Life Sciences), 2.5 g/L of ammonium sulfate (Roth), 0.5 g/L of $FeSO_4 \times 7H_2O$ (Glentham Life Sciences), 140 g/L of glucose and 10 mL of micronutrient stock solution were dissolved in 1000 mL of dH_2O. For the micronutrient stock solution, 0.061 g of H_3BO_3, 0.196 g of $MnSO_4 \times H_2O$, 0.287 g of $ZnSO_4 \times 7H_2O$, 0.0025 g of $CuSO_4 \times 5H_2O$ and 0.0125 g of $(NH_4)6Mo_7O_{24} \times 4H_2O$ were dissolved in 1000 mL of dH_2O and filtered through a 0.2-micron filter. All the components for the micronutrient stock solution were ordered from Glentham Life Sciences.

Plasmid cloning and transformation

All the genes used in this study were codon-optimized for expression in Y. lipolytica and designed for the Golden Gate assembly system [55]. The homologs of the PaCrtZ enzyme were found using InterPro (https://www.ebi.ac.uk/interpro/) and BLASTP (https://blast.ncbi.nlm.nih.gov/Blast.cgi) online tools. Protein sequences of the PaCrtZ homologs were aligned using the MAFFT web server (https://mafft.cbrc.jp), and the phylogenetic tree, from which we selected the homologs for testing, was created and visualized using the iTOL tool (https://itol.embl.de/). The list of all PaCrtZ homologs used in this work is presented in Table 1.

Table 1 List of all PaCrtZ homologs used in this study

Name	Organism	Origin	Accession №
CaBCH	*Chondromyces apiculatus*	Bacteria	WP_044242546.1
CcBCH	*Chondromyces crocatus*	Bacteria	WP_063796230.1
EuBCH	*Euhalothece natronophila*	Bacteria	WP_146296647.1
FnBCH	*Flavobacterium sp.*	Bacteria	WP_184167177.1
MfoBCH	*Massilia forsythiae*	Bacteria	QJD99361.1
NkBCH	*Niastella koreensis*	Bacteria	WP_014223153.1
PaCrtZ	*Pantoea ananatis*	Bacteria	CRH31697.1
PsBCH	*Paracoccus sp. N81106*	Bacteria	P54973.1
PvBCH	*Pseudescherichia vulneris*	Bacteria	WP_313106347.1
PzBCH	*Paracoccus zeaxanthinifaciens*	Bacteria	WP_022708004.1
OrBCH	Cyanobiont of *Ornithocercus magnificus*	Cyanobacteria	GCE64578.1
SpBCH	*Spirulina sp.* SIO3F2	Cyanobacteria	NEO87414.1
DsBCH	*Dunaliella salina*	Green algae	APW83732.1
CmBCH	*Cucurbita moschata*	Plants	XP_022929023.1
CsBCH	*Crocus sativus*	Plants	CAC95130.2
GlBCH	*Gentiana lutea*	Plants	B3SGL0.1
GrBCH	*Gossypium raimondii*	Plants	KJB52188.1
HuBCH	*Herrania umbratica*	Plants	XP_021275943.1
PvuBCH	*Phaseolus vulgaris*	Plants	AEL29210.1
TeBCH	*Tagetes erecta*	Plants	AAG10430.1
ZmBCH1	*Zea mays*	Plants	PWZ54187.1
ZmBCH2	*Zea mays*	Plants	PWZ46576.1

The coding sequences of the PaCrtZ homologs and the sequences for RIDD and RIAD elements, together with the flexible GS-rich linker, were codon-optimized for expression in Y. lipolytica and synthesized by Twist Bioscience (California, USA). Sequences encoding KDEL (Lys-Asp-Glu-Leu) and SKL (Ser-Lys- Leu) amino-acids were added using primers ABP2153 and ABP6534 and ABP2153 and ABP6535, respectively (Table S1). The primers used for PCR amplification were synthesized by Macrogen-Europe BV (Amsterdam, Netherlands). The polymerase used for PCR reactions was Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher Scientific™, USA), following the manufacturer's instructions. BCHs were expressed under the constitutive promoter of Y. lipolytica P_{TEF1} and synthetic T_{SYN25} terminator. Gene ura3, expressed under the control of the P_{EXP1} promoter and T_{CYC1} terminator, was used as a selection marker. For integration into the Y. lipolytica genome, we used zeta homologies [29]. Restriction enzymes and T4 DNA Ligase Buffer were ordered from Thermo Fisher Scientific™; T7 DNA ligase was ordered from New England Biolabs®. The plasmids were amplified by electroporation into E. coli strain DH10β, whereby the transformants were plated onto LB media plates with a suitable antibiotic for selection and cultivated for 24 h at 37 °C. The plasmids were isolated using GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific™). All plasmids were sequenced to confirm the correct assembly before being linearized using the NotI FastDigest™ restriction enzyme (Thermo Fisher Scientific™). Linearized DNA fragments were isolated from agarose gel using GeneJET Gel Extraction Kit (Thermo Fisher Scientific™) by following the manufacturer's instructions. Using the LiAc/SS-Carrier DNA/PEG transformation protocol [56], linear fragments were transformed into the CH_931 strain. All reagents for the transformation mixture were purchased from Sigma-Aldrich. A list of all homologs used in this study is provided in Table 1, a list of all assembled expression cassettes can be found in the Supporting information in Table S3, and a list of all strains used in this study can be found in the Supporting information in Table S4.

Carotenoid extraction and quantification

For the carotenoid extraction, a 20 µL sample of the fermented broth was mixed with 160 µL of butyl acetate (VWR) with 0.05% of butylated hydroxytoluene (Sigma-Aldrich) and 200 µL of glass beads (Thermo Fisher Scientific, 500–750 µm). The mixture was then homogenized using a FastPrep®-96 instrument at 1,800 rpm for 5 min. After homogenization, the samples were centrifuged at

14,000 rpm for 10 min, and the resulting supernatant was carefully transferred to glass vials for further analysis.

Quantification of carotenoids was performed using the Thermo Accela 600 HPLC system. The column used for separation was AkzoNobel Kromasil 100-5-C18, 50×4.6 mm with 5 µm particle size. The mobile phase consisted of a gradient of 100% dichloromethane (VWR) and 80% methanol (J.T.Baker®). The flow rate was set at 0.5 mL/min, and the column oven temperature was maintained at 25 °C. Detection of carotenoids was achieved using a photodiode array (PDA) detector (Thermo Fisher Scientific™). β-carotene and βcryptoxanthin were detected at the wavelength of 455 nm; zeaxanthin was detected at the wavelength of 476 nm. All analytical standards were ordered from Sigma-Aldrich.

Results and discussion

Construction of BCX and zeaxanthin-producing strains

Testing of β-carotene hydroxylases

A previously engineered β-carotene-producing Y. lipolytica strain CH_931, with βcarotene production of 855±45 mg/L, was used as the parent strain for constructing zeaxanthin-producing strains. This strain incorporates a cassette for β-carotene synthesis and features overexpression of the native genes of Y. lipolytica's mevalonate pathway. The list of all genes used in the development of the CH_931 strain is shown in the Supporting information in Table S2, and the list of all strains used in this study can be found in the Supporting information in Table S4. BCHs catalyze zeaxanthin biosynthesis from β-carotene. BCH enzyme from P. ananatis (PaCrtZ) is known for converting β-carotene into zeaxanthin in Y. lipolytica, and it is the best-performing BCH reported to date [14]. In this study, we evaluated 21 homologs of PaCrtZ protein to find additional BCHs that are functional in Y. lipolytica YB-392. The homologs were selected from various species across different taxonomical groups (Fig. 2), including non-photosynthetic bacteria, cyanobacteria, green algae, and higher plants. This selection was based on existing literature data on zeaxanthin production by these species and/or the amino acid sequence similarity of the selected proteins compared to PaCrtZ. Our results showed that out of the 21 screened PaCrtZ homologs, six were able to convert β-carotene into zeaxanthin: PzBCH from Paracoccus zeaxanthinifaciens, PsBCH from Paracoccus sp. N81106, PvBCH from Pseudescherichia vulneris, EuBCH from Euhalothece natronophila, MfoBCH from Massilia forsythiae and NkBCH from Niastella koreensis; and additional two homologs were able to convert βcarotene only into

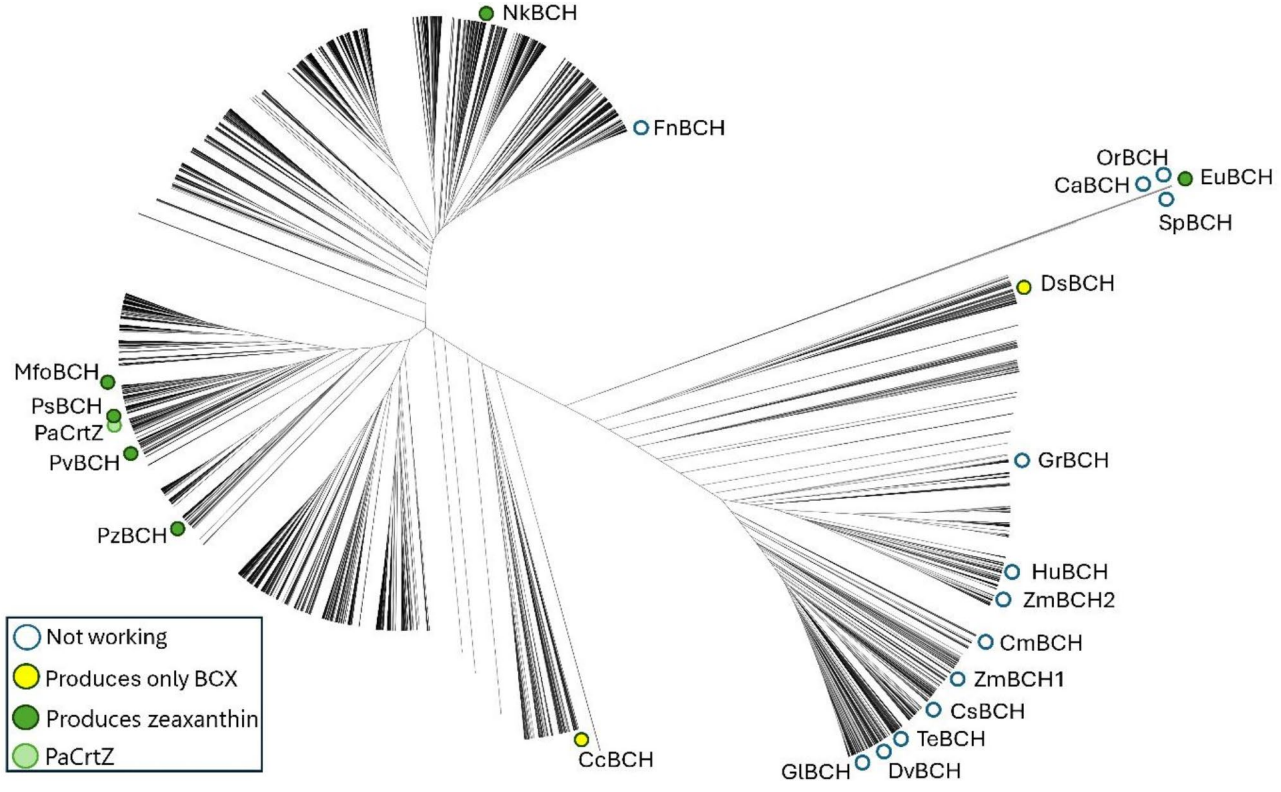

Fig. 2 Phylogenetic tree of PaCrtZ homologs. The tree was created from 5590 protein sequences of PaCrtZ homologs, found using the InterPro and BLASTP online tools, and visualized from the protein alignment using the iTOL tool. The source organisms of the selected homologs and the sequence accession numbers can be found in Table 1

the BCX, which is intermediate between β-carotene and zeaxanthin: CcBCH from Chondromyces crocatus and DsBCH from Dunaliella salina.

Interestingly, all homologs capable of converting β-carotene to zeaxanthin originate from bacteria, yet they are grouped into separate phylogenetic branches. BCX-producing enzymes are also present in distinct phylogenetic branches, but one of them (CcBCH) has a bacterial origin, while the other one (DsBCH) originates from the green algae. This suggests that evolutionary divergence has led to the development of enzyme variations in different organisms, each with unique characteristics regarding substrate specificity and efficiency. Investigating this diversity could enable the identification of novel BCHs with enhanced or specialized functions, potentially uncovering enzymes that are better suited for specific applications, such as high-yield production of BCX or zeaxanthin.

The highest zeaxanthin titer among tested BCHs was achieved with the Y. lipolytica strain ZE_7 expressing the PacrtZ gene, producing 278±48 mg/L of zeaxanthin and achieving a 25±5% conversion rate (Fig. 3). Among the tested PaCrtZ homologs, the highest zeaxanthin titer of 179±21 mg/L and a 15±3% conversion rate was obtained by strain ZE_10 expressing the PzBCH gene. Originating from the zeaxanthin-producing marine bacteria P. zeaxanthinifaciens, PzBCH is classified as a sterol desaturase family protein and shares 66.5% similarity with the amino acid sequence of PaCrtZ. The relatively high efficiency of PzBCH is also evident in the production of BCX. The ZE_10 strain produced 88% (195±16 mg/L) on average more BCX than the ZE_7 strain (104±43 mg/L) and more than the strains expressing other tested homologs.

Strain ZE_8, expressing the PsBCH gene, achieved the second-highest zeaxanthin titer among the tested homologs, producing 145±40 mg/L of zeaxanthin with a 13±5% conversion rate. Originating from the bacterium Paracoccus sp. N81106 (formerly known as Agrobacterium aurantiacum), PsBCH is highly similar to PaCrtZ (91.4% identity at the amino acid level), as shown in the phylogenetic analysis (Fig. 2), and its hydroxylating activity has been confirmed in the context of astaxanthin biosynthesis [57]. Although strain ZE_8 did not produce as much zeaxanthin as strain ZE_7, it achieved a similar BCX titer as the ZE_10 strain, reaching 184±38 mg/L, indicating its potential as a high-performing enzyme.

The high amino acid similarity between PaCrtZ and its homologs is not the primary indicator of the enzyme's activity in hydroxylating β-carotene. Namely, the strain ZE_3, expressing the EuBCH gene from the bacterium

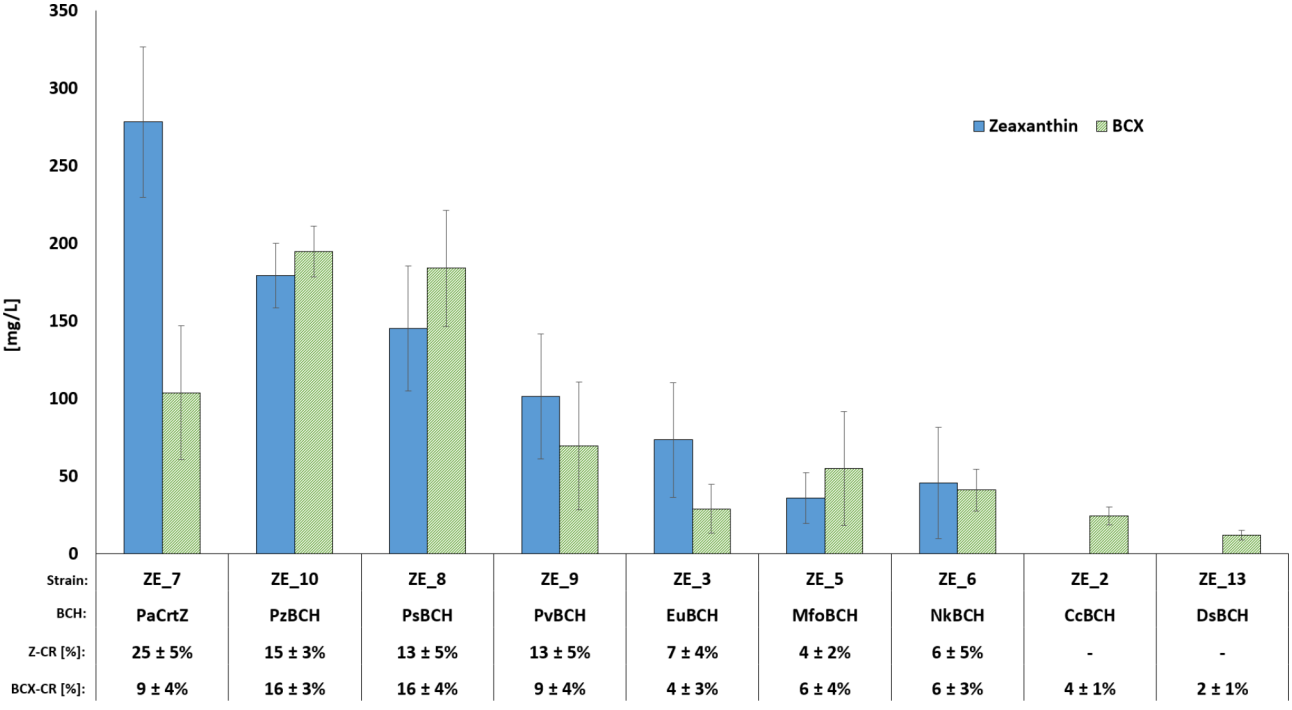

Fig. 3 Zeaxanthin and BCX titers by strains expressing BCHs. ZE_7: strain expressing *PacrtZ* from *Pantoea ananatis*; ZE_10: strain expressing *PzBCH* from *Paracoccus zeaxanthinifaciens*; ZE_8: strain expressing *PsBCH* from *Paracoccus sp*. N81106; ZE_9: strain expressing *PvBCH* from *Pseudescherichia vulneris*; ZE_3: strain expressing *EuBCH* from *Euhalothece natronophila*; ZE_5: strain expressing *MfoBCH* from *Massilia forsythiae*; ZE_6: strain expressing *NkBCH* from *Niastella koreensis*; ZE_2: strain expressing CcBCH from *Chondromyces crocatus*; ZE_13: strain expressing *DsBCH* from *Dunaliella salina*. Error bars represent the standard deviation ($N=5$ culture replicates for each strain). BCH: β-carotene hydroxylase. Z-CR - zeaxanthin conversion rate; BCX-CR: β-cryptoxanthin conversion rate. Residual β-carotene titers are presented in Figure S1

Euhalothece natronophila, produced zeaxanthin. Despite both PaCrtZ and EuBCH enzymes having a bacterial origin, EuBCH is approximately 60% longer in amino acid sequence than PaCrtZ, with only 27% similarity. While strain ZE_3 produced over five times less zeaxanthin than the top-producing strain expressing the PaCrtZ gene (strain ZE_7), these findings suggest that enzymes capable of hydroxylating β-carotene can be found among phylogenetically distant organisms, even when protein sequences of BCHs show low similarity to enzymes already confirmed to catalyze the conversion of β-carotene to zeaxanthin. The other tested strains expressing PaCrtZ homologs that successfully converted β-carotene to zeaxanthin demonstrated less than 30% of the zeaxanthin production efficiency compared to strain ZE_7 or did not produce zeaxanthin or BCX at all.

It is important to note that some hydroxylases preferentially hydroxylate carotenoids other than β-carotene. For example, HpCrtZ from Haematococcus lacustris is known to hydroxylate canthaxanthin into astaxanthin [47], but there is also evidence that HpCrtZ does not hydroxylate β-carotene into zeaxanthin or BCX [30]. The lack of β-carotene conversion to BCX or zeaxanthin by strains expressing some of the tested BCHs could be because the consequence of the enzyme's hydroxylating preference. Similar to HpCrtZ's preference for hydroxylating canthaxanthin to astaxanthin, but not β-carotene to zeaxanthin, some of the tested enzymes, as well as the ones which expression did not led to BCX or zeaxanthin production in our study, may have a preference for hydroxylating carotenoids other than β-carotene, a hypothesis that should be explored in future studies.

Out of the eight active BCHs we evaluated, two of them were able to biosynthesize BCX, but not the zeaxanthin: CaBCH from bacteria Chondromyces apiculatus and DsBCH from green algae Dunaliella salina. The highest BCX titer of 24±6 mg/L was achieved by strains ZE_2 expressing the CcBCH gene, with a 3±1% conversion rate of β-carotene to BCX (Fig. 3). Despite the low titer, this is the first report of BCX production in Y. lipolytica without subsequent zeaxanthin formation, highlighting the need for further work to optimize and enhance the production process. Optimizing gene expression through the use of stronger promoters, increasing gene copy numbers, and applying modifications previously used to enhance zeaxanthin titer, such as targeting the enzyme to the endoplasmic reticulum or peroxisomes, as well as creating a multienzyme complex, could lead to an increase in BCX titerThe amount of residual β-carotene in the tested strains suggests that β-carotene was not a rate-limiting for zeaxanthin or BCX production (β-carotene conversion into zeaxanthin is not limited to an equilibrium), which implies that CH_931 was a good enough β-carotene producer for this study (Figure S1).

Targeting PaCrtZ to subcellular compartments

The ER plays a crucial role in the synthesis of triacylglycerides (TAGs) and is also the essential cellular compartment for the synthesis and storage of β-carotene. Synthesized TAGs aggregate into lipid droplets (LDs), which not only store TAGs but are also vital for maintaining lipid homeostasis [58]. TAGs stored in LDs can be hydrolyzed to the free fatty acids (FFA), which are co-transported to peroxisome and converted into acetyl-CoA through βoxidation. During FFA transport from LDs to peroxisomes, β-carotene stored in LDs is also transported [32]. This indicates that both the ER and peroxisomes are essential for the synthesis and storage of carotenes. As a cytosolic protein, PaCrtZ has only limited access to βcarotene that is stored in different cell compartments (such as the ER and peroxisomes), so an increase in the amount of enzyme does not significantly affect the conversion rate. Previous studies have shown that targeting enzymes involved in astaxanthin biosynthesis to the ER or peroxisomes leads to increased astaxanthin production [32]. However, the effect of such targeting on zeaxanthin production has not been explored. Due to its superior zeaxanthin synthesis capabilities compared to its homologs, we therefore selected the PaCrtZ enzyme to test whether its targeting the ER or peroxisomes would affect zeaxanthin production.

We added the amino acid sequence KDEL, responsible for ER-targeting, to the Cterminus of the PaCrtZ enzyme, assembling the construct PaCrtZ_KDEL. This construct was expressed in the CH_931 strain, creating the ZE_30 strain. The aim was to bring the enzyme to the ER and make the stored β-carotene more accessible (Fig. 1A). The ZE_30 strain produced zeaxanthin at a concentration of 383±90 mg/L, making a 54% increase in the titer compared to the ZE_7 strain expressing the original, unmodified PacrtZ, which produced 249±70 mg/L. Compared to the 27±6% conversion rate of the ZE_7, the conversion efficiency was significantly improved in the ZE_30 strain, achieving 47±7% (Fig. 4).

We also evaluated the effect of targeting the PaCrtZ enzyme to peroxisomes. The peroxisome-targeting sequence SKL was added to the C-terminus of PaCrtZ, assembling the construct PaCrtZ_SKL. This construct was expressed in the CH_931 strain, creating the ZE_29 strain (Fig. 1B). The ZE_29 strain produced 412±34 mg/L of zeaxanthin, which is a 66% increase in the titer of zeaxanthin compared to the control strain ZE_7. Additionally, ZE_29 showed a 48±3% conversion rate, a significant increase compared to the 29±6% conversion rate of the ZE_7 strain expressing the unmodified PacrtZ gene (Fig. 4). These results suggest that enhanced conversion efficiency of β-carotene to zeaxanthin can be achieved by strategically distributing BCHs to the ER

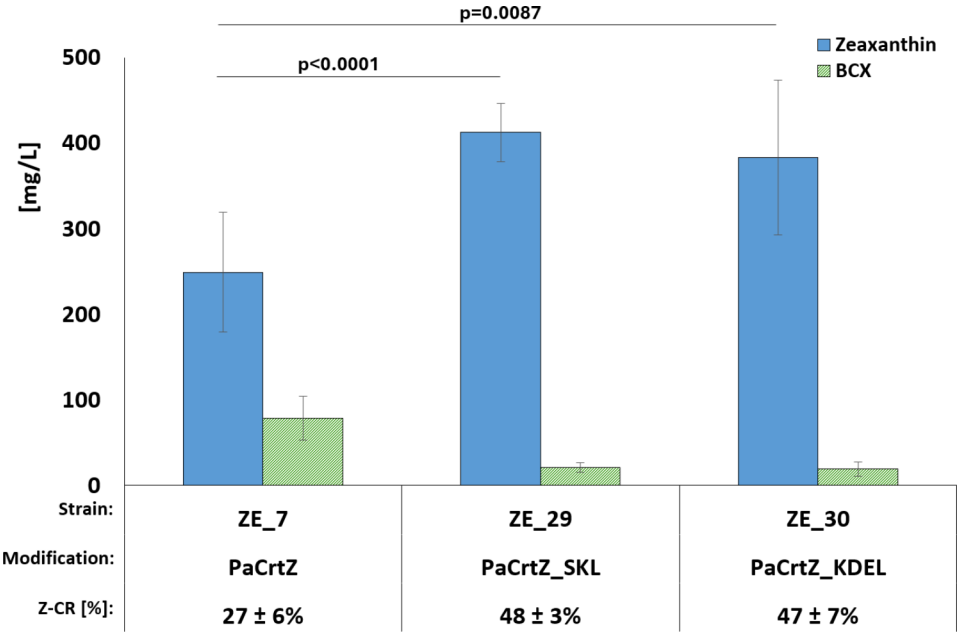

Fig. 4 Targeting PaCrtZ to subcellular compartments. Production of zeaxanthin and BCX is shown: ZE_7 – strain expressing unmodified *PacrtZ*; ZE_29 – strain expressing gene encoding PaCrtZ_SKL for targeting to peroxisomes; ZE_30 – strain expressing gene encoding PaCrtZ_KDEL for targeting to peroxisomes. p – *p*-value. Z-CR - zeaxanthin conversion rate. Error bars represent standard deviation ($N = 10$ culture replicates for each strain)

and peroxisomes, matching the localization of βcarotene within these cellular compartments.

Multienzyme PaCrtZ complex

Previous studies have shown that increasing the production of carotenoids can be achieved by forming complexes of the enzymes involved in their biosynthesis. The RIDD-RIAD-mediated multienzyme complexes have been proven effective in producing β-carotene and astaxanthin in Y. lipolytica [14, 47] and lycopene in S. cerevisiae [50]. Since no data is available on employing the multienzyme BCH complexes for zeaxanthin production in Y. lipolytica, we aimed to explore this approach in our study.

RIDD and RIAD are peptides that belong to the dock-and-lock peptide interacting family. We individually fused them to the C-terminus of the PaCrtZ enzyme using 21 amino acids long, flexible GS-rich linker, resulting in the hybrid proteins PaCrtZ_RIDD and PaCrtZ_RIAD, respectively. Because of the 2:1 binding stoichiometry of RIDD and RIAD peptides [50], we decided to test the effect of combinatorial PaCrtZ_RIDD and PaCrtZ_RIAD expression by designing the PaCrtZ_DAD encoding cassette that resulted in the expression of two copies of PaCrtZ_RIDD and one copy of PaCrtZ_RIAD, creating the strain ZE_33. To test the effect of the fusion of each peptide, we separately expressed three copies of PaCrtZ_RIDD and three copies of PaCrtZ_RIAD, creating the ZE_31 and ZE_32 strains, respectively. For the control, we expressed three copies of the unmodified PaCrtZ enzyme (strain ZE_34).

The strains with RIDD and RIAD modifications produced zeaxanthin, indicating that modifying the C-terminus of the PaCrtZ enzyme with those peptides does not negatively affect enzyme functionality (Fig. 5). The control strain ZE_34 produced 264±14 mg/L of zeaxanthin, with a 24±2% conversion rate. Increasing the copy number of the PacrtZ gene in the ZE_34 strain did not significantly increase the zeaxanthin titer compared to the ZE_7 strain (278±48 mg/L), which expressed a single copy of the PacrtZ gene. Zeaxanthin production of the ZE_31 strain was similar to the ZE_34 control strain. The ZE_32 strain, which expressed three genes encoding PaCrtZ_RIAD, achieved a zeaxanthin titer of 321±44 mg/L, a 21.6% improvement over the control ($p = 0.011$). Moreover, the conversion rate also improved to 39±4%. The highest zeaxanthin titer was achieved by the ZE_33 strain, which expressed both PaCrtZ_RIDD and PaCrtZ_RIAD hybrid proteins. The strain ZE_33 produced 369±64 mg/L of zeaxanthin with 53±7% conversion rate, which is a 39% increase in zeaxanthin titer ($p < 0.001$) and a 28% increase in conversion rate compared to the control strain ($p < 0.001$).

Analyzing the results, we observed that modifying the PaCrtZ enzyme by equipping it with RIDD and RIAD sequences positively affected β-carotene conversion to zeaxanthin, resulting in higher zeaxanthin titers. Adding the RIAD peptide to the C-terminus of PaCrtZ increases the number of helical structures in the hybrid

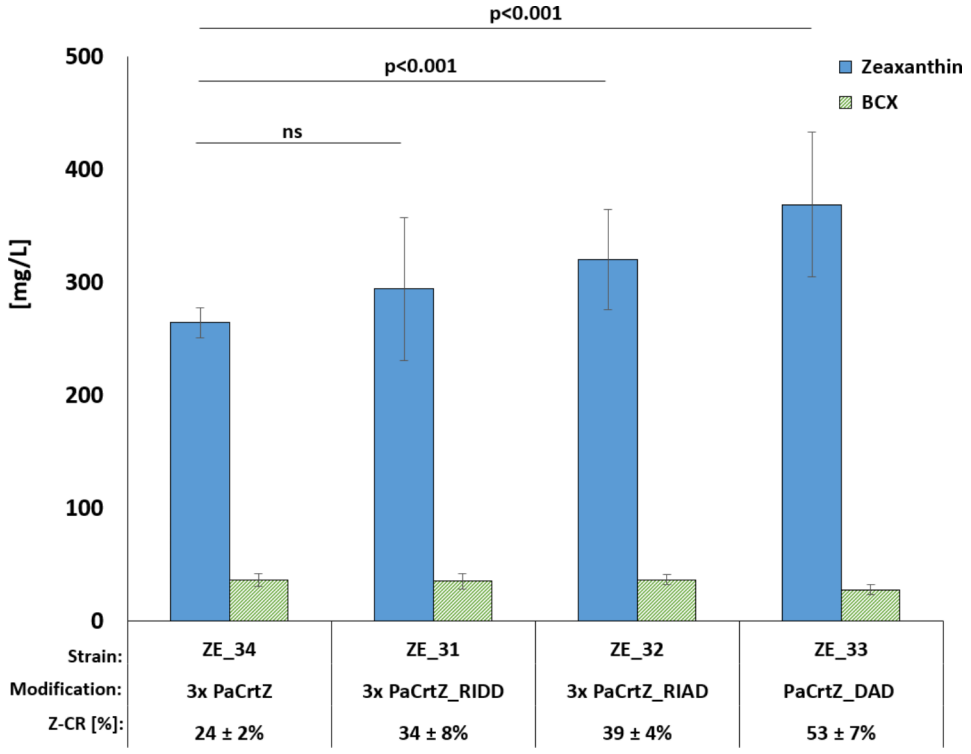

Fig. 5 Production of zeaxanthin and BCX by creating multienzyme PaCrtZ complex with RIDD and RIAD peptides connected to enzyme's C-terminus. ZE_34 – strain expressing three copies of unmodified *PacrtZ* gene; ZE_31 – strain expressing three copies of *PacrtZ* gene, modified with sequence for linker and RIDD peptide; ZE_32 – strain expressing three copies of *PacrtZ* gene, modified with the sequence for linker and RIAD peptide; ZE_32 – strain expressing two copies of *PacrtZ* gene, modified with sequence for linker and RIDD peptide and one copy of *PacrtZ* gene, modified with sequence for linker and RIAD. p – *p*-value; ns – statistically non-significant *p*-value ($p > 0.05$). Z-CR - zeaxanthin conversion rate. Error bars represent standard deviation ($N = 10$ culture replicates for each strain)

protein, potentially enhancing its anchoring to the membranes of cell compartments containing β-carotene. This enhanced anchoring could result in a higher conversion rate of β-carotene into zeaxanthin. However, this effect was not observed in the strain expressing PaCrtZ_RIDD. The dimerization of RIDD peptides may prevent them from acting as effective protein anchors, resulting in unchanged enzyme localization compared to the unmodified enzyme. PaCrtZ_RIDD and PaCrtZ_RIAD form a homotrimer complex when expressed in the same strain [59, 60]. Although the anchoring effect of the homotrimer complex is not evident, the specific protein orientation due to the complex formation could contribute to a significant increase in zeaxanthin titer and conversion rate.

These findings represent the first demonstration that modifying BCH with RIDD and RIAD peptides positively affects zeaxanthin production in Y. lipolytica, especially when a multienzyme homotrimer complex of BCHs is created.

Conclusions

Given the adverse impacts of plant extraction and chemical synthesis of zeaxanthin on the environment, economy, and human health, leveraging metabolic engineering to produce zeaxanthin by microorganisms has emerged as a promising approach. In this study, we evaluated 21 β-carotene hydroxylase (BCH) homologs of PaCrtZ enzyme for their ability to convert β-carotene into zeaxanthin in the β-carotene-producing strain of Y. lipolytica. In our study, we identified six novel BCHs capable of producing zeaxanthin and two that can produce only β-cryptoxanthin (BCX) - the intermediate between β-carotene and zeaxanthin. Notably, utilizing BCH from Chondromyces crocatus, we produced 24±6 mg/L of BCX, which is the first report of BCX production in Y. lipolytica. Our study further showed that targeting PaCrtZ to specific organelles, specifically the ER or peroxisomes, enhances zeaxanthin production. Localization to the ER resulted in a 54% increase in zeaxanthin titer compared to the control strain, whereas targeting PaCrtZ to peroxisomes yielded a 66% increase in zeaxanthin titer compared to the control. Furthermore, by engineering multienzyme complex with RIDD and RIAD peptides linked to the C-terminus of PaCrtZ, we observed notable

improvements in both the conversion rate and zeaxanthin production. The zeaxanthin titer of 369±64 mg/L was achieved, representing a 39% increase in zeaxanthin titer and a 28% increase in the conversion rate compared to the control strain, expressing unmodified PaCrtZ. Our findings suggest that the efficiency of zeaxanthin synthesis can be significantly improved through strategic spatial organization of BCHs, consistent with the cellular localization of β-carotene. Identifying new BCHs capable of producing zeaxanthin demonstrates the potential for discovering high-performance enzymes suitable for industrial production and emphasizes the versatility of microbial production in generating various carotenoids. Further studies are needed to evaluate the potential synergistic effects of these modifications in combination with other BCHs, enhancing gene expression by increasing gene copy number and/or utilizing stronger promoters. Additionally, optimizing cultivation conditions and media could lead to further increases in zeaxanthin or BCX titer and/or improvements in the conversion rate.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Author contributions
M.S. contributed to the conceptualization, research, data analysis, and visualization of the results and prepared the original draft. T.M. and A.J.K. were involved in the conceptualization and formal analysis of the study. V.M., G.K., and Š.F. were responsible for conceptualization, providing resources, supervising the project, administering the project, reviewing and editing the manuscript. M.K. contributed to the conceptualization and provided supervision. J.H. contributed to the methodology development and validation. U.P. was involved in conceptualization, writing (review and editing), and provided supervision.

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Data availability
Data is provided within the manuscript or supplementary information files.

Declarations

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