1. Introduction

Astaxanthin is a keto carotenoid with strong antioxidant activity, which is widely used in food, medicine, cosmetics and other industries [1,2]. At present, commercial astaxanthin is synthesized by chemical synthesis methods [3]. However, there are significant differences between chemically synthesized astaxanthin and natural astaxanthin in structure and function, and the oxidation resistance and stability of chemically synthesized astaxanthin are far lower than natural astaxanthin [4,5]. At present, the large-scale production of astaxanthin in the market still depends on the extraction of natural products [1]. Natural synthesis of astaxanthin by engineering microbial chassis has become a promising alternative at present [3,6].

Haematococcus pluvialis is the microorganism with the highest content of natural astaxanthin, so it is the algae that is most used to produce astaxanthin at present [7,8]. However, microalgae grow very slowly and need continuous power and light supply, which makes the cost of algae production equipment higher. In recent years, synthetic biologists have developed a variety of tools using Saccharomyces cerevisiae as a model microbial chassis [9]. It has excellent potential to produce heterologous metabolites due to its high biomass and mature genetic modification technology [10]. Recently, astaxanthin has been successfully produced in Saccharomyces cerevisiae [11–14] by introducing exogenous biosynthesis into chassis strains. Astaxanthin biosynthesis pathway can be divided into two modules: First, glucose is converted to β-carotene hydroxylase (crtZ) and β-carotene ketolase (crtW) decreases the content of the astaxanthin. Here, we exploited directed evolution of the fusion of crtZ and crtW for improving astaxanthin biosynthesis in Saccharomyces cerevisiae. The results demonstrated that the fusion enzyme of crtZ-crtW with 2 X GGGGS peptides linker can effectively reduce the accumulation of intermediates and improves the content of astaxanthin. Compared with the control strain, the fusion enzyme of ketase and hydroxylase reduced zeaxanthin and canthaxanthin by 7 and 14 times and increased astaxanthin by 1.6 times, respectively. Moreover, 9 variant fusion mutants with improved astaxanthin production were generated through directed evolution. Combining these dominant mutants generated a variant, I595S + I606L, which increased the astaxanthin content of 3.8 times than the control strain. The AlphaFold2 assisted structural analysis indicated that these two mutations alter the interaction between the substrate and the enzymes pocket. Our research provided an efficient idea to reduce the accumulation of the intermediate products in complex biosynthesis pathway.
hydroxylated or ketolated products as substrates. Therefore, the production of astaxanthin by engineering microbial cells is often accompanied by the accumulation of unwanted intermediate zeaxanthin and canthaxanthin [13,24], which will directly reduce the production of astaxanthin.

In this study, we reported the fusion strategy of crtZ and crtW, which can reduce the accumulation of intermediates in the process of astaxanthin biosynthesis. In order to further improve the content of astaxanthin, directed evolution of the fusion enzyme was performed to improve the catalysis activity (Fig. 1). We used the AlphaFold2 to analyze the structural of the mutated fusion enzyme and found that these mutations alter the interaction mutated between the substrate and the enzymes pocket. Overall, our results show that the directed evolution of the fusion enzyme can greatly reduce the content of intermediates and increase the production of astaxanthin.

2. Methods and materials

2.1. Strains, plasmids and media

The yeast strains used in this study are shown in Table S1. The selected medium for astaxanthin producing strains is SC-Leu-Ura-His (complete medium without leucine, histidine and uracil synthesis, glucose 22 g/L), added 20 g/L agar powder to all yeast solid media. Plasmid transformation was carried out by E. coli Trans1-T1 purchased from Beijing Biotechnology Co., Ltd. The culture condition of E. coli is Culture in LB medium (10 g/L tryp-one, 5 g/L yeast extract, 10 g/L NaCl and 100 μg/mL ampicillin), added 15 g/L agar powder to LB solid medium.

2.2. Construction of plasmids for fusion enzyme

The crtZ gene and crtW gene module were amplified by PCR using primers pDYW-Fuse-F and pDYW-Fuse-R (Table S2). The homologous arm of pRS416 vector was introduced at the 5′end of crtZ gene, and the designed linker was introduced at the 3′end as the homologous arm connected with crtW gene. Similarly, the primers were designed to amplify the crtW gene with linker at the 5′end and the homologous arm of pRS416 vector at the 3′end, after the PCR purified fragment and linearized vector were assembled through BM, the large intestine was connected with the pRS416 vector by BM assembly. The mutant library was transferred to strain yDYW000 and cultivated at 30 °C in SC-Leu-Ura-His medium.

2.3. Cloning the crtZ-crtW gene and constructing its mutant library

The mutant library of the crtZ-crtW gene was constructed by error-prone PCR. Taq DNA polymerase purchased from Beijing Tiangen Biochemical Technology Co.LTD. Configure mutagenic buffer (1.423 g magnesium chloride hexahydrate (70 mM), 3.728 g potassium chloride (500 mM), 1.211 g Tris (100 mM) and 0.1 g gelatin (0.1 W/V) dissolved in 100 mL ddH2O, adjust pH to 8.3 with 8 mM HCl solution and store at –20 °C), and MnCl2 solution (0.0495 g manganese chloride was dissolved in 1 mL ddH2O to make 250 mM MnCl2 solution, then diluted 25 times), 5 × EPdNTPs: 5 mM dCTP, 5 mM dTTP, 1 mM dATP and 1 mM dGTP. Error prone PCR of fusion enzyme using primers pDYW-F (ATGACTAACTTCTTTGATGGTTGTT) and pDYW-R (TTATGAAAATAGAACCCACCAAGGC), subsequently, the purified PCR product was connected to the pRS416 vector by BM assembly. The mutant library was further transferred into E. coli Trans1-T1 and cultured on LB plate containing 100 μg/mL ampicillin. All the obtained E. coli plasmids were digested by NotI and the digested fragments were recovered. The mutant library was transferred to strain yDYW000 and cultivated at 30 °C in SC-Leu-Ura-His medium.

2.4. Yeast transformation and assembly

The yeast colonies were incubated in 5 mL YPD overnight at 30 °C with shaking at 220 rpm and transferred to fresh medium the next day. The cultures were incubated at 30 °C and 220 rpm until the OD600 reached 0.5–0.8. Cells were washed once with sterile H2O, resuspended in 0.1 M LiAc solution and placed on ice until needed. The yeast transformation system contained 620 μL of 50% polyethylene glycol (PEG-3350), 40 μL of salmon sperm DNA (100 mg/mL), 90 μL of 1 M LiAc and 150 μL of a mixture of plasmids or fragments and cells. The system was incubated at 30 °C for 30 min, followed by the addition of 90 μL of DMSO and then heat shock at 42 °C for 18 min. The cells were resuspended in 5 mM CaCl2 for 5 min and incubated on solid SC-Leu-Ura-His mediums for 2–4 days.

2.5. Shake flask cultivation for astaxanthin production

Select the single colony of the strain to be fermented and mark it on the corresponding solid defect medium. After the colony grows, select the single colony and inoculate it into 5 mL SC defect liquid medium as shown in Fig. S2 and Fig. S3. The construction of crtW-crtZ fusion enzyme plasmids also used the above methods.
the primary seed liquid. After incubating at 220 rpm at 30 °C until the OD600 reaches 6–8 (about 20–24 h), inoculate with 0.2 of the initial OD600 into a new 5 mL SC defective medium as the secondary seed solution, continue to incubate until the OD600 reaches 5–6 (about 14–16 h) and then transfer with 0.1 of the initial OD600 into 50 mL of fresh YPD-40 medium (40 g/L glucose, 20 g/L tryptone and 1 g/L yeast extract). Grow in a shaking table at 250 rpm and 30 °C for 60 h. Each sample was performed on technical triplicates.

2.6. Analysis of carotenoids production by HPLC

Total carotenoids were extracted as described below. Cells from 2 mL culture were collected and washed with distilled water, resuspended in 1 mL of 3 mol/L HCl, boiled for 5 min, and subsequently cooled in an ice bath for 5 min. Afterward, the cell debris was washed twice with distilled water and resuspended in 0.5 mL of acetone containing 1% (w/v) butylated hydroxytoluene. The mixture was thereafter vortexed until it became colorless (approximately 20 min) and incubated at 30 °C for 10 min. This was followed by centrifugation at 13,800 × g for 5 min. The acetone phase containing the extracted astaxanthin was filtered through a 0.22-μm membrane for HPLC analysis. The standard products of astaxanthin (7542-45-2), zeaxanthin (144-68-3), canthaxanthin (514-78-3), beta-carotene (7235-40-7) and lycopene (502-65-8) were purchased from Sigma-Aldrich. The extracted products were analyzed by HPLC (Waters2695, Waters Corp., USA) equipped with a BDS HYPERSIL C18 column (150 mm × 4.6 mm, 5 μm, Thermo Fisher Scientific, USA) and a UV/VIS detector (Waters 2489) at 470 nm. The mobile phase consisted of acetonitrile-water (91:1 v/v) and methanol-2-propanol (3:2 v/v) with a flow rate of 1 mL per min. The column temperature was set at 25 °C. A portion of each sample was harvested and dried at 70 °C for measurement of the dry cell weight. Each sample was performed on technical triplicates.

2.7. Homology modeling, molecular docking, and structural analysis

The three-dimensional structure model of the fusion enzyme crtZ-crtW and its mutant were constructed according to the amino acid sequence by the AlphaFold2 program [25], and the structural information of the fusion enzyme was obtained. And AlphaFold2 program was used to minimize the energy of the simulated protein structure model. The 3D structure file of β-carotene was downloaded from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Then the molecular structure of β-carotene was linked with the fusion enzyme by the AutoDock software (Olson, 2018), and a molecular docking system between crtZ-W fusion enzyme and β-carotene molecular structure model was established. The β-carotene structure file was retrieved from PDB library. The docking cluster analysis is carried out in the AutoDock software environment. Clustering is characterized by binding energy (kcal/mol). Establish the link between β-carotene and the corresponding amino acids is then minimized. PyMOL software (Delano, 2010) is used for the analysis of complex structures. The software can also be used to analyze the mutations of specific amino acid sites to explore the spatial and molecular interactions between amino acids.

3. Results

3.1. Fusion of the β-carotene hydroxylases and ketolases to reduce the intermediate by-products

Our previous study showed that crtZ from Agrobacterium aurantiacum (Aa crtZ) and crtW from Brevundimonas vestitilis DC263 (BDC263 crtW) had a very positive effect on astaxanthin synthesis in yeast [26,27]. The chassis β-carotene producing strain yJBH0012 was a S. cerevisiae yeast containing a β-carotene biosynthesis pathway integrated at the synV YEL063C/CAN1 site [28]. To increase the production of β-carotene, a tHMG1 gene was integrated at the HO site of yJBH0012 to generate the chassis strain yDYW000 [29]. Then the crtZ and crtW genes with URA3 tags were integrated into the Ty1 retrotransposon site of the strain to generate the initial strain yDYW001 (Fig. S4). The fermentation assay showed that the yDYW001 produced 0.49 mg/g DCW astaxanthin, 0.35 mg/g DCW zeaxanthin and 0.41 mg/g DCW canthaxanthin. It is possible that the imbalanced pathway flux caused the accumulation of the intermediate zeaxanthin and canthaxanthin reduced the astaxanthin. Inspired by natural organization of enzymes into specific complexes, fusion enzymes can connect catalytic sites to generate enzyme-to-enzyme channeling and reduce the mass transfer distance of the reactive intermediates [30,31]. As an important part of the fusion enzyme, linker peptides (linker) can significantly affect the biological functions of proteins [32,33]. It is reported that the linker lengths may affect the catalytic efficiency of the fusion enzyme and the accumulation of intermediates [22]. In this study, the flexible and hydrophilic amino acid GGGGSG (the corresponding gene sequence is gggggagggaggagaag) were selected as the connecting peptide, which made the two enzymes close to each other in the spatial structure and can increase the domain interaction between the two enzymes [24]. To test the effect of different linker length and fusion orders of the two enzymes, we constructed six fusion enzymes by combination of three linkers and two different genes orders: crtZ-linker-crtW (ZL1W), crtZ-2linker-crtW (ZL2W), crtZ-3linker-crtZ (ZL3W), crtW-linker-crtZ (WL1Z), crtW-2linker-crtZ (WL2Z) and crtW-3linker-crtZ (WL3Z). The expression cassettes of the fusion enzymes were individually integrated into the Ty1 site on the β-carotene producing strain yDYW000, resulting in strains yDYW002 ~ yDYW007 (Fig. 2A). The above strains were cultivated in shake flask containing YPD medium and the products were extracted for analysis after 60 h of fermentation. As shown in Fig. 2B, the astaxanthin content of the six fusion enzyme strains were 0.59 mg/g DCW, 0.25 mg/g DCW, 0.76 mg/g DCW, 0.31 mg/g DCW, 0.68 mg/g DCW and 0.21 mg/g DCW. It was evident that the strain expressing the crtZ-W fusion order produced more astaxanthin than the corresponding crtW-Z expressing strain, with the highest content obtained as yDYW004 (ZL2W), which was 1.6-fold compared to yDYW001 (Fig. 2B). And compared with the control strain, zeaxanthin and canthaxanthin, as the main intermediates, changed significantly before and after fusion (Fig. S5). The content of intermediate products of the fusion enzyme expressed in the form of crtZ-W fusion was significantly reduced. ZL2W has the lowest intermediate product content, the content of canthaxanthin is 14 times lower than the control group, and the content of zeaxanthin is 7 times lower than the control group. However, the content of the protein intermediates expressed as a fusion with crtW-Z did not decrease significantly, and the content of canthaxanthin increased significantly. Unexpectedly, the production of astaxanthin of the strain fused by crtW-Z decreased significantly. These results indicated that the order of protein ligation and the length of linker can affect the structure and catalytic efficiency of the fusion enzyme. Specifically, the crtZ-2linker-crtW (ZL2W) of fusion enzyme can decrease the intermediates and increase the astaxanthin content.

3.2. Improve astaxanthin production by directed evolution

In order to further improve the production of astaxanthin, directed evolution strategy was used to increase the catalysis activity of the fusion enzyme. Theoretically, improving the adaptability of the fusion enzyme to the substrate molecule may increase the transformation rate of astaxanthin. Therefore, random mutagenesis of ZL2W fusion enzyme were performed by error prone PCR (Fig. 3A). By changing the concentration of MnCl₂ in the reaction system to control the mutation efficiency, several different mutation rates were designed. In this way, random mutations can occur in the whole sequence of the fusion enzyme through a stable mutation rate. Previous studies reported that mutating 1–2 sites per gene is highly advantageous for protein evolution, while too much mutations easily inactivate the enzyme [35]. By designing different gradients of MnCl₂ concentrations in the reaction system, the
Fig. 2. (A) Construction of astaxanthin producing strain. The \textit{crtE}, \textit{crtI}, \textit{crtYB} module genes are integrated into the \textit{CAN1} site of synV, the \textit{dHMG1} is integrated in the \textit{HO} site and the \textit{crtZ} and \textit{crtW} are integrated into the retrotransposon of \textit{Ty1} through different fusion forms (linker is GGGGS, where X = 1,2,3). (B) Astaxanthin and intermediate production analysis after fermentation of the fusion enzyme strains. The error bar represents the standard deviation of three independent experiments. (Student’s t-test; NS, not significant; *P < 0.05, **P < 0.01).

Fig. 3. (A) The mutant library of ZL2W (\textit{crtZ}GGG\textit{G}G\textit{G}G\textit{SS}2\textit{crtW}) fusion enzyme was constructed for directed evolution. The mutant library was transformed into the carotene producing strain yDYW000. After the colonies grew, the colonies with darker color were selected. (B) Yeast colonies with different color were grew on the selective plates. (C) Carotenoid content analysis: content of mutant astaxanthin, zeaxanthin, canthaxanthin, lycopene and \(\beta\)-carotene, total carotenoids and percentage of astaxanthin in total carotenoids. The error bar represents the standard deviation of three independent experiments. (Student’s t-test; **P < 0.01).
PCR products obtained at different concentrations were purified and BamHI assembled with the vector. And transformed into E.coli Trans1-T1 competent cells. After the transformants grow, 24 single colonies were selected from each concentration mutation library plate for sequencing analysis to determine the mutation rate of each concentration, as shown in Table S3. It is known from the table that the concentration of manganese chloride in the system does affect the probability of the occurrence of the mutation, with the general trend that the higher the concentration, the higher the mutation rate. Finally, the 0.15 mM MnCl₂ concentration was used for generating random mutation of the ZL2W fusion enzyme. Transformation of the mutated library into the strain yDYW000 resulted in colonies with different colors on the defective plates (Fig. 3B). Single colonies with darker color were selected from the library, and after two rounds of purification and marking, shake flask fermentation was carried out to analyze the content of carotenoids. In about 10000 mutant libraries, 48 strains with darker colonies were obtained through color screening. After fermentation of these strains and analysis of astaxanthin production, 9 strains with increased astaxanthin production were obtained. As shown in Fig. 3C, the astaxanthin content of mutants I87V, L276A, L95S and I206L increased significantly. Compared with the starting strain yDYW001, the astaxanthin content of mutants I87V, L276A, L95S and I206L increased from 0.49 mg/g DCW to 1.21 mg/g DCW, 1.28 mg/g DCW, 1.36 mg/g DCW and 1.52 mg/g DCW, which increased by 2.5–3.1 times respectively. At the same time, it can be seen that the total carotenoids and the percentage of astaxanthin in the total carotenoids of these dominant mutants have also increased, and some β-carotene have not been completely transformed. This result demonstrated the directed evolution of the fusion enzyme successfully increased the astaxanthin content production in Saccharomyces cerevisiae.

3.3. Combining different beneficial mutations further increases astaxanthin production

To further improve the production of astaxanthin, we combined these point mutations through different design protocols on the fusion enzyme genes. As shown in Fig. 4, the combined mutant L95S + I206L increased astaxanthin content by 1.87 mg/g DCW, which was higher than that of L95S (1.36 mg/g DCW) and I206L (1.52 mg/g DCW). In addition to mutant L95S + I206L, other combined base mutations did not generate higher astaxanthin production than that of the single base mutations. For example, I87V + I206L, L276A + I95S, L276A + I87V + L95S, produced 0.78 mg/g DCW, 1.09 mg/g DCW, 0.85 mg/g DCW, 1.05 mg/g DCW and 0.09 mg/g DCW, respectively. Specially, the two mutation sites involved in I87V + L95S were both in the crtZ domain, and the distance between the two sites is only 8 amino acids. It is possible that the combination of the two mutation sites disrupted the catalytic function of the crtZ, which decreased the astaxanthin production. This result indicated that combining different beneficial mutations in the fusion enzyme can generate strains with increase or decrease of target products, which can be used for selection of strains with better performance.

3.4. Protein structure analysis of the mutated fusion enzyme

In order to analyze the interaction between the substrates and the mutated fusion enzyme, the AlphaFold2 was used to produce homology models of the crtZ, crtW, ZL2W fusion enzyme and its mutant ZL2W-L95S + I206L. Compared with the structures of crtZ and crtW, the merged of crtZ-W had no observable effect on protein structure (Fig. S6). Then Autodock software was used to dock the protein and substrate for analysis. We analyzed the size of substrate pocket on the surface before and after protein mutation. As shown in Fig. 5A, the binding pocket between the substrate and protein molecules was compressed after mutation. This made the substrate molecules better embedded in the binding pocket when interacting with proteins, which greatly improved the binding ability between substrate molecules and proteins. The docking results of protein and molecule indicated that there was an additional interaction between the serine residue of mutant L95S and the substrate molecule carotene. It is possible that the interaction improved the structural stability of the mutant complex, and shorten the distance between the substrate molecule and the protein, which increased the catalytic activity of the enzyme on the substrate.

For mutant L95S + I206L, as shown in Fig. 5B. When S95 and I206 replaced L95 and I206, the hydrogen bonding between the mutant and the substrate molecules was significantly enhanced. This is very beneficial to the formation of polar groups such as carbonyl and hydroxyl. It is worth noting that in the wild-type, the hydroxyl binding position of carotene molecule was outside the electron cloud, and there was almost no hydrogen bonding around. However, in the mutant, the position where carotene molecules bind hydroxyl groups was perfectly wrapped by electron clouds, and the hydrogen bonding effect had been significantly improved, making hydroxyl groups easier to generate.

4. Discussion

At present, the low catalytic activity of hydroxylase and ketolase and the accumulation of intermediates is a challenge in microbial production of astaxanthin. In this study, construction of the fusion enzyme significantly reduced the content of intermediates in the process of converting
β-carotene to astaxanthin, meanwhile the output of astaxanthin is increased from 0.49 mg/g DCW to 0.76 mg/g DCW. Secondly, directed evolution of the fusion enzyme by error prone PCR generated 9 mutants with significantly increased astaxanthin production. The content of mutant I206L reached 1.52 mg/g DCW, which was 3.1-times higher than that of the original strain. In order to further improve the astaxanthin production, we reproduced these point mutations on the fusion enzyme gene through different design combinations, and then fermented these strains to detect their astaxanthin content. The mutant L95S + I206L was obtained, and its content reached 1.87 mg/g DCW, which was further increased by 1.23-times and 1.38-times compared with the single point mutant I206L and L95S astaxanthin. We determined the total amount of carotenoids for individual strains and calculated the proportion of astaxanthin in them. As shown in Figs. 2B, 3C and 4, the trend of the total amount of strain carotenoids increased step by step with increasing astaxanthin content. Notably, the amount of intermediate product after mutation of the fusion protein remained much lower than in controls. However, as the carotenoid content increased, the proportion of astaxanthin in them also rose gradually, especially the mutants L95S + I206L, and finally the content of astaxanthin to total carotenoids reached 66.5%.

Through Alphafold2 assisted 3D modeling and analysis of \( \text{crtZ} \), \( \text{crtW} \) and \( \text{crtZ-W} \) fusion enzymes, we found the beneficial mutations occurred near the active pocket of protein binding to substrate molecules (Fig. S7). After docking analysis with the substrate molecules, we found that the hydrogen bonding between the mutant and the substrate molecules has been significantly strengthened. This made the binding between substrate molecules and proteins more stable. Therefore, we speculated that these beneficial mutations change the size of the protein active bag bound to the substrate molecule, thereby enhancing the binding ability between substrate molecules and proteins.

Previous study indicated that fusing \( \text{crtZ} \) and \( \text{crtW} \) genes from different sources can improve the yield of astaxanthin in \textit{Escherichia coli}. [36]. Nadja A. Henke et al. constructed a membrane bound \( \text{crtZ} \) and \( \text{crtW} \) fusion protein, which significantly improved the astaxanthin production of the strain at high sugar concentration [37]. Interestingly, these
studies have proved that the fusion mode of crtZ-W is obviously better than that of crtW-Z when it is used to fuse crtZ and crtW for the purpose of improving astaxanthin production. This is consistent with our results. It is possible that the catalytic active region of crtZ-crtW is located on both sides of the fusion protein. This protein fusion mode can not only ensure the proximity effect of the enzyme, but also enable the intermediate to be guided from one active site to the next site in a very short distance. Thus, the fusion enzyme of crtZ-W reduced the accumulation of intermediate products. In addition, the length of linker will also directly affect the distance between the active sites of two proteins. If the linker is too short, the active regions of two proteins may block with each other. While the linker is too long, the distance of intermediates transport may reduce the reaction efficiency. The structures of fusion enzymes were composed by the state-of-the-art de novo structural modeling algorithm, AlphaFold2. It could be clearly seen that while the linker and a small part of crtW were folded into a loop structure, the main body of enzymes had distinct helix structures. Compared with independent enzymes, two parts of the fusion enzyme are closer in space, which therefore reduces the diffusion of intermediates. We modeled the position of the carotene in the enzyme by docking.

In the industrial application of microbial enzymatic reaction for synthesizing valuable products, some enzymes are potentially inhibited by high level of substrates, causing the accumulation of substrates or intermediate products. For example, substrate inhibition of lycopene cyclase was found to be the main limitation in carotenoid biosynthesis in *Yarrowia lipolytica*, Yongshuo Ma et al. removed the lycopene substrate inhibition by structure-guided protein engineering [19]. It is possible that the hydroxylase and ketolase were inhibited by the high level of β-carotene. In this study, the directed evolution of the fusion enzyme of crtZ and crtW may decrease the β-carotene substrate inhibition of the hydroxylase and ketolase by reducing the distance of intermediates transport and improving the enzyme catalytic activity. It is worth noting that the color become dark red with the accumulation of astaxanthin, which can be used for quick screening of high astaxanthin content yeast. Due to the limitation of human eye ability, this is still a very laborious work. Therefore, it is still a challenge to the high-throughput screening of evolutionary phenotypes, the development of biosensors and high-throughput screening equipment may solve this problem [38]. At the same time, the genetic diversity produced by random evolution makes it a very time-consuming and laborious work to screen dominant mutants. It is worth noting that most of the mutation sites of dominant mutants occur near the active pocket of protein substrate binding (Fig. S7). Therefore, it is a very feasible scheme to evolve fusion protein for screening the dominant mutants by rational design, which will greatly reduce the screening cost required to obtain the dominant mutants. Moreover, this strategy could be extended to many other microbial chias cell, such as *Yarrowia lipolytica* and *Yarrowia lipolytica* [19]. To sum up, this study provided a new idea to improve the accumulation efficiency of heterologous metabolites in yeast by combining the two ideas of reducing intermediates and improving substrate conversion.

CRediT authorship contribution statement

**Yong-Wen Ding**: Methodology, Validation, Investigation, Data curation, Visualization, Writing – original draft. **Chuan-Zhen Lu**: Experimen, Investigation. **Yan Zheng**: Validation, Investigation, Modeling analysis. **Han-Zhang Ma**: Experiment, Investigation. **Jin Jin**: Methodology, Validation, Investigation. **Bin Jia**: Supervision, Funding acquisition, Project administration, Writing – review & editing, Validation. **Ying-Jin Yuan**: Supervision, Funding acquisition, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2022.10.005.

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