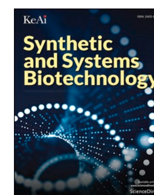
Synthetic and Systems Biotechnology 10 (2025) 58–67

Contents lists available at ScienceDirect

Synthetic and Systems Biotechnology

journal homepage: www.keaipublishing.com/en/journals/synthetic-and-systems-biotechnology



Original Research Article

Systematic metabolic engineering enables highly efficient production of vitamin A in *Saccharomyces cerevisiae*

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A R T I C L E   I N F O

Keywords:
Vitamin A
Isozyme
Retinol
Metabolic engineering
Cofactor engineering
Saccharomyces cerevisiae

A B S T R A C T

Vitamin A is a micronutrient critical for versatile biological functions and has been widely used in the food, cosmetics, pharmaceutical, and nutraceutical industries. Synthetic biology and metabolic engineering enable microbes, especially the model organism *Saccharomyces cerevisiae* (generally recognised as safe) to possess great potential for the production of vitamin A. Herein, we first generated a vitamin A-producing strain by mining β-carotene 15,15′-mono(di)oxygenase from different sources and identified two isoenzymes *Mbblh* and *Ssbco* with comparable catalytic properties but different catalytic mechanisms. Combinational expression of isoenzymes increased the flux from β-carotene to vitamin A metabolism. To modulate the vitamin A components, retinol dehydrogenase 12 from *Homo sapiens* was introduced to achieve more than 90 % retinol purity using shake flask fermentation. Overexpressing *POS5Δ17* enhanced the reduced nicotinamide adenine dinucleotide phosphate pool, and the titer of vitamin A was elevated by almost 46 %. Multi-copy integration of the key rate-limiting step gene *Mbblh* further improved the synthesis of vitamin A. Consequently, the titer of vitamin A in the strain harbouring the Ura3 marker was increased to 588 mg/L at the shake-flask level. Eventually, the highest reported titer of 5.21 g/L vitamin A in *S. cerevisiae* was achieved in a 1-L bioreactor. This study unlocked the potential of *S. cerevisiae* for synthesising vitamin A in a sustainable and economical way, laying the foundation for the commercial-scale production of bio-based vitamin A.

1. Introduction

Vitamin A is a fat-soluble vitamin necessary for the normal growth, development, and metabolic activities of the human body. It was discovered in the 19th century by American scientists Elmer Mc Collum and Margaret Davis [1]. Vitamin A is not a single compound but a series of retinol derivatives [2], which are composed of three structural parts: β-ionone ring, isoprene-like backbone, and functional group. Depending on the side chain joining functional groups, vitamin A includes retinol, retinal, retinoic acid, and retinyl palmitate. Deficiency in vitamin A can cause night blindness [3], reduced immunity, and restricted bone development [4]. According to statistics, nearly 300 million preschool children are deficient in vitamin A [5], seriously affecting their growth, development, and physiological activities [6]. Moreover, vitamin A has tremendous commercial value in the food, cosmetic, pharmaceutical, and nutraceutical industries.

Many valuable natural products are obtained by extraction from plants or animals [7]. Vitamin A can be extracted from animal tissues

Peer review under responsibility of KeAi Communications Co., Ltd.

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https://doi.org/10.1016/j.synbio.2024.08.004
Received 11 May 2024; Received in revised form 14 August 2024; Accepted 14 August 2024
Available online 16 August 2024
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and cod liver oil, but it faces problems such as scattered sources, cumbersome production process, high cost, and low yield. Currently, the main production method of vitamin A is based on the chemical synthesis of petroleum-derived substances such as acetone and acetylene. Although chemical synthesis can achieve large-scale production, it is not easy to accurately screen vitamin A components, and it causes environmental pollution problems. Therefore, engineering microbes to produce vitamin A is an attractive alternative to meet the growing market demand. The central cleavage of β-carotene is a critical step in the biosynthesis of vitamin A. β-carotene produces two molecules of retinal under the cleavage of β-carotene 15,15′-oxygenase, and retinal is oxidised or reduced to form retinoic acid or retinol [8]. β-carotene 15, 15′-mono(di)oxygenase is a cytosolic enzyme that was first discovered in the rat liver and intestine [9]. Dovery et al. [10] obtained β-carotene 15, 15′-dioxygenase from guinea pig intestinal mucosal extracts, which could cleave 1 mol of β-carotene into 1.5–2 mol of retinal, confirming the enzyme's central cleavage mechanism.

Researchers have successfully constructed vitamin A biosynthesis pathways in various heterologous chassis cells, such as *Escherichia coli*, *Yarrowia lipolytica*, and *Saccharomyces cerevisiae*. Expression of β-carotene 15,15′-dioxygenase from *uncultured marine bacterium 66A03* (*Mbblh*) in *E. coli* exhibits the highest activity toward β-carotene with a vitamin A titer of 136 mg/L [11]. Yong-Su Jin et al. [12] achieved the first production of vitamin A in *S. cerevisiae* by introducing the exogenous *Mbblh* gene and using xylose as a carbon source with *in situ* dodecane extraction to produce 3350 mg/L vitamin A, including 2094 mg/L of retinal and 1256 mg/L of retinol. However, no previous study has investigated β-carotene 15,15′-mono(di)oxygenases from other sources except for *Uncultured marine bacterium 66A03* in *S. cerevisiae*. Although exogenous enzymes from different sources act on the same substrate, there are some differences in substrate specificity and catalytic activity [13]. Moreover, host-cell compatibility may also affect the expression and performance of exogenous enzymes [14]. For instance, yeast chassis cells po1f that expressed phytoene dehydrogenase and the bifunctional phytoene synthase/lycopene cyclase from *Mucor circinelloides* (CarB and CarRP, respectively) produced 4.12-fold more β-carotene than the enzymes from *Xanthophyllomyces dendrorhous* (CrtI and CrtYB, respectively) [15]. Cai et al. [16] found that the enzyme activity and the overall expression level of lipase isozymes, which catalyse the same reaction in *Komagataella phaffii*, were increased through co-expression of isozymes from different sources. Therefore, screening genes suitable for the chassis expression system is essential to improve the metabolic flux of the heterologous pathway.

Since retinol has extensive application values, there is a need to regulate the components in vitamin A biosynthesis [17]. There are three different types of enzymes that convert retinal to retinol, including alcohol dehydrogenases (ADHs), aldoketo reductases, and retinol dehydrogenases (RDHs) [18]. Zhou et al. [19] found that the accumulation of fatty alcohols decreased to varying degrees by knocking out the endogenous *SfA1* and *ADH5* genes in yeast, respectively. *ADH6* and *ADH7* were considered the most important genes encoding vanillin reductase activity in *S. cerevisiae* [20]. For selective retinol biosynthesis, yeast endogenous *ENV9* with the *E. coli ybbO* were co-expressed in engineered yeast, resulting in the retinol titer reaching 2479.34 mg/L in fed-batch fermentation [17]. Recently, the engineered yeast SR8A has exhibited highly selective retinol production by identifying and introducing the human *RDH12* gene [21]. Moreover, an insufficient supply of cofactors is a common problem during natural product biosynthesis, especially terpenoids. By adding different concentrations of metal ions in vitro experiments, the researchers found that the activity of *Mbblh* was the highest when adding Fe^{2+} [22]. Reduced nicotinamide adenine dinucleotide phosphate (NADPH), the reduced form of nicotinamide adenine dinucleotide phosphate ($NADP^{+}$), is the cofactor for vitamin A biosynthesis pathway enzymes. Therefore, tailored cofactor engineering strategies may drive vitamin A production. Moreover, multi-copy integration of key rate-limiting step genes may help attenuate metabolic obstruction [23]. Park et al. [24] increased the retinol titer by 22-fold upon butylated hydroxytoluene treatment by integrating 11 copies of *Mbblh* expression cassettes into multiple ribosomal DNA sites in *Y. lipolytica*. To date, vitamin A yield in *S. cerevisiae* was increased to 3350 mg/L. However, the vitamin A yield was still much lower than that in *Y. lipolytica*. Therefore, systematic metabolic engineering of *S. cerevisiae* with a heterologous pathway may offer an effective solution to enhance vitamin A yield.

In this study, to rebuild the vitamin A pathway with good output, an integrated approach should be proposed accordingly by source screening β-carotene 15,15′-oxygenase, combinatorial expression of isozymes, manipulating regulation genes of vitamin A components, cofactor engineering and multi-copy integration strategy (Fig. 1). Ultimately, an engineered *S. cerevisiae* with the highest reported titer of vitamin A was achieved successfully, demonstrating the great potential for biosynthesis of high-value products like vitamin A and driving the commercial-scale production of bio-based vitamin A.

2. Materials and methods

2.1. Strains, culture media, and reagents

E. coli TOP10 cells used for gene cloning and plasmid amplification were purchased from Biomed Gene Technology Co., Ltd. (Beijing, China) and cultured at 37 °C in Luria−Bertani medium (1 % tryptone, 0.5 % yeast extract, and 1 % NaCl) supplemented with 50 μg/mL kanamycin or 100 μg/mL ampicillin for selection.

S. cerevisiae strain SyBE_SC0014CY03 was selected as the background strain for this study. Yeast strains for normal cultivations were cultured in YPD medium (2 % glucose, 2 % peptone, and 1 % yeast extract). All engineered yeast strains carrying plasmids with the Ura3 marker were selected and cultured in synthetic complete dropout medium (2 % glucose, 0.67 % yeast nitrogen base, and 0.2 % amino acid mix without uracil) at 30 °C for selection. YPDG medium (4 % glucose, 2 % peptone, 1 % yeast extract, and 1 % D-(+)-galactose) was used for shake-flask fermentations.

Vazyme (Nanjing, China) provided Phanta Max Super-Fidelity DNA Polymerase and Green Taq Mix used for polymerase chain reaction. All restriction endonucleases were purchased from New England Biolabs. Standards of retinal and retinol were purchased from Macklin (Shanghai, China). Dodecane and acetone were purchased from Kermel (Tianjin, China). BHT was purchased from Sigma (Sigma-Aldrich). The Amplite colorimetric NADP/NADPH ratio assay kit was purchased from AAT Bioquest.

2.2. Construction of plasmids and strains

All engineered yeast strains involved in this study are listed in Table 1. All plasmids used in this study are listed in Table S1. All the primers synthesised by Tsingke Biotechnology Co., Ltd. (China) are listed in Table S2. Bco from *Uncultured marine bacterium 66A03* (*Mbblh*), *Homo sapiens* (*Hsbco*), *Gallus* (*Ggbco*), *Sus scrofa* (*Ssbco*) and *Halobacterium salinarum NRC-1* (*H.NRCbrp*), *ybbO* from *E. coli*, and *RDH12* from *H. sapiens* were codon-optimised for expression in *S. cerevisiae* (Table S3) and synthesised by Tsingke Biotechnology Co., Ltd. (China). The CEN.PK2–1C genome was used to amplify the promoters, terminators, and endogenous genes through Phanta Super Fidelity DNA Polymerase. The core components in gene expression cassettes were ligated with 20 bp homologous arms of adjacent fragments through the BM seamless cloning kit (Biomed, China). The genome was modified through the CRISPR/Cas9 system. The genomic RNA target sequences were designed using CRISPOR (http://crispor.tefor.net/crispor.py) [25, 26]. All transformations were performed using the LiAc/SS carrier DNA/PEG method [27].

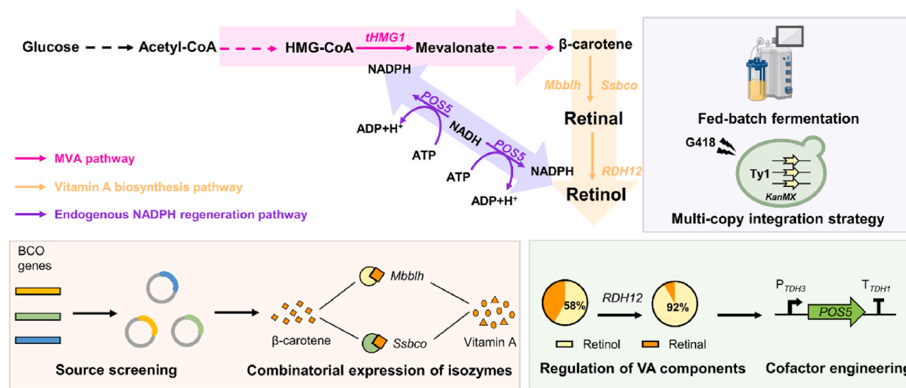

Fig. 1. Overview of vitamin A biosynthetic pathway and the systematic engineering strategies applied in this study. Acetyl-CoA, acetyl coenzyme A; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-CoA; tHmg1, truncated 3-hydroxy-3-methyl-glutaryl reductase; *Mbblh*, β-carotene 15,15′-dioxygenase from *Uncultured marine bacterium 66A03*; *Ssbco*, β-carotene 15,15′-monooxygenase from *Sus scrofa*; *RDH12*, retinol dehydrogenase from *Homo sapiens*; *POS5*, the NADH kinase.

Table 1
Strains used in this study.

Strains	Description	Source
CEN.PK2–1C	MATa, ura3-52, trp1-289, leu2-3,112, his3Δ1, MAL2-8C, SUC2	Invitrogen
SyBE_SC0014CY03	CEN.PK2–1C, Δgal1Δgal7Δgal10::nat-T$_{ERG13}$-ERG13-P$_{GAL7}$-T$_{ERG19}$-ERG19-P$_{GAL10}$-P$_{GAL1}$-ERG10-T$_{ERG10}$, Δypl062w::DR-T$_{CYC1}$-BtCrtI-P$_{GAL10}$-P$_{GAL1}$-PaCrtB-T$_{PGK1}$, trp1::TRP1_T$_{CYC1}$-BtCrtI-P$_{GAL10}$-P$_{GAL1}$-PaCrtB-T$_{PGK1}$, leu2::LEU2_T$_{TDH2}$-DR-T$_{CYC1}$-BtCrtI-P$_{GAL3}$-T$_{ACT1}$-tHMG1-P$_{GAL10}$-P$_{GAL1}$-TmCrtE-T$_{GPM1}$, his3::HIS3-T$_{ENO2}$-T$_{ACT1}$-tHMG1-P$_{GAL10}$-P$_{GAL1}$-(BTS1-ERG20)-T$_{FBA1}$, YGLCtau3::hphA-T$_{IDI1}$-IDI1-P$_{GAl7}$-T$_{PDC1}$-SaPMK-P$_{GAL10}$-P$_{GAl1}$-SaMK-T$_{HXT7}$, YMRWdelta15::UAS-P$_{GAL1}$-PaCrtY-T$_{ADH1}$-DR	This lab
yWXH01	SyBE_SC0014CY03, pRS416K-T$_{ACT1}$-P$_{GAL1}$-Mbblh-T$_{PGI1}$	This study
yWXH02	SyBE_SC0014CY03, pRS416K-T$_{ACT1}$-P$_{GAL1}$-Hsbco-T$_{PGI1}$	This study
yWXH03	SyBE_SC0014CY03, pRS416K-T$_{ACT1}$-P$_{GAL1}$-Ggbco-T$_{PGI1}$	This study
yWXH04	SyBE_SC0014CY03, pRS416K-T$_{ACT1}$-P$_{GAL1}$-H. NRCbrp-T$_{PGI1}$	This study
yWXH05	SyBE_SC0014CY03, pRS416K-T$_{ACT1}$-P$_{GAL1}$-Ssbco-T$_{PGI1}$	This study
yWXH06	SyBE_SC0014CY03, DAK2::P$_{GAL1}$-Mbblh-T$_{PGI1}$	This study
yWXH07	SyBE_SC0014CY03, DAK2::P$_{GAL1}$-Ssbco-T$_{PGI1}$	This study
yWXH08	yWXH06, TKL2::P$_{GAL1}$-Mbblh-T$_{PGI1}$	This study
yWXH09	yWXH07, TKL2::P$_{GAL1}$-Ssbco-T$_{PGI1}$	This study
yWXH10	yWXH06, TKL2::P$_{GAL1}$-Ssbco-T$_{PGI1}$	This study
yWXH11	yWXH10, pRS416K-T$_{ADH2}$-P$_{TEF1}$-SFA1-T$_{GPD}$	This study
yWXH12	yWXH10, pRS416K-T$_{ADH2}$-P$_{TEF1}$-ADH5-T$_{GPD}$	This study
yWXH13	yWXH10, pRS416K-T$_{ADH2}$-P$_{TEF1}$-ADH6-T$_{GPD}$	This study
yWXH14	yWXH10, pRS416K-T$_{ADH2}$-P$_{TEF1}$-ENV9-T$_{GPD}$	This study
yWXH15	yWXH10, pRS416K-T$_{ADH2}$-P$_{TEF1}$-RDH12-T$_{GPD}$	This study
yWXH16	yWXH10, pRS416K-T$_{ADH2}$-P$_{TEF1}$-ybbO-T$_{GPD}$	This study
yWXH17	yWXH10, HO::T$_{ADH2}$-P$_{TEF1}$-RDH12-T$_{GPD}$	This study
yWXH18	yWXH17, pRS416K-T$_{HXT7}$-P$_{TDH3}$-ZNF1-T$_{TDH1}$	This study
yWXH19	yWXH17, pRS416K-T$_{HXT7}$-P$_{TDH3}$-POS5Δ17-T$_{TDH1}$	This study
yWXH20	yWXH17, pRS416K-T$_{HXT7}$-P$_{TDH3}$-GND1-T$_{TDH1}$	This study
ySY08	yWXH17, delta22::T$_{HXT7}$-P$_{TDH3}$-POS5Δ17-T$_{TDH1}$-P$_{URA3}$-URA3-T$_{URA3}$	This study
ySY10	ySY08, Ty1::T$_{ENO2}$-P$_{GAL1}$-Mbblh-T$_{PGI1}$-P$_{pyc1}$-KanMX-T$_{TEF1}$	This study

2.3. Fermentation in shake flasks and fermenters

For shake-flask fermentation, a single colony of transformants picked from the YPD or SD agar plates was inoculated into 3 mL of YPD or SD medium for 12–16 h (30 °C, 220 rpm). Then, the primary seed was inoculated into 5 mL of the same fresh medium with an initial optical density at 600 nm (OD_{600}) of 0.2 for further cultivation until the exponential phase (30 °C, 220 rpm). The secondary seed was transferred to a 250-mL flask containing 50 mL of fresh YPDG fermentation medium with an initial OD_{600} of 0.1 (30 °C, 250 rpm). After 24 h, a Fe^{2+} solution filtered through a 0.22-μm aqueous filter membrane was added to the culture with a final concentration of 1.6 mM to enhance Bco catalytic activity. Moreover, 20 % (v/v) dodecane containing 1 % (w/v) BHT was added to the culture to capture the products. In two-phase fermentation, the vitamin A titer was calculated based on the volume of the aqueous. The whole fermentation process continued for 96 h until harvesting.

For fed-batch fermentation, seed cultures were prepared by inoculating into a 250-mL shake-flask containing 40 mL of fresh YPD medium for approximately 16 h to an OD_{600} of 8–10 (30 °C, 220 rpm), and then 5 mL of precultures were inoculated into a 1-L shake-flask containing 200 mL of YPD and subcultured for an additional 8 h to an OD_{600} of 8–10 (30 °C, 250 rpm). Then, 10 % (v/v) of seed cultures were transferred to a 1-L bioreactor (Man Sen, China) containing 400 mL of YPD medium supplemented with 10 mL of concentrated trace metal solution. The trace element solution formulations refer to previous studies [12]. The fermentation temperature was conducted at 30 °C, and pH was controlled at 5.5 by automatically feeding ammonium. The dissolved oxygen was kept at ≥30 % by adjusting the agitation speed from 300 to 1200 with an airflow rate of 2–3 vvm.

According to the employed galactose-inducible system for vitamin A biosynthesis [28], fed-batch fermentation was divided into two stages: cell growth and vitamin A production. During the first stage, 500 g/L of glucose solution was fed periodically at 2–3 g/(L·h) to keep the glucose concentration below 2 g/L after the initial 20 g/L glucose was depleted. For nitrogen source feeding, 300 g/L yeast extract solution was added to the fermenter at a rate of 1.5–2.5 g/(L·h) during the cell growth stage. In the second stage, a final concentration of 40 g/L lactose hydrolysate was added to induce vitamin A biosynthesis. Moreover, 160 mL of dodecane containing 1 % (w/v) BHT and Fe^{2+} solution were added to the culture. An appropriate volume of ethanol was fed periodically to maintain an ethanol concentration of 0–10 g/L until the end of fermentation. For fed-batch fermentation, the production of vitamin A was calculated based on the volume of the aqueous solution, considering the volume variation due to feeding and sample collection.

2.4. NADPH/NADP$^+$ ratio analysis

The NADPH/NADP$^+$ ratio was measured using the Amplite Colorimetric NADP$^+$/NADPH ratio assay kit. Samples were collected at 24 h intervals during the fermentation process. The NADPH/NADP$^+$ ratio was calculated by detecting the absorbance at 460 nm using a

microplate reader (Varioskan LUX, Thermo Scientific).

2.5. Analytical methods

Cell growth was monitored by measuring OD_{600} using a spectrophotometer (TU-1810, Beijing Puxi Instruments Co., P.R. China). After harvesting, the fermentation broth was centrifuged, and the dodecane phase was collected. Then, the organic phase was diluted 20–100 times with acetone. After filtration with a 0.22-μm organic filter membrane, the sample was ready for high-performance liquid chromatography (HPLC) analysis. The reference compounds were dissolved and diluted with acetone when preparing retinal and retinol standards.

An HPLC system (Shimadzu SPD-20A, Shimadzu) equipped with an ultraviolet detector and a BDS Hypersil C18 column (250 mm × 4.6 mm, 5 μm, Thermo Scientific) was used to analyse the retinoids. The detection method was as follows: (1) the mobile phase A was acetonitrile-water containing 2 % (v/v) formic acid (7:3 v/v); the mobile phase B was methanol-isopropanol (3:2 v/v); (2) the column temperature was 40 °C; (3) the flow rate was 1 mL/min. The signals of retinal and retinol were detected at 325 and 370 nm, respectively. The chromatographic program was as follows: 100 % phase A for 5 min, 5–10 min gradient to 10 % phase A and 90 % phase B for 15 min, 25–30 min gradient back to 100 % phase A, and hold for 10 min.

2.6. Copy number estimation

For genomic DNA extraction, cells were cultured in YPD medium until mid-log phase and extracted according to the manufacturer's protocol of the genome extraction kit (Tsingke, Beijing, China). Quantitative real-time polymerase chain reaction (qPCR) was performed on an Applied Biosystems QuantStudio 3™ Real-Time PCR System (Thermo Fisher Scientific, California, USA) by using ArtiCan^{CEO} SYBR qPCR Mix (TSE401) reagents (Tsingke, Beijing, China), and all assays were performed in triplicate. Oligonucleotide primers for qPCR are listed in Table S2. ALG9 gene on the chromosome was chosen as the reference gene. Plasmids containing fragments of the target gene were constructed for the preparation of standard curves of linear correlation between CT value and copy number. Then, the copy numbers were quantified using a

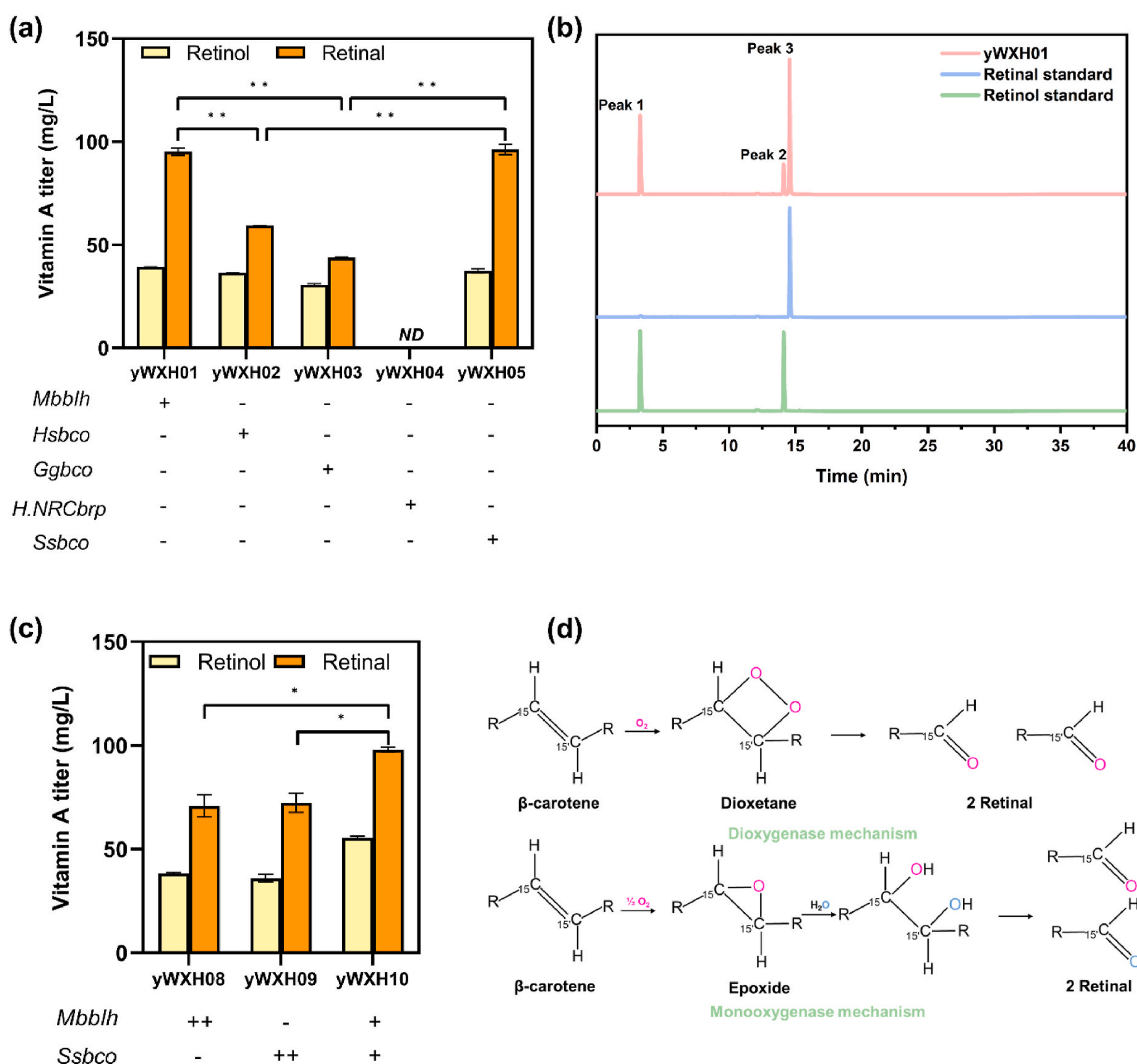

Fig. 2. Construction of vitamin A biosynthesis pathway in *S. cerevisiae*. (a) Effect of exogenous enzymes from different sources on vitamin A production. *Mb, Uncultured marine bacterium* 66A03; *Hs, Homo sapiens*; *Gg, Gallus gallus*; *H.NRC, Halobacterium salinarum NRC-1*; *Ss, Sus scrofa.* (b) HPLC analysis of standard retinal, standard retinol, and the extracts of the initial strain. Acetone (peak 1), retinol (peak 2), and retinal (peak 3) are shown in yWXH01. The retinal and retinol eluted at approximately 14.0 min and 14.5 min. (c) Increasing the copy number of Bcos in the engineered yeast. (d) Catalytic mechanisms of mono(di)oxygenase. The error bars represent the standard deviation calculated from triplicate experiments, * represents p-value <0.05, ** represents p-value <0.01, and *** represents p-value <0.001.

previously described method [29].

3. Results and discussion

3.1. Construction of the vitamin A biosynthesis pathway in S. cerevisiae

β-carotene is one of the direct precursors of vitamin A biosynthesis [30]. SyBE_SC0014CY03, a β-carotene-producing strain, was selected as the chassis cell to reconstruct the vitamin A biosynthetic pathway, which overexpressed six genes between acetyl-CoA and farnesyl pyrophosphate to provide sufficient precursors for terpenoids synthesis (Fig. S1). Thus, strain SyBE_SC0014CY03 was selected as the initial strain for further study.

The β-carotene 15,15′-oxygenase gene family was divided into two families, the Brp/Blh beta-carotene dioxygenase and carotenoid oxygenase families, according to phylogenetic tree analysis (Fig. S2). To screen β-carotene 15,15′-oxygenase with excellent performance and compatibility with our chassis, five Bco genes belonging to two families were selected for vitamin A biosynthesis, respectively, from *Uncultured marine bacterium 66A03* (*Mbblh*), *H. sapiens* (*Hsbco*), *G. gallus* (*Ggbco*), *S. scrofa* (*Ssbco*) and *H. salinarum NRC-1* (*H.NRCbrp*). The plasmid containing the BCO expression cassettes was transformed into SyBE_SC0014CY03. Strains yWXH01, yWXH02, yWXH03, and yWXH05 were able to convert β-carotene to vitamin A, which was confirmed by the retention time of HPLC chromatograms (Fig. 2a and b). *Ssbco*-expressing yWXH05 produced 37.38 ± 1.06 mg/L retinal and 96.34 ± 2.55 mg/L retinol comparable to that of the *Mbblh*-expressing strain. Therefore, the enzymes derived from *Uncultured marine bacterium 66A03* and *S. scrofa* were selected for further optimisation.

Considering the limited storage capacity of cells for lipophilic compounds like vitamin A and the potential toxicity of the products to cells, we selected the hydrophobic solvent dodecane for *in situ* extraction of vitamin A accumulated in cells. Herein, we investigated the effect of the dodecane addition ratio on vitamin A production. We found that by adjusting 50 % (v/v) dodecane to 20 % (v/v), the vitamin A production of yWXH01 and yWXH05 strains was increased to 171.1 mg/L and 163.9 mg/L, which was 27.46 % and 22.38 % higher than the control, respectively (Fig. S3). The experimental results coincide with the research of Stark's team. Stark et al. found that the organic phase extraction of PEA was an ideal way < to improve productivity, but it affected the viability of yeast to a certain extent, and the presence of the product had a synergistic inhibitory effect on yeast [31]. Therefore, we speculated that when the ratio of the organic phase is too high, the production of yeast cells may be affected by dissolved oxygen and other aspects.

3.2. Effect of isoenzymes combinatorial expression on vitamin A production

Taking the strain stability and the spontaneous loss of plasmid during passages into consideration [32], *Mbblh* and *Ssbco* genes were integrated into the chromosome of the strain SyBE_SC0014CY03 by homologous recombination and two copies of *Mbblh* or *Ssbco* were integrated as control strains simultaneously, generating strains yWXH10, yWXH08, and yWXH09, respectively. Compared to adding one copy of the same gene, the co-expression enzymes of different families were relatively more advantageous, and vitamin A production reached 153.51 ± 2.04 mg/L (Fig. 2c). Furthermore, we found that the β-carotene 15,15′-oxygenase derived from *Uncultured marine bacterium 66A03* is β-carotene 15, 15′-dioxygenase, while the β-carotene 15,15′-oxygenase derived from *S. scrofa* is β-carotene 15,15′-monooxygenase. The oxygen atom of the monooxygenase product is supplied by molecular oxygen and water, whereas the dioxygenase product is supplied by molecular oxygen, which is added to the double bond that forms a dioxetane intermediate and subsequently decays to the products (Fig. 2d) [33,34]. The two enzymes are isoenzymes catalysing the same reaction but differ in amino acid sequence, molecular mass, binding form, reaction mechanism, and substrate specificity. Therefore, considering the complementary advantages of the two enzymes to drive the production of vitamin A, yWXH10 was used as the candidate for further optimisation. Enzymatic cofactors are indispensable for conducting highly effective biocatalytic activities [35]. We found that *Mbblh* and *Ssbco* require Fe^{2+} as a cofactor. By adding different concentrations of Fe^{2+} at the beginning of the cultivation, we found that vitamin A production was maximised when 1.6 mM Fe^{2+} was added, including 106.06 ± 6.79 mg/L retinol and 76.70 ± 1.58 mg/L retinal (Fig. S4). We found that the proportion of retinol increased somewhat after adding Fe^{2+}, suggesting that yeast endogenous aldehyde reductase may also need Fe^{2+} as a cofactor.

3.3. Regulation of vitamin A components by manipulating dehydrogenases

Although there is no natural vitamin A metabolism pathway in *S. cerevisiae*, the co-production of retinal and retinol upon introducing β-carotene 15,15′-mono(di)oxygenase suggests the presence of endogenous non-specific dehydrogenases in yeast. Mo et al. [36] found that four alcohol dehydrogenases (*ADH6*, *ADH7*, *SFA1*, and *GRE2*) in *S. cerevisiae* might be involved in the conversion of retinal to retinol. Moreover, other previous studies reported that both *ADH6* and *ADH7* are NADP-dependent alcohol dehydrogenases with similar structure and functional activity [37], and *GRE2* catalyses the irreversible reduction of the cytotoxic compound methylglyoxal (MG, 2-oxopropanal) rather than retinal [38]. Therefore, we selected *ADH6*, *SFA1* and two other endogenous enzymes (*ADH5*, *ENV9*) as representatives to mine the potential retinol dehydrogenase in yeast [19]. Four yeast endogenous dehydrogenases, *SFA1*, *ADH5*, *ADH6*, and *ENV9*, were overexpressed under the control of P_{TEF1} to promote the conversion of retinal to retinol. Compared with the control retinol accounting for 58.03 % of the total vitamin A, strains yWXH11, yWXH12, yWXH13, and yWXH14, which overexpressed *SFA1*, *ADH5*, *ADH6*, and *ENV9*, reached 65.39 %, 62.25 %, 64.39 % and 75.01 %, respectively (Fig. 3a). *ENV9* is a member of the yeast endogenous short-chain dehydrogenase superfamily encoding enzymes involved in lipid droplet morphology, which plays an important role in lipid metabolism and biofilm regulation [39] and thus might have a role in fat-soluble vitamin A biosynthesis.

Vitamin A is an essential micronutrient for the human body. RDHs in retinal rod cells catalyse all-trans retinal to all-trans retinol reduction, thereby maintaining normal human retinal function [40], in which *RDH12* has been expressed in a heterologous host to regulate retinol biosynthesis [21]. Moreover, Han et al. [41] found that the deletion of the *ybbO* gene encoding a promiscuous aldehyde reductase in *E. coli* resulted in metabolic flow to retinoic acid instead of retinol, while increasing retinoic acid production by 2.4-fold, suggesting the presence of RDH activity in the *ybbO*-encoded enzyme. Yeast codon-optimised *RDH12* and *ybbO* were introduced under the control of the P_{TEF1}. The strains yWXH15 and yWXH16, which introduced the exogenous genes *RDH12* and *ybbO*, had a retinol percentage of 91.72 % and 64.3 %, respectively (Fig. 3b). The retinol titer of the strain yWXH16 reached 190.39 ± 9.51 mg/L, which was 1.9-fold higher than that of the control. The Michaelis constant (K_m) of the enzyme encoded by *RDH12* from *H. sapiens* for the reduction of retinal to retinol was lower than that for oxidising retinol to retinal (0.04 μM **vs.** 0.40 μM), suggesting that the enzyme has a higher catalytic efficiency for retinal [21].

3.4. Improvement of vitamin A production by regenerating NADPH and adding BHT

Synthesis of retinol from retinal involves a reaction that consumes NADPH to produce $NADP^+$. In addition, NADPH is required for synthesising mevalonate, the upstream precursor of vitamin A. The key genes encoding glucose-6-phosphate dehydrogenase (*ZWF1*), 6-phosphogluconate dehydrogenase (*GND1*) and the NADH kinase (*POS5*)

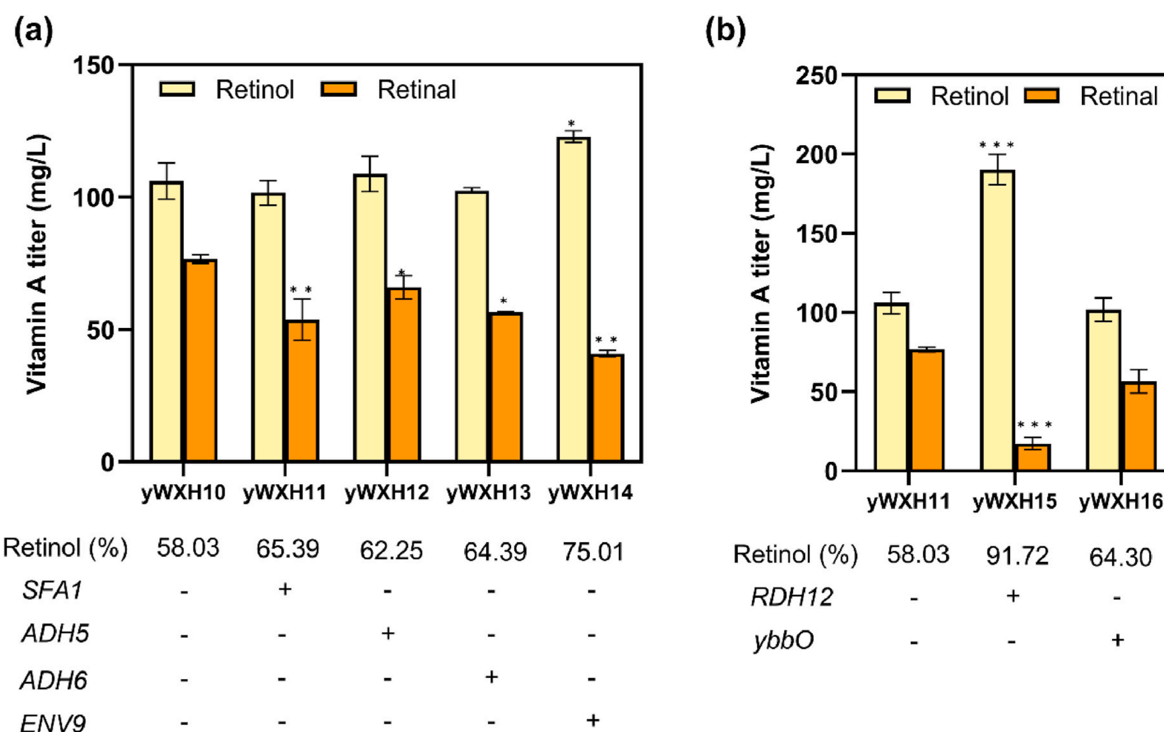

Fig. 3. Regulation of vitamin A components by manipulating dehydrogenases. (a) Effect of manipulating yeast endogenous dehydrogenases. (b) Effect of manipulating exogenous dehydrogenases. The error bars represent the standard deviation calculated from triplicate experiments, * represents p-value <0.05, ** represents p-value <0.01, and *** represents p-value <0.001.

are efficient sources of NADPH supply in yeast [42]. The NADH kinase encoded by *POS5* phosphorylates NADH to form NADPH [43]. Specifically, *POS5Δ17* (lacking the mitochondrial target signal) can efficiently regenerate the cytosolic NADPH from NADH by utilizing ATP [17,44,45]. To promote NADPH regeneration, the *GND1*, *ZWF1*, and *POS5-Δ17*genes, which were cloned into plasmid pRS416K under the control of P_{TDH3}, were transformed into yWXH17, obtaining the strains yWXH18, yWXH19, and yWXH20 respectively. Compared with the control strain, there was no significant difference in vitamin A yield between the two strains overexpressing *GND1* or *ZWF1*, whereas the titer of vitamin A was improved by almost 46 % in yWXH19, demonstrating that individual expression of *POS5Δ17* enhanced vitamin A production (Fig. 4a). Moreover, we evaluated the $NADPH/NADP^+$ ratio of engineered yeast strains. At 48 h, the strain overexpressing *POS5Δ17* had a 66 % higher $NADPH/NADP^+$ ratio than the control, which enhanced vitamin A production (Fig. 4b). At 72 h, there was no significant difference in the $NADPH/NADP^+$ ratio among the four strains mentioned above, suggesting that routine utilisation of NADPH as a reducing agent during the production and the stationary phases (Fig. S5) [46]. *POS5Δ17* overexpression enhanced the NADPH pool, which supported the fact that introducing the vitamin A biosynthetic pathway into host cells would result in an insufficient supply of NADPH cofactors.

Retinol, the key active component of vitamin A, is susceptible to oxidation or isomerisation when exposed to light, heat, and air [47]. Adding antioxidant butylated hydroxytoluene (BHT) was previously reported to be an effective method to maintain retinol stability [21,24]. To investigate the effect of the antioxidant BHT supplementation on vitamin A production, we added 0.05 % and 1 % BHT to the dodecane layer of the medium during cultivation. The titer of retinol with 0.05 % and 1 % BHT reached 317.51 ± 19.52 and 365.06 ± 0.51 mg/L, respectively, which were 13.26 % and 29.44 % higher than without BHT treatment (Fig. 4c). Therefore, we added 1 % BHT to reduce oxidative loss of retinol in subsequent experiments. Simultaneously, to achieve a stable passage of strains, we integrated the *POS5Δ17* and *Ura3* genes into the YORWdelta22 locus of yeast chromosome using Ura3 as auxotrophic markers. Retinol production was increased to 453.55 ± 11.86 mg/L from 365.06 ± 0.51 mg/L (Fig. 4d). We speculate that the further increase in retinol production was due to a more stable expression of *POS5Δ17* after it was integrated into the yeast chromosome, and the recovery of the *Ura3* gene was conducive to cell growth for maximal retinol production [48,49].

3.5. Elevation of vitamin A production through multi-copy integration *Mbblh*

The reaction catalysed by *Mbblh* to produce retinal is a rate-limiting step [17]. A similar phenomenon was observed in this study, where yWXH01 still accumulated a large amount of β-carotene after the introduction of *Mbblh*, and the conversion rate of β-carotene to vitamin A was only 30 % (Fig. S6). However, integrating critical pathway genes in single or few copies, particularly those encoding rate-limiting steps, is often insufficient to maintain high metabolic fluxes. There are various long terminal repeats in the *S. cerevisiae* genome, one of which is called the Ty retrotransposons, and the copy number is approximately 20–50, so these Ty sites are ideal multi-copy integration sites [50]. Previous studies showed that the integration of key genes at the δ-sites of retrotransposons (Ty) elements could optimize metabolic flux and promote protein overproduction [51–53].Since ySY08 is no longer an auxotrophic strain after the recovery of the *Ura3* gene, we constructed a multi-copy *Mbblh* expression cassette using the G418 resistance gene (*KanMX*) as a selection marker under the control of the weak promoter P_{pcy1} (Fig. 5a). Expressing the *KanMX* gene using a weak promoter can intensify the screening pressure to increase the copy number of the exogenous gene. To further improve retinol production, *Mbblh* expression cassettes were randomly integrated into multiple Ty1 sites of ySY08. The recombinant strains may possess different copy numbers during the integration by homologous recombination. With the increase of the screening pressure, the copy number of *KanMX* resistance genes of transformants that can survive on the plate may increase, while the copy number of exogenous genes is also increased. When the concentration of

Fig. 4. Improvement of vitamin A production by regenerating NADPH and adding BHT. (a) Production of vitamin A by overexpressing *ZWF1*, *POS5Δ17*, and *GND1* in plasmids. (b) The NADPH/NADP$^+$ ratio in the corresponding engineered yeast strain at 48 h. (c) Effect of adding BHT on vitamin A production. (d) Effect of integrating *POS5Δ17* and recovering the *Ura3* gene on vitamin A production. The error bars represent the standard deviation calculated from triplicate experiments, * represents p-value <0.05, ** represents p-value <0.01, and *** represents p-value <0.001.

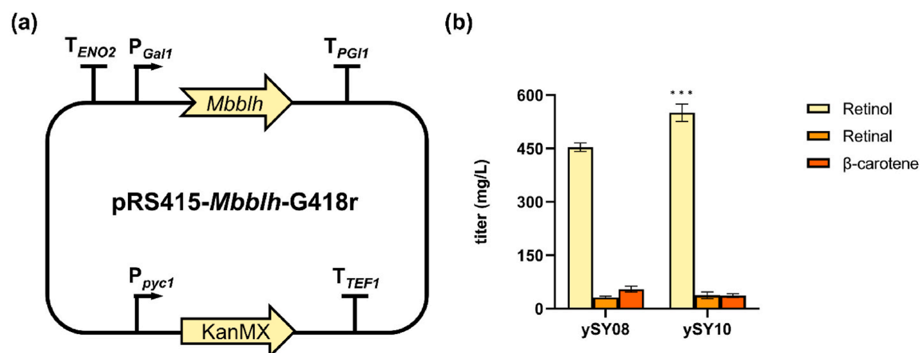

Fig. 5. Elevation of vitamin A production through multi-copy integration *Mbblh*. (a) Construction of recombinant yeast with a high copy of *Mbblh*. (b) Effect of multi-copy integration of *Mbblh* on vitamin A production. The error bars represent the standard deviation calculated from triplicate experiments, * represents p-value <0.05, ** represents p-value <0.01, and *** represents p-value <0.001.

G418 was increased to 1200 mg/L, there were only about 3–5 monoclonals on the plate. Therefore, we verified the production capacity of all transformants by fermentation and found that their yield levels were comparable, with retinol production was increased to 550.29 ± 24.44 mg/L in ySY10 (Fig. 5b). The production of retinal did not increase significantly, suggesting that the chassis possessed a better retinal reduction capacity after introducing the *RDH12* gene. To confirm that the increase in product level was correlated with gene dosage, we evaluated the single-cell copy number of *Mbblh* in the ySY10 strain (Fig. S7), and 7.45 copies of *Mbblh* was detected after multi-copy integration, which confirmed the increase in *Mbblh* copy number drives vitamin A production. We also evaluated the precursor β-carotene content of the final strain ySY10, which showed a certain decrease in titer, proving that increasing the copy number of *Mbblh* can improve the metabolic pull force and promote the efficient synthesis of the target product. Therefore, ySY10 was selected for subsequent fed-batch fermentation.

3.6. *The highest reported titer of vitamin A in yeast through fed-batch fermentation*

To evaluate the performance of the final engineered strain ySY10, fed-batch fermentation was conducted in a 1-L bioreactor (Fig. 6). After the initial glucose was exhausted in approximately 8 h, the glucose solution was fed periodically to maintain the rapid growth of the strain. At 18 h, lactose hydrolysate was injected to induce vitamin A production, while 40 % dodecane containing 1 % (w/v) BHT and 1.6 mM Fe^{2+} solution were added after induction. Then, the strain ySY10 entered the vitamin A accumulation stage and ethanol was utilised as the carbon source when the rest of the glucose was depleted. During ethanol consumption, vitamin A production was continuously increased until it reached a maximum at 67 h. A total titer of 5.21 g/L vitamin A containing 4.12 g/L retinol and 1.09 g/L retinal was achieved, the highest reported titer in eukaryote cells. The vitamin A production obtained in a 1-L bioreactor was approximately 8.86-fold higher than that in the shake-flask level. We found that the proportion of retinol dropped to 79.1 % after scale-up, while the shake flask reached 93.6 %. We speculated that one possible reason is that there was sufficient dissolved oxygen in the bioreactor compared with the shake flask, resulting in partial oxidation of retinol forming retinal [54]. Furthermore, the oxygen supply caused global changes in yeast metabolism [55], and the reaction catalysed by Bco to retinal requires oxygen participation, so sufficient oxygen promoted the overproduction of retinal, which may not be converted in yeast in time to be extracted into the dodecane. We believed that the retinol production by our engineered strain would be further improved by continuous efforts in fermentation optimisation.

4. Conclusions

In summary, a comprehensive approach consisting of a systematic metabolic engineering strategy was employed to promote vitamin A production in *S. cerevisiae.* Herein, combinational expression of two excellent β-carotene 15,15′-oxygenases *Mbblh* and *Ssbco* increased the flux from β-carotene to vitamin A metabolism. *RDH12* gene was introduced into the chassis to selectively synthesise retinol, accounting for more than 90 %. Then, tailored cofactor engineering was developed for the vitamin A biosynthesis pathway through supplementation with Fe^{2+} and NADPH. The addition of the antioxidant BHT reduced the oxidative loss of retinol, which was enriched in dodecane. Moreover, multi-copy integration of the key rate-limiting step gene β-carotene 15,15′-oxygenase further improved vitamin A synthesis. As we expected, the titer of vitamin A in the final strain harbouring the Ura3 marker was increased to 588 mg/L in shake-flask fermentation. Finally, the highest reported titer of 5.21 g/L vitamin A in *S. cerevisiae* was achieved in the 1-L fermenter.

CRediT authorship contribution statement

Yi Shi: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Shuhuan Lu:** Resources, Project administration. **Xiao Zhou:** Resources. **Xinhui Wang:** Visualization, Validation, Investigation, Formal analysis, Data curation, Conceptualization. **Chenglong Zhang:** Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Nan Wu:** Validation, Formal analysis, Conceptualization. **Tianyu Dong:** Validation, Methodology, Formal analysis, Conceptualization. **Shilong Xing:** Formal analysis. **Ying Wang:** Supervision, Resources, Project administration, Methodology, Funding acquisition. **Wenhai Xiao:** Supervision, Resources, Project administration, Methodology, Funding acquisition. **Mingdong Yao:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition.

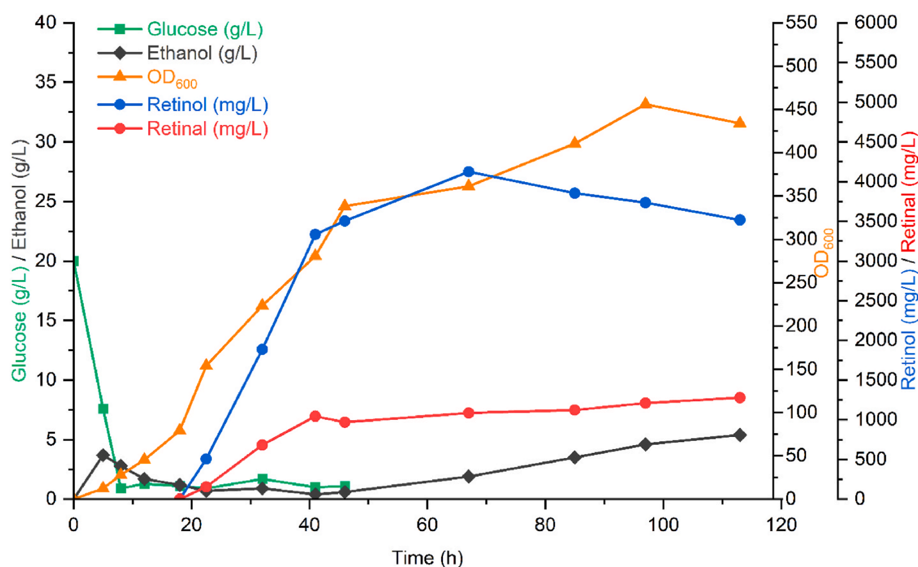

Fig. 6. Fed-batch fermentation of the final strain ySY10 in a 1-L bioreactor. The curves depict the changes in cell growth, glucose concentration, ethanol concentration, and vitamin A production during fed-batch fermentation.

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.

Acknowledgements

This work was supported by the National Key Research and Development Program (2023YFF1103701), the Key-Area Research and Development Program of Guangdong Province (2020B0303070002), the National Key Research and Development Program of China (2018YFA0900702), and the National Natural Science Foundation of China (22178261).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2024.08.004.

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