ENGINEERING YEAST TO PRODUCE PLANT NATURAL PRODUCTS

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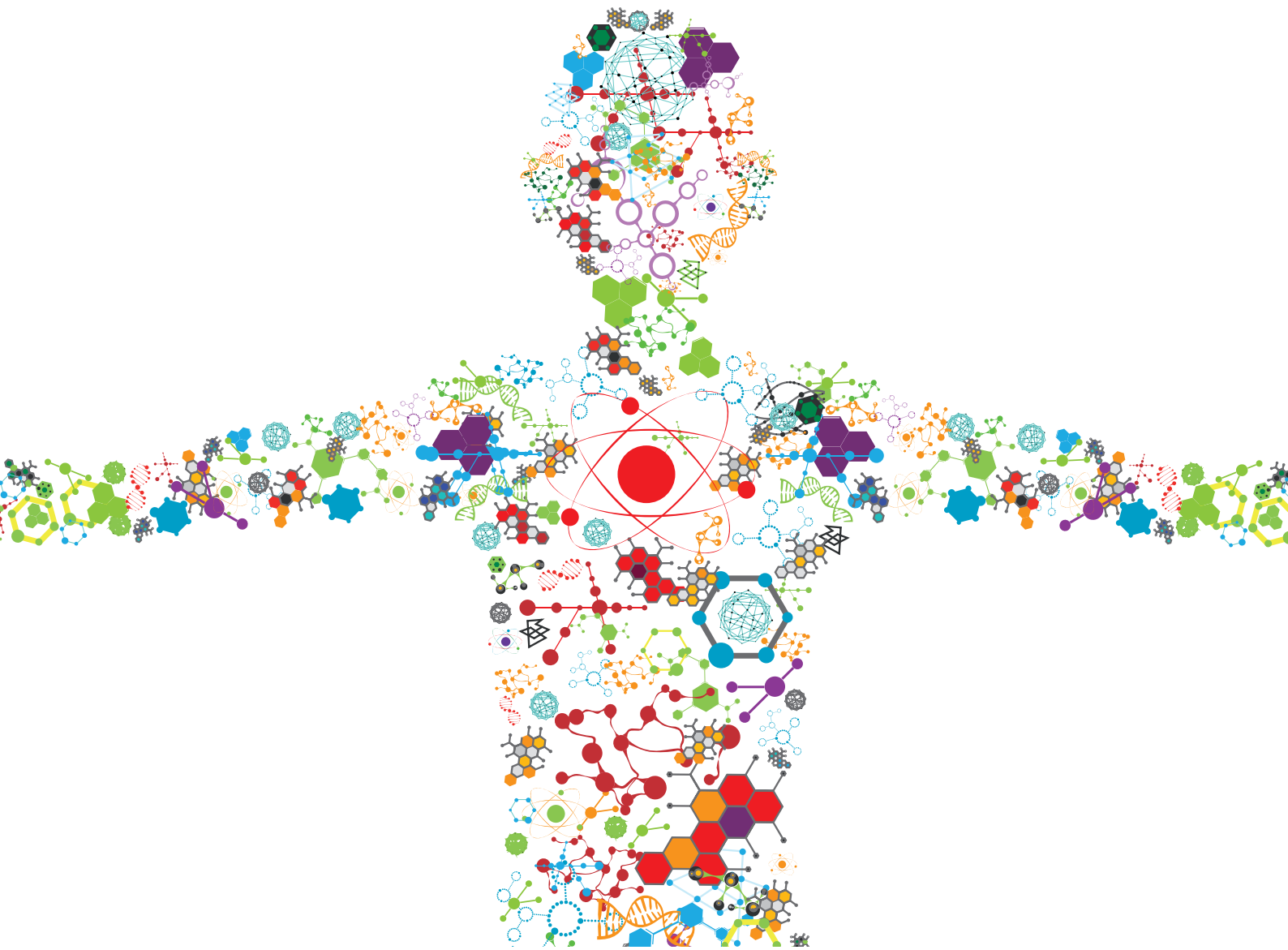

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ENGINEERING YEAST TO PRODUCE PLANT NATURAL PRODUCTS

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EDITORIAL
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Editorial: Engineering Yeast to Produce Plant Natural Products

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Keywords: synthetic biology, yeasts, plant natural products, metabolic engineering, *Saccharomyces cerevisiae*

Editorial on the Research Topic

Engineering Yeast to Produce Plant Natural Products

Plants produce diverse natural products, and some of them are drugs or drug precursors. Especially, traditional Chinese medical herbs and other medical plants have the capacity to produce a wide range of bioactive compounds. The extraction of these natural products from plants requires substantial time, land and water usage, and they often produce with low yields. In addition, production can be variable, being affected by pests or climate changes. These factors result in the limited supply of plant bioactive compounds at affordable costs. Therefore, it is necessary to develop efficient and eco-friendly alternative production strategies.

Many yeasts can be genetically manipulated, and thanks to efficient tools and strategies of molecular biology, their metabolism can be reprogrammed (Nielsen and Keasling, 2016; Ji et al., 2020). Achievements of microbial engineering, such as the production of artemisinin (Paddon et al., 2013) and ginsenosides (Wang et al., 2015), have suggested that yeasts can be ideal microbial cell factories for the synthesis of plant natural products. However, understanding the plant biosynthetic pathways and engineering yeasts for production present some bottlenecks and challenges, such as the current limitations on enzyme discovery tools and high-throughput engineering strategies.

In this research topic, the tools and strategies for yeast engineering, and their applications for the production of several plant natural products were described. The discovery and application of CRISPR/Cas systems in microorganisms is revolutionizing the strain design (Pickar-Oliver and Gersbach, 2019). In this topic, Shan et al. summarized the different optimized strategies for CRISPR/Cas systems and their applications in the construction of non-conventional yeast-based cell factories. Zhang and Shi reviewed the recent applications of transcription factor (TF) based biosensors to dynamically control the production of natural products in yeasts. The biosensors targeting to intermediates in natural product synthesis pathways (i.e., fatty acid synthesis, shikimate pathway, and mevalonate pathway) can be further implemented for improving the biosynthetic efficiency of plant natural products. Recent studies have shown that metabolic mass transfer is one of important factors to improve the heterologous production in microorganisms (Ma et al., 2021a). In relation to this, Xue et al. summarized the diverse strategies used for metabolic mass transfer during the production of plant natural products using engineered yeasts, and covered how properly refining/balancing metabolic flux with the metabolic mass transfer strategies would further enhance the biosynthesis efficiency. Tan et al. introduced their approach using efficient selection scheme for the incorporation of non-canonical amino acids into

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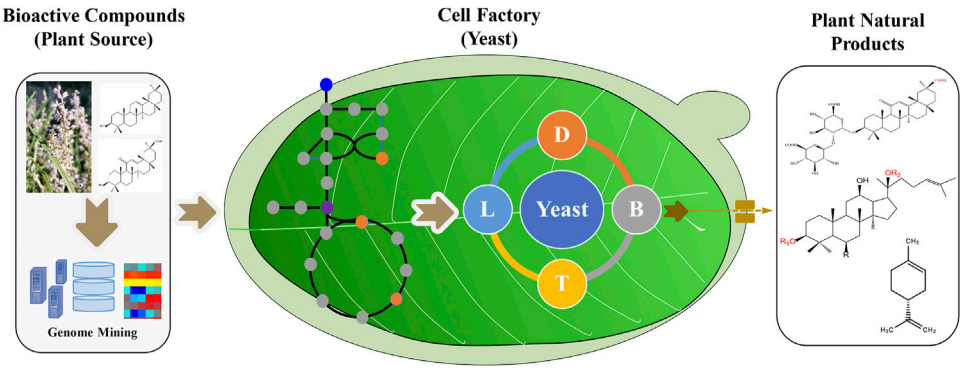

FIGURE 1 | Yeast-based production of valuable plant natural products by metabolic engineering and synthetic biology strategies. The genes can be screened from plant omics data, and the synthetic biology tools help to engineer yeasts with the recovered enzymes and metabolism. The scale up strategies will further increase the titer, yield and rate of plant natural products.

Saccharomyces cerevisiae proteins. This efficient selection scheme will expand the application of non-canonical amino acids for protein engineering in yeast cell factories.

Plant terpenoids are one of the main sources of bioactive compounds with pharmaceutical applications. Several bioactive terpenes have been produced using engineered *S. cerevisiae* and other yeasts (Yang et al., 2020; Ma et al., 2021b). Guan et al. described the discovery of glycyrrhetinic acid biosynthetic pathway and summarized the development of its biosynthesis using engineered *S. cerevisiae*, which serves as an eco-friendly example for producing compounds from traditional Chinese medical plants. Among the terpenes, monoterpenoids are usually bioactive compounds in plant essential oils. Gao et al. have reviewed the production of monoterpenoids using engineered yeasts. They especially described the application of protein engineering and structural biology strategies used to optimize key enzymes of the pathway. In the future, structural biology may enable highly efficient production of plant natural products using engineered yeasts (Cravens et al., 2019). Another application case is cocoa butter, which is the main component of chocolate. The application of yeasts for the biosynthesis of cocoa butter constituents has been achieved in the past few years, such as heterogeneously expression of cocoa lipid metabolic genes in *S. cerevisiae* (Wei et al., 2017a; Wei et al., 2017b; Wei et al., 2018). Wang et al. summarized recent development of producing cocoa butter equivalents using yeast, which might lead to the future production of yeast chocolate. RNA interference has been used for disease treatment and pest control, and yeast is one of the best hosts for the production of low-cost double-stranded RNA (dsRNA) for RNA interference applications (Zotti et al., 2018; Ahn et al., 2019). Guan et al. reviewed the advances in microbial dsRNA production systems including the use of yeast cells for their expression. Abbasi et al. updated recent advances in the production of sugar alcohols and functional sugars in *Yarrowia lipolytica*. Xylose is one of the most abundant sugars in nature and present in the side streams of lignocellulose processing (Wang et al., 2021). Zha et al. described the advances in the use of xylose as the substrate to produce natural products. Moreover, future challenges for the commercial production of natural products from xylose using engineered yeasts were discussed.

Tools and strategies to engineer yeasts for plant natural products are still in development. This research topic not only covers the synthetic biology technologies used for the production of natural products in yeast but also details several examples of valuable plant natural products produced in this host. This compendium of articles provides valuable insights for future developments. From our point of view, the integration of omics technologies, metabolic engineering, and synthetic biology strategies will accelerate the commercial production of bioactive plant compounds in the yeasts (**Figure 1**). In summary, this research topic highlights both the state of the art and the future perspectives of the biotechnological production of plant-derived natural products in yeast.

AUTHOR CONTRIBUTIONS

YW wrote the draft of the manuscript, BJ, X-JJ, TC, and RL-A revised the manuscript. All authors approved the final version of the submitted version.

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Efficient Selection Scheme for Incorporating Noncanonical Amino Acids Into Proteins in *Saccharomyces cerevisiae*

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With the advances in the field of expanded genetic code, the application of non-canonical amino acid (ncAA) is considered an effective strategy for protein engineering. However, cumbersome and complicated selection schemes limit the extensive application of this technology in *Saccharomyces cerevisiae*. To address this issue, a simplified selection scheme with confident results was developed and tested in this study. Based on a mutation library derived from *Escherichia coli* tyrosyl-tRNA synthetase (*Ec*TyrRS), a logic gate in synthetic biology was used to optimize screening procedures. We found that an "and" gate was more suitable than an "or" gate for isolating aminoacyl-tRNA synthetase from *S. cerevisiae*. The successful incorporation of *O*-methyltyrosine (OMeY) proved the utility and efficiency of this new selection scheme. After a round of positive selection, several new OMeY-tRNA synthetase (OMeYRS) mutants were screened, and their incorporation efficiency was improved. Furthermore, we characterized the insertion of several tyrosine analogs into Herceptine Fab and discovered that OMeYRS and its mutants were polyspecific. One of these mutants showed an optimal performance to incorporate different ncAAs into recombinant proteins in *S. cerevisiae*; this mutant was cloned and transfected into mammalian cells, and the results proved its functionality in HEK293 cells. This study could expand the application of ncAA in *S. cerevisiae* to construct efficient yeast cell factories for producing natural and synthetic products.

Keywords: expanded genetic code, non-canonical amino acid, protein engineering, cell factory, *Saccharomyces cerevisiae*

INTRODUCTION

Most proteins are built from 20 proteinogenic amino acids. For decades, scientists have focused on modifying proteins with ncAA via solid-phase peptide synthesis (Merrifield, 1969), expressed protein ligation (Ambrogelly et al., 2007), cell-free translation (Martin et al., 2018), and *in vivo* expression using genetic code expansion (Liu and Schultz, 2010). Genetic code expansion enables the incorporation of ncAA into target proteins at specific sites upon recognition of nonsense codons

by orthogonal aminoacyl-tRNA synthetase and its cognate tRNA (aaRS/tRNA) in living cells. Proteins with ncAA may acquire new physicochemical, biological, and pharmacological properties. Furthermore, incorporated ncAA can be used as probes to investigate protein structure or function (Liu and Schultz, 2010).

To ensure the orthogonality and fidelity, engineered aaRS must aminoacylate its cognate tRNA with the corresponding ncAA, and no endogenous tRNA or native amino acid must be recognized. Moreover, the evolved orthogonal tRNA should not be recognized by endogenous aminoacyl-tRNA synthetases carrying native amino acids (Chatterjee et al., 2012; Cervettini et al., 2020). Several orthogonal aaRS/tRNA pairs have been developed, such as *Methanococcus janaschii* tyrosyl-tRNA synthetase (*Mj*TyrRS/tRNA^{CUA}), *Escherichia coli* tyrosyl-tRNA synthetase (*Ec*TyrRS/tRNA^{CUA}), and pyrrolysyl-tRNA synthetase (PylRS/tRNA^{CUA}) from certain *Methanosarcina*. PylRS/tRNA^{CUA} enables the incorporation of lysine analogs in prokaryotes and eukaryotes. However, *Mj*TyrRS/tRNA^{CUA} is orthogonal in prokaryotes such as *E. coli* but not in eukaryotes, whereas *Ec*TyrRS/tRNA^{CUA} is orthogonal in eukaryotes rather than in prokaryotes (Chin, 2014). Even though numerous ncAAs of tyrosine analogs have been efficiently encoded in *E. coli*, only a limited set of structurally simple ncAAs can be inserted in *Saccharomyces cerevisiae* and mammalian cells. Thus, the development of an efficient evolution system for *Ec*TyrRS engineering is important.

The translation machinery of *S. cerevisiae* is highly homologous to that from other eukaryotes, including mammalian cells. Besides, due to features such as a well-defined genetic background, mature genetic manipulation, easiness to cultivate, *S. cerevisiae* is suitable for studying eukaryotic evolution of orthogonal *Ec*TyrRS/tRNA^{CUA} encoding tyrosine analogs. Site-saturated mutagenesis of *Ec*TyrRS and general selection schemes have been developed in *S. cerevisiae.* Using this method, several specific *Ec*TyrRS mutants have been successfully "cut and pasted" into mammalian cells to incorporate ncAA into proteins (Liu et al., 2007; Chatterjee et al., 2013). To establish the selection scheme, transcription factor Gal4p was used as an expression regulator of three reporter genes, *URA3*, *HIS3* and *LacZ*. Amber stop codons were introduced at two permissive sites of Gal4p. The *URA3* and *HIS3* were used for positive selection involving the suppression of the auxotrophic phenotype. The protein encoded by the *HIS3* gene is related to cell tolerance to 3-aminotriazole (3-AT) in a dose-dependent manner. Moreover, the expression of LacZ turns colonies blue in the presence of X-gal, a chromogenic substrate for β-galactosidase (Chin et al., 2003b). In addition, the expression of *URA3* leads to cell death in the presence of 5-fluoroorotic acid (5-FOA), since Ura3p converts 5-FOA into a toxic product. Thus, *URA3*/5-FOA was used as a negative selection system. In the first round of selection, cells were cultivated in the presence of target ncAA without uracil (Ura) or histidine (His) to screen active aaRS that can recognize read-through amber codons with ncAA or canonical amino acids. Surviving cells were grown in the presence of 5-FOA without ncAA to remove aaRS that could incorporate canonical amino acids. In general, three cycles of positive and negative selections yield specific aaRS that incorporate target ncAA into proteins (Chin et al., 2003a; Cropp et al., 2007). Although this selection scheme is efficient, it remains cumbersome and time-consuming (Grasso et al., 2019).

S. cerevisiae is widely used for producing bioproducts, addressing biological processes and displaying libraries of surface antibodies or enzymes (Nevoigt, 2008; Cherf and Cochran, 2015). The application of ncAA in *S. cerevisiae* is limited, due to the inefficiency of current orthogonal pairs. It was reported that the incorporation efficiency of ncAA increases significantly in *S. cerevisiae* upon optimization of tRNA expression cassette and interception of nonsense-mediated mRNA decay pathway, which plays an essential role in the rapid degradation of mRNA containing premature stop codons (Wang and Wang, 2008). However, optimization of aaRS efficiency is still relatively rare in *S. cerevisiae*. In addition, some aaRSs are polyspecific to several ncAAs in bacterial and mammalian cells (Young et al., 2011; Chatterjee et al., 2013). Thus, the availability of an efficient aaRS with polyspecificity is attractive in *S. cerevisiae* because a single construct could be used to incorporate different ncAAs.

In this study, a selection scheme of specific aaRS was optimized using the *Ec*TyrRS mutation library to incorporate OMeY. The traditional three-cycles of positive and negative selection was simplified to one round of positive selection. As a result, five novel OMeYRS mutants were selected and their charging efficiency was determined. In addition, the incorporation efficiency of several tyrosine analogs including *O*-Propargyl-L-tyrosine (OPrY), *O*-Bromoethyl-L-tyrosine (BetY), and *p*-2′-fluoroacetyl-L-phenylalanine (FpAcF) into Herceptine Fab was compared with that from OMeYRS mutants. One OMeYRS mutant was cloned and transfected into mammalian cells, and the results demonstrated that this mutant works in HEK293 cells.

MATERIALS AND METHODS

Chemical Synthesis of Non-canonical Amino Acids

Solvents and reagents used for chemical synthesis were purchased from Sigma-Aldrich, TCI and Bide Pharmatech. The 0.25 mm silica gel 60-F254 was used for analytical thin layer chromatography, using UV light treatment and vanillin or ninhydrin staining. Flash column chromatography was carried out on silica gel (particle size 200–300 mesh, purchased from Qingdao Haiyang Chemical Co.). ^{1}H and ^{13}C NMR spectra were analyzed using Bruker AVANCE III-400 spectrometers. All ncAAs were synthesized as previously described (Tang et al., 2018).

Strains and Media

Escherichia coli Trans5α was used for plasmid construction and propagation. Recombinant *E. coli* strains were grown in LB medium (5 g/L yeast extract, 10 g/L peptone, 10 g/L NaCl). *S. cerevisiae* MAV203 was used to express recombinant proteins with or without ncAA incorporation; yeasts cells were cultivated in YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose). The recombinant yeast strains expressing enhanced

green fluorescent protein (EGFP) and Fab were grown in SC-SCAA medium as previously reported (Wittrup and Benig, 1994). The strains containing aaRS libraries were cultivated, screened, and identified using selective media: SC-Leu-Trp (1.7 g/L YNB, 0.65 g/L SC-Leu-Trp-His-Ura, 5 g/L $(NH_4)_2SO_4$, 20 mg/L His, 20 mg/L Ura, 20 g/L glucose), SC-Leu-Trp-His-Ura + 3-AT + X-Gal + 10 mM OMeY (1.7 g/L YNB, 0.65 g/L SC-Leu-Trp-His-Ura, 5 g/L $(NH_4)_2SO_4$, 20 g/L glucose, 10 mM or 30 mM 3-AT, 1% X-Gal, 10 mM OMeY), SC-Leu-Trp-His-Ura + 3-AT + X-Gal and SC-Leu-Trp + TRP + 0.5% 5-FOA.

Plasmid Construction

Recombinant plasmids were constructed by Gibson assembly (New England Biolabs) using the primers listed in **Supplementary Table S1**. The gene coding for EGFP-Y39TAG was amplified from plasmid pEGFP-Y39TAG (Tang et al., 2018) and cloned into vector pYX242WS; the resulting plasmid was pYX242WS- EGFP-Y39TAG. The light chain of Fab and the signal peptide *SUC2* from yeast invertase Suc2p were cloned into pYX242WS between promoter *TPI1* and terminator *PGK1*, the recombinant plasmid was named pYX242WS-Lc. The heavy chain of Fab, the signal peptide *SUC2*, and the anchoring motif *SED1* from yeast cell wall protein Sed1p were cloned into pYX242WS-Lc between promoter *TEF1* and terminator *PolyA,* and the recombinant plasmid was named pYX242WS-Fab. Site-directed mutagenesis of pYX242WS-Fab with QuickChange method resulted in pYX242WS-Fab-G123TAG. Plasmids encoding an evolved aaRS/tRNA gene cassette were extracted from library. OMeYRS and its mutant B were constructed into vector pAcBac1, and the recombinant plasmids were named pAcBac1-OmeYRS and pAcBac1-B, respectively. To express recombinant proteins with incorporated ncAA, plasmids with aaRS/tRNA gene cassette and pYX242WS-EGFP-Y39TAG or pYX242WS-Fab-G123TAG were co-transformed into MAV203. HEK293T cells were co-transfected with pCMV-EGFP-Y39TAG and pAcBac1-OMeYRS or pAcBac1-B.

Library Selection Procedure

Yeast EcTyrRS mutation library for OMeYRS mutant selection was a gift from the Peter Schultz Lab at the Scripps Research. Yeast cells were cultivated in SC-Leu-Trp with 1 mM OMeY for 4 h, harvested and washed twice with 0.9% NaCl buffer. Cell pellets were suspended in 0.9% NaCl buffer, then plated onto SC-Leu-Trp-His-Ura + 10 mM 3-AT + 1% X-Gal + 10 mM OMeY and incubated at 30°C for 48–60 h. To identify OMeY synthetase variants, colonies were transferred into 96 well blocks containing 500 μL of SC-Leu-Trp and cultivated at 30°C for 24 h. Strains were plated onto SC-Leu-Trp-His-Ura + 30 mM 3-AT + 1% X-Gal + 10 mM OMeY, SC-Leu-Trp-His-Ura + 30 mM 3-AT + 1% X-Gal, and SC-Leu-Trp + TRP + 0.5% 5-FOA and grown at 30°C for 48 h.

Flow Cytometry Analysis

Strains expressing EGFP were cultivated for 24 h, collected and washed twice with phosphate-buffered saline solution (PBS), pH 7.0. Cells were suspended in sterile water to an OD_{600} of 0.5 for fluorescence activating cell sorter (FACS) analysis using a Cytoflex flow cytometer (Beckman). Strains expressing Fab were harvested after 24 h of cultivation and washed twice with PBS. Cell pellets were suspended in PBS containing 1 mg/mL of bovine serum albumin to an OD_{600} of 0.5; then anti-Fab antibody was added to 1:1000 [Alexa Fluor® 647 AffiniPure F(ab')$_2$ Fragment Goat Anti-Human IgG, Jackson 109-606-006]. After incubation at room temperature for 1 h, cells were centrifuged and washed twice with PBS, then suspended in sterile water before FACS analysis.

Identification of ncAA Incorporation Into Proteins From HEK293

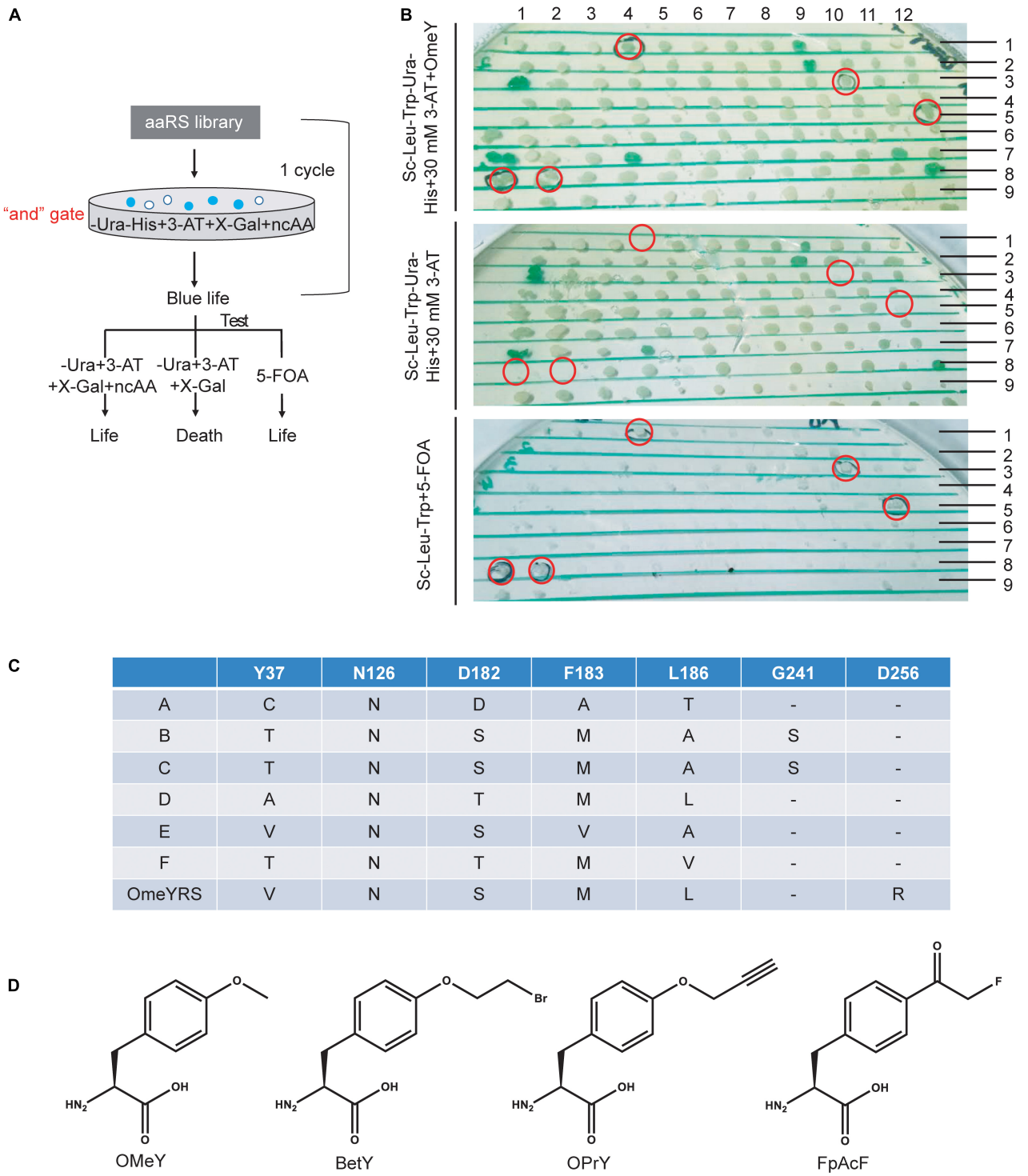
HEK293 cells were cultured in DMEM medium, supplemented with 10% FBS, and incubated at 37°C in 5% CO_2 to reach 80–90% confluency. Cells were transfected with plasmid pCMV-EGFP- Y39TAG and plasmid pAcBac1-OMeYRS or pAcBac1-B, using PEI reagent (Polysciences). After 4 h of transfection, culture medium was replaced with fresh medium with or without 0.25 mM OMeY for 48 h. Images were taken by immunofluorescence microscopy (Olympus, Japan).

RESULTS

Optimization of Screening Scheme for aaRS Selection

Screening procedures were rationally optimized for selecting specifically active aaRS. In a previous study, an "or" gate was applied for positive selection in the selection scheme (**Supplementary Figure S1A**), in which cells were cultured without Ura or without His but with 3-AT. Both cultures for the next step selection. Survived cells were collected and mixed to grow in the presence of 5-FOA without ncAA to remove aaRS that could incorporate canonical amino acids. This strategy may generate larger bias and aggravate the screening burden. Here, we changed the "or" gate to an "and" gate, which enriched blue colonies capable of growing in medium without Ura and His by adding target ncAA, 3-AT and X-gal. Cells from blue colonies were tested under three conditions as shown in **Figure 1A**.

The selection of an OMeYRS mutant from the *Ec*TyrRS mutation library for incorporating OMeY into proteins was used in this study as an example, so new ncAA incorporations can be developed based on this method. The structure of wild-type *Ec*TyrRS is shown in **Supplementary Figure S1B**. The five sites Y37, N126, D182, F183, and L186 (**Supplementary Figure S1B**, labeled in red) in the catalytic domain were chosen to perform site-saturation mutagenesis. In the selection procedure, the concentration of 3-AT was optimized to 10 mM. After one round of selection, 192 of blue colonies were picked and identified. As shown in **Figure 1B**, cells expressing active OMeYRS mutants grew well on media SC-Leu-Trp-Ura-His + 30 mM 3-AT + OMeY and SC-Leu-Trp + 5-FOA, which were used for negative selection. Conversely, these mutants did not grow on SC-Leu-Trp-Ura-His + 30 mM 3-AT. An example of presumptive colony is shown in **Figure 1B** (first row and the fourth column).



	Y37	N126	D182	F183	L186	G241	D256
A	C	N	D	A	T	-	-
B	T	N	S	M	A	S	-
C	T	N	S	M	A	S	-
D	A	N	T	M	L	-	-
E	V	N	S	V	A	-	-
F	T	N	T	M	V	-	-
OmeYRS	V	N	S	M	L	-	R

FIGURE 1 | Optimization of a ncAA selection approach *in S. cerevisiae*. **(A)** Diagram of selection scheme and experimental testing in yeast. **(B)** Identification of screened phenotypes of OMeYRS mutants. The presumptive colonies were labeled in red cycles. **(C)** Sequence information of OMeYRS mutants. **(D)** Structure of the ncAAs OMeY, BetY, OPrY, and FpAcF used in this study.

We found that approximately 4% of cells were positive, all of which were sequenced and named A, B, C, D, E, F (**Figure 1C**). The OMeYRS reported previously was used as control (Chin et al., 2003a). However, G241 of B and C mutated to serine, which was not contemplated in the original design. This change could have been caused by random mutation to

improve cell adaptation to culture conditions under enhanced selection pressure.

Incorporation of OMeY Into Recombinant Proteins by OMeYRS Mutants

The site-specific insertion of OMeY was verified to demonstrate that the observed phenotypes were due to the five new orthogonal OMeYRS mutants. EGFP response to the UAG condon at position 39 was used as a reporter. As shown in **Figure 2**, control strain with empty plasmids cultured with or without OMeY did not produce fluorescence signal, whereas the strain expressing OMeYRS showed fluorescence only in the presence of OMeY, revealing that EGFP containing an amber codon could be used for verifying ncAA incorporation. The FACS analysis of the OMeYRS mutant-expressing strains showed that 3.54%(A), 9.78% (B), 7.94% (D), 9.84% (E), and 6.50% (F) of the population positively expressed EGFP by incorporating OMeY, and that EGFP was not expressed in the absence of OMeY (**Figure 2**). These results showed that several OMeYRS mutants displayed a varied incorporation efficiency, and the strains expressing B, C and E showed a stronger fluorescence signal than that from strains expressing OMeYRS (6.66%), indicating that the expression of EGFP containing OMeY was improved. Thus, the five novel OMeYRS mutants incorporated OMeY and were orthogonal in *S. cerevisiae.*

Polyspecificity of OMeYRS and Its Mutants

EGFP expressed in cytoplasm and its folding and post-translational modification mechanisms are different for secretion proteins. Antibody libraries are often secreted on cell surface by yeast surface-display platform for antibody-antigen screening. Incorporated ncAA could endow antibodies with new and stable properties (Bidlingmaier and Liu, 2015; Liu et al., 2016). Thus, Heceptin Fab was used as a reporter protein for evaluating aaRS incorporation in *S. cerevisiae*. Yeast cell wall protein Sed1p was selected for anchoring Fab on the yeast cell surface, and the signal peptide from invertase Suc2p was used for driving secretion. Fab was detected using an antibody labeled with Alexa Fluor® 647, so red fluorescent cells were analyzed by FACS. As shown in **Supplementary Figure S2**, the strain expressing wild type Fab showed a peak shift, whereas both control strain with an empty plasmid and the strain expressing only the light chain of Fab showed a background peak. These results proved that Heceptin Fab was expressed on yeast cell surface, and was a good candidate to characterize the function of aaRS.

Furthermore, the mutant Fab with an amber codon located at complementarity-determining regions was co-expressed with each aaRS. In the presence of ncAA, mutant Fab should anchor on the cell surface as well as wild type Fab, whereas no Fab is present on the cell surface in the absence of ncAA (**Figure 3A**). As shown in **Figure 3B** and **Supplementary Figure S3**, the insertion of OMeY into Fab was achieved by OMeYRS and its mutants. The fluorescence of OMeYRS mutant-expressing strains was higher than that observed in OMeYRS-expressing strains, and the trend was similar in cells expressing EGFP with OMeY. The background fluorescence of Fab-expressing strains was slightly higher than that of EGFP-expressing strains in the absence of ncAA, and this effect could be due to the various properties of different reporters.

To identify the polyspecificity of OMeYRS and its mutants, three additional ncAAs, OPrY, FpAcF, and BetY were tested (**Figure 1D**), since they can be used for cross-linking reaction (Tang et al., 2018) and bioconjugation (Maza et al., 2016). The FACS analysis showed that the three ncAAs were inserted into Fab by OMeYRS with different incorporation efficiencies. BetY had the highest incorporation efficiency among the three ncAAs, whereas no expression was observed without the addition of ncAA. Strains expressing OMeYRS mutants also showed distinguishable fluorescence peaks upon addition of ncAAs, whereas no sharp peaks were observed without the addition of ncAA, revealing that these mutants charged the three additional ncAAs into Fab instead of canonical amino acids (**Figure 3B** and **Supplementary Figure S3**). Except for mutant A, all OMeYRS mutants efficiently inserted OPrY into Fab and 15.94% (B), 16.25% (D), 15.21% (E), and 15.61% (F) of the population stained positively. A (20.79%), D (16.61%), E (15.13%), and F (16.61%) were more suitable for charging FpAcF into Fab compared with OMeYRS (8.58%) and B (7.71%). For the incorporation of BetY, the surface-display efficiency of Fab in strains expressing B (29.08%) and D (25.59%) was about half than that observed in wild type Fab (53.00%, **Supplementary Figure S2**). These results indicated that OMeYRS and its mutants were polyspecific and were able to charge all four selected ncAAs with minimal background expression in the absence of ncAA. Based on these findings, we concluded that mutant D was the best candidate for inclusion into a single construct.

Verification of the Function of a New OMeYRS Mutant in Mammalian Cells

The OMeYRS construct was transfected into mammalian cells, and it worked efficiently with strict orthogonality. Thus, we sought to determine whether OMeYRS mutants could be used for incorporating ncAA into mammalian cells. OMeYRS and mutant D were used as an example and cloned into a plasmid following CMV promoter; the plasmid contained eight copies of *Bacillus stearothermophilus* $tRNA^{CUA}$ driven by H1 promoter. HEK293T cells were co-transfected with a recombinant plasmid containing OMeYRS or mutant D and a plasmid containing EGFP. The amber stop codon was assigned at the position 39 of EGFP (**Figure 4A**). Bright green fluorescence was detected in both strains expressing OMeYRS and the mutant D in the presence of OMeY (**Figure 4B**), whereas no fluorescence was observed in the absence of OMeY, suggesting that the expression of full-length EGFP was achieved by both OMeYRS and the mutant D. These results demonstrated that the novel OMeYRS mutants selected by our optimized selection scheme work well in mammalian cells.

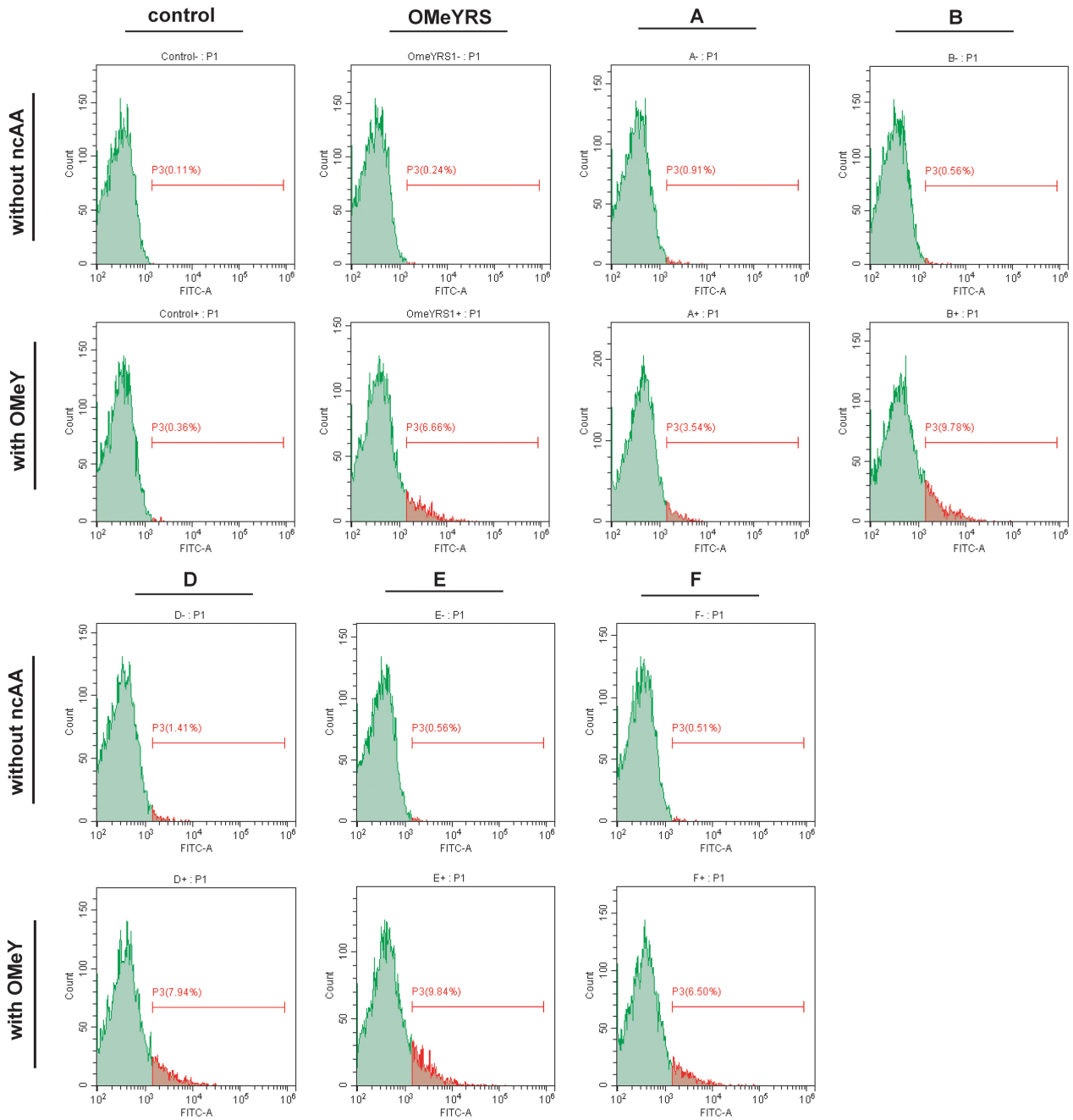


FIGURE 2 | FACS analysis of EGFP with OMeY incorporated by several OMeYRS mutants. Control represents the strain containing empty plasmids. The green peak represented the population of cells that did not express EGFP and had no fluorescence. The control without ncAA was used as background to gate and the population shown in red was gated which expressed EGFP and had fluorescence. The red number represented the proportion of fluorescent cells in total cells. Data analysis was performed using CytExpert Software.

DISCUSSION

*Ec*TyrRS was the first enzyme evolved for site-specific incorporation of tyrosine analogs into proteins in eukaryotic organisms. It has been applied for industrial-scale production of therapeutic antibodies modified by a tyrosine analog due to its high incorporation efficiency (Tian et al., 2014). Even though the selection scheme has been developed in *S. cerevisiae* with practicability and reliability for two decades, only a few structurally simple ncAAs can be incorporated, since the complex and time-consuming screening process remains as one of the limiting factors. To improve the utility of this enzyme by inserting an expanded set of ncAAs with diverse structures, we rationally designed a selection process by using

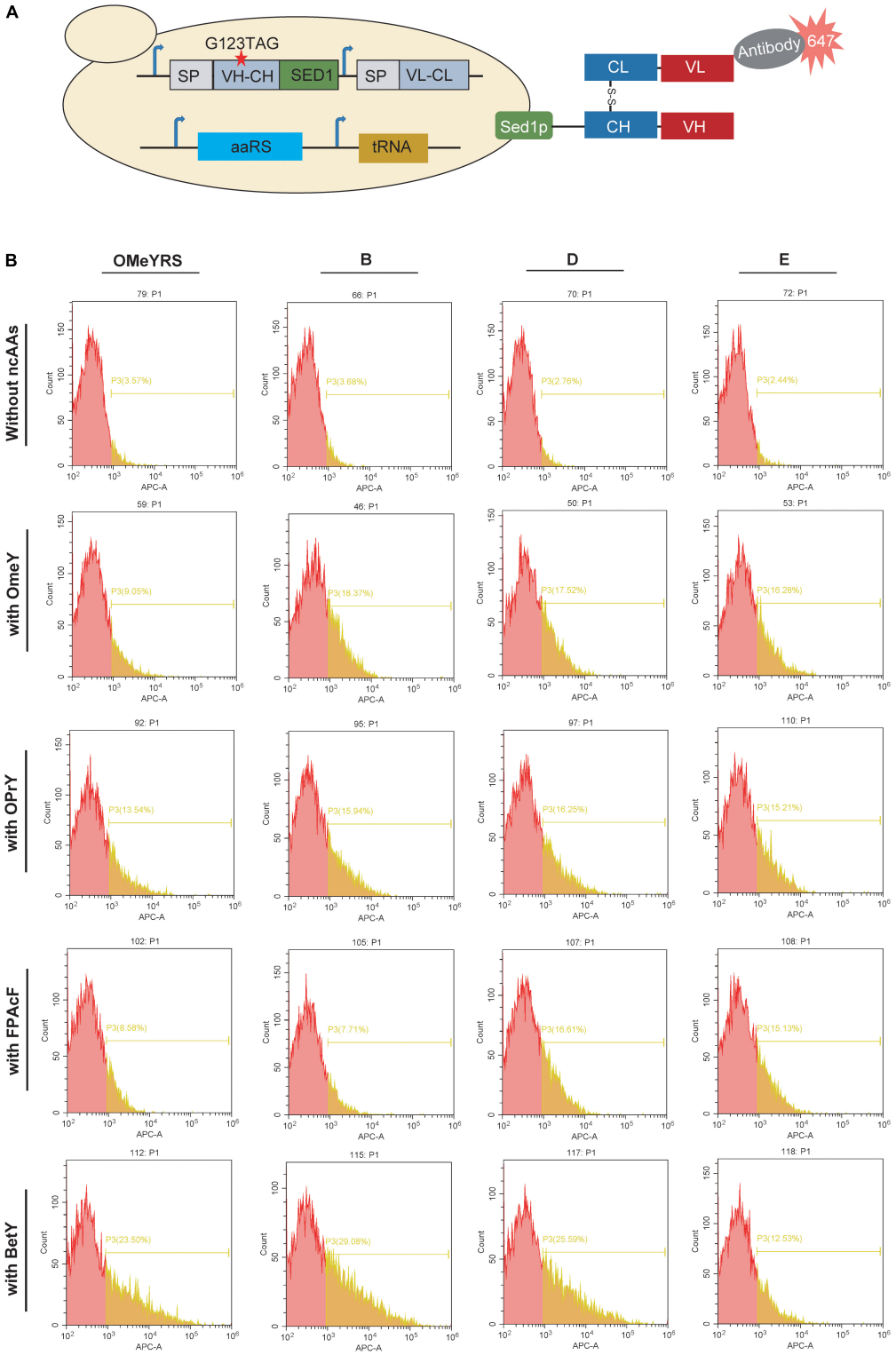


FIGURE 3 | Incorporation of several ncAAs into surface-displayed Fab. **(A)** Schematic representation of surface-display Fab and its detection. SP represented the signal peptide from yeast invertase Suc2p. VH-CH: a variable region of heavy chain (VH) and a constant region of heavy chain (CH); VL-CL: a variable region of light chain (VL) and a constant region of light chain (CL). The red star indicated the mutation site. **(B)** FACS analysis of Fab with ncAAs incorporated by several OMeYRS mutants. Strains expressing Fab were harvested, washed and suspended in PBS containing 1 mg/mL of bovine serum albumin to an OD600 of 0.5, and then anti-Fab antibody was added by 1:1000. After incubation at room temperature for 1 h, cells were centrifuged and washed for FACS analysis. The red peak represented the population of cells that did not stain positively. The yellow number represented the proportion of fluorescent cells in total cells.

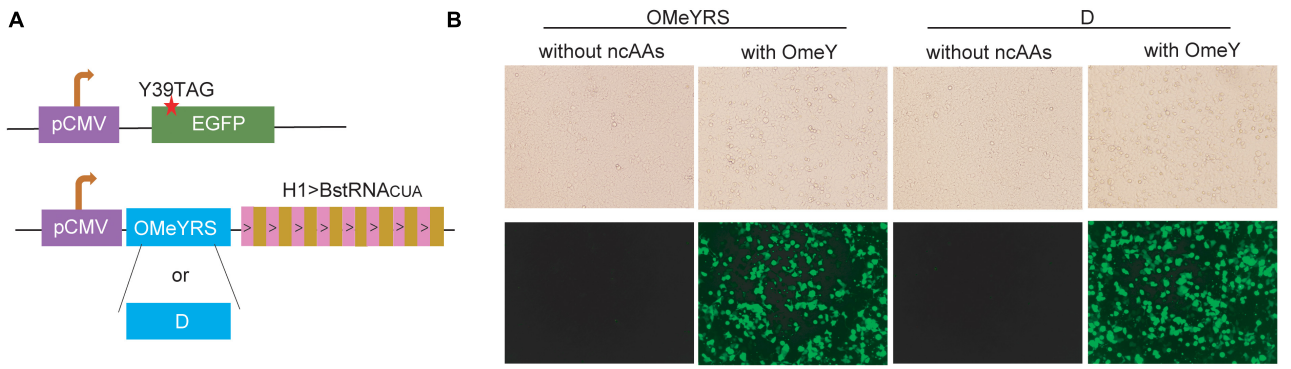


FIGURE 4 | Expression in mammalian cells of EGFP mutants containing OMeY. **(A)** Constructs of *EGFP* and aaRS/tRNA expression cassette. The red star indicated the mutation site. **(B)** Microscopy detection of green fluorescence signal of HEK293 cells transfected with the mammalian expression plasmids. Four hours after transfection, the medium was changed with or without 0.25 mM OmeY, and images were taken at 48 h.

an "and" gate to substitute the commonly used "or" gate. In this strategy, three screening standards including two auxotrophic selection conditions and one blue-white screening method were combined for positive selection, and this selection step is the enrichment process which produce few false positive clones. By adjusting the concentration of 3-AT from 30 to 10 mM, the selection could be accomplished by one round of positive selection and the grown blue clones could be directly picked for verification. Certainly, this positive selection could be combined with negative selection to reduce false positive aaRS, which utilize native amino acids instead of ncAA. The optimized selection scheme used in this study is less time-consuming and simplifies the screening process, which has the potential to be used for rapid aaRS evolution on an extended scale.

The expression of heterologous proteins in *S. cerevisiae* often results in low activity, due to the instability and non-target properties, which limits the production of bioproducts. The introduction of ncAA with specific features could assist protein evolution to produce functional proteins with improved traits, including enhanced stability, high activity, and strong regioselectivity (Jackson et al., 2006; Budisa et al., 2010; Kolev et al., 2014; Ravikumar et al., 2015). For example, the activity of prodrug activator nitroreductase mutants expressed in *E. coli* was improved significantly by incorporating multiple ncAAs, such as *p*-aminophenylalanine, naphthylalanine, *p*-nitrophenylalanine; the latter showed a 30-fold increased activity (Jackson et al., 2006). Thus, an efficient incorporation of several ncAAs would be a desired strategy for enzyme engineering to construct yeast cell factories. In *S. cerevisiae*, the incorporation of lysine analogs by pyrrolysyl-tRNA synthetase/tRNA^{CUA} has been characterized for applications in click chemistry and photocaged and photo-cross-linking reactions (Hancock et al., 2010). Although several structurally simple ncAAs of tyrosine analogs have been incorporated into proteins by *Ec*TyrRS mutants in *S. cerevisiae* (Chin et al., 2003a), their applications are yet to meet the requirements for protein engineering.

To verify the incorporation of several tyrosine analogs in *S. cerevisiae*, we identified the insertion of several ncAAs into recombinant proteins and found that OMeYRS and its mutants screened by a new selection scheme were polyspecific, which was similar to that in mammalian cells (Chatterjee et al., 2013). It is worth noticing that mutant D showed a better performance than OMeYRS for incorporating these ncAAs. Enzyme-substrate docking models suggest that Y37A and F183M mutations increased the binding pocket to accommodate larger sidechains, especially for the *para* position substitution of tyrosine analogs. The F183M mutation, existing in both OMeYRS and mutant D, maintains the hydrophobicity but provides more flexibility to adjust different groups. In addition, the docking results suggest that the substitution groups of OMeY, OPrY, BetY, and FpAcF all pointing toward Y37, which might explain why mutant D (Y37A) outperforms OMeYRS (Y37V) (**Supplementary Figure S4**). Overall, these results support our data and help to explain the substrate polyspecificity and high incorporation efficiency of mutant D. To the best of our knowledge, this is the first report showing the incorporation of OPrY, BetY, and FpAcF into recombinant proteins in *S. cerevisiae*.

CONCLUSION

This study demonstrated that an optimized selection scheme can increase the screening efficiency of aaRS for incorporating specific ncAA into proteins from *S. cerevisiae*. The traditional three-cycle of positive and negative selections was condensed into one round of positive selection, which showed an improved performance and selection efficiency, compared with those obtained by traditional selecting cycles. Several OMeYRS mutants with higher incorporation efficiency were reported using this scheme, among which mutant D was found to be functional after transfection into HEK 293T cells. Additionally, we found that OMeYRS and its mutants are polyspecific to several ncAAs of tyrosine analogs such as OPrY, BetY, and FpAcF. This is the first study addressing the incorporation of OPrY, BetY, and FpAcF into recombinant proteins in *S. cerevisiae*. We consider that this

study can be used as a strategy to extend the genetic application of ncAA in *S. cerevisiae* and mammalian cells.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

LT, TL, and HT designed the experiments. LT, ZZ, YX, WK, and ZD carried out the experiments. LT, YX, TL, and HT wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2020.569191/full#supplementary-material

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frontiers in Bioengineering and Biotechnology

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Advances in Metabolic Engineering of *Saccharomyces cerevisiae* for Cocoa Butter Equivalent Production

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Cocoa butter is extracted from cocoa beans, and it is mainly used as the raw material for the production of chocolate and cosmetics. Increased demands and insufficient cocoa plants led to a shortage of cocoa butter supply, and there is therefore much interesting in finding an alternative cocoa butter supply. However, the most valuable component of cocoa butter is rarely available in other vegetable oils. *Saccharomyces cerevisiae* is an important industrial host for production of chemicals, enzyme and pharmaceuticals. Advances in synthetical biology and metabolic engineering had enabled high-level of triacylglycerols (TAG) production in yeast, which provided possible solutions for cocoa butter equivalents (CBEs) production. Diverse engineering strategies focused on the fatty acid-producing pathway had been applied in *S. cerevisiae*, and the key enzymes determining the TAG structure were considered as the main engineering targets. Recent development in phytomics and multi-omics technologies provided clues to identify potential targeted enzymes, which are responsible for CBE production. In this review, we have summarized recent progress in identification of the key plant enzymes for CBE production, and discussed recent and future metabolic engineering and synthetic biology strategies for increased CBE production in *S. cerevisiae*.

Keywords: cocoa butter equivalents, *Saccharomyces cerevisiae*, metabolic engineering, synthetic biology, lipid biosynthesis

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INTRODUCTION

Cocoa butter (CB) is mainly extracted from cocoa beans of cocoa tree (*Theobroma cacao*), and is usually used as food flavor and cosmetics additive (Jahurul et al., 2013). With the economic development, global demands of chocolate and other CB-based products increase. As the main sources of CB, the cocoa tree can only grow in the tropical area with limited planting area, and the large-scale cocoa farming would occupy the rain forest space and thus threat the global food supplies (Clough et al., 2009). Moreover, the pest damage and infectious disease of cocoa tree might reduce CB yields (Drenth and Guest, 2016). Therefore, CB supply is quite limited, and an alternative CB supply is of interest.

CB is mainly composed of three different kinds of triacylglycerols (TAG), including 1,3-dipalmitoyl-2-oleoyl-glycerol (POP, C16:0–C18:1–C16:0), 1-palmitoyl-3-stearoyl-2-oleoyl-glycerol (POS, C16:0–C18:1–C18:0), and 1,3-distearoyl-2-oleoyl-glycerol (SOS, C18:0–C18:1–C18:0) (Lipp and Anklam, 1998; Vieira et al., 2015). SOS is the key CB flavor composition, and it is the most

valuable composition in CB (Jahurul et al., 2013). Cocoa butter equivalents (CBEs) are lipids that have similar physicochemical properties as CB, and vegetable oils are often used as CBEs (Lipp and Anklam, 1998; Sonwai et al., 2014). Though the POP and POS contents are high in some vegetable oils, such as coconut oil and palm oil, the SOS content in vegetable oils is low (Lipp and Anklam, 1998). The properties of these vegetable oils-derived CBEs normally are different with CB, since they have lower melting temperature and the mouth feeling is different with CB (de Silva Souza and Block, 2018). Shea butter and a few other tropical butters are ideal CBEs (Zeng et al., 2020), but their supply is limited due to their limited distribution in the tropical area.

Yeasts are attractive choices for industrial-scale microbial oleochemical production (Zhou et al., 2016b; Spagnuolo et al., 2019). The accumulation of TAGs is a way for carbon storage in yeast, and the main TAGs of yeast are composed of C16 and C18 fatty acids (Klug and Daum, 2014). Many yeasts are generally recognized as safe (GRAS) species and can be used in the food and cosmetic industry, enabling yeasts as potential CBE production hosts. Several different wild-type yeast species had been used for CBE production (Wei et al., 2017b), and *Saccharomyces cerevisiae* is the most widely used microbial cell factories. However, no SOS was detected in the lipidome of *S. cerevisiae* BY4741 (Ejsing et al., 2009), and the SOS content of *S. cerevisiae* CEN.PK113-7D was low (Ejsing et al., 2009). Therefore, metabolic engineering and other synthetic biology strategies need to be applied to increase CBE production of *S. cerevisiae*, especially the SOS production. Metabolic engineering of *S. cerevisiae* for CBE or SOS production requires understanding the lipid biosynthetic pathway of *S. cerevisiae* and plants.

FATTY ACID BIOSYNTHESIS IN *S. cerevisiae*

In *S. cerevisiae*, glucose is converted into Glycerol-3-phosphate (G-3-P) through glycolysis, which is the precursor of TAG backbone. Part of G-3-P is further converted into pyruvate, which is used to synthesize acetyl-CoA by pyruvate dehydrogenase complex (PDHC) in mitochondria (Krivoruchko et al., 2015). Most acetyl-CoA generated in mitochondria is consumed in the tricarboxylic acid (TCA) cycle. In the cytosol, the pyruvate dehydrogenase (PDH) bypass converts pyruvate to acetyl-CoA via three steps catalyzed by pyruvate decarboxylase (PDC), acetaldehyde dehydrogenase and two acetyl-CoA synthetase (ACS1 and ACS2) (**Figure 1A**; Zhang et al., 2018).

In an initial step, acetyl-CoA is carboxylated by the addition of CO_2 to malonyl-CoA with Acetyl-CoA carboxylase (Acc1p). Malonyl-CoA is used as building blocks, and acetyl-CoA is used as the precursor in the fatty acid biosynthesis. The fatty acid biosynthesis is catalyzed by fatty acid synthase, a multienzyme complex consisting of Fas1p and Fas2p. The fatty acid types of *S. cerevisiae* are determined by the genetic background, carbon source and other effects, which usually contain C16 and C18 fatty acids with one or none double bond (Wei et al., 2017b). The *ACC*1, *FAS1*, and *FAS2* in wild-type yeast strains were replaced with same strong constitutive promoter in order to produce fatty acid consistently (Tang et al., 2015). The fatty acyl-CoA length is decided by the elongases of Elo1p, Elo2p, and Elo3p (Tehlivets et al., 2007). Usually, Elo1p controls medium length-chain fatty acid synthesis (C12 to C16), while Elo2p and Elo3p are responsible for long-chain fatty acid synthesis (up to C26) (Rossler et al., 2003; Wenning et al., 2017). Approximately 70–80% of the total fatty acids in yeasts are monounsaturated fatty acid in a wide range of cultivation conditions, which are synthesized from saturated fatty acyl-CoA precursors by the △9-fatty acid desaturase of Ole1p (Martin et al., 2007).

TAG BIOSYNTHESIS IN *S. cerevisiae*

Phosphatidic acid (PA) is a vital component in the acylglycerol lipid metabolism. PA is synthesized by two different pathways, the G-3-P pathway and the dihydroxyacetone phosphate (DHAP) pathways (**Figure 1A**). The conversion between G-3-P and DHAP can reversely be catalyzed by glycerol-3-phosphate dehydrogenase (Gpd1p and Gut2p). The acyl-CoA is transferred to the *sn*-1 position of glycerol-3-phosphate (G-3-P) to form 1-acyl-G-3-P (LPA) which is mainly catalyzed with the enzyme of acyl-CoA:glycerol-*sn*-3-phosphate acyl-transferase (GPAT, Sct1p, and Gpt2p) or to DHAP which is catalyzed with acyl-CoA:DHAP acyltransferase (DHAPAT). G-3-P pathway is the main pathway for lysophosphatidic Acid (LPA) formation in *S. cerevisiae* (Klug and Daum, 2014; Fakas, 2017). Subsequently, an acyl chain was added to the *sn*-2 position by lysophophatidate acyl-transferase (LPAT, Slc1p, Ale1p, or Loa1p) to yield PA. PA can either be dephosphorylated to diacylglycerol (DAG) by phosphatidic acid phosphatase (Pah1p), or converts to phospholipids by Phosphatidate cytidylyltransferase (Cds1p). Phospholipids can further convert to TAGs via the acyltransferase Lro1p. DAG is the substrate for TAG synthesis by acyl-CoA:diacylglycerol acyl-transferase (DGAT) of Dga1p (Coleman and Lee, 2004; de Kroon et al., 2013). Besides, PA can also be used for other lipids synthesis, such as phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylinositol (PI) (de Kroon et al., 2013). TAG can be degraded to free fatty acids (FFA) with triacylglycerol lipase (Tgl3p, Tgl4p, and Tgl5p). The FFA is further converted to acetyl-CoA via Faa1p, Faa4p, Pox1p and other enzymes in peroxisome (**Figure 1A**).

LIPID BIOSYNTHESIS IN PLANT

The *de novo* lipid biosynthetic pathway of plant and *S. cerevisiae* is different (**Figure 1**; Bates et al., 2013; Fakas, 2017). In plant, the major biochemical reactions for TAG biosynthesis includes plastid fatty acid synthesis step, acyl editing step and TAG synthesis step, which mainly occur in plastid, mitochondria and endoplasmic reticulum (ER). The precursors for plant fatty acid synthesis are Acetyl-CoA and malonyl-ACP, which are converted to fatty acids-ACP through catalysis by ketoacyl-ACP synthase (KAS) and several other enzymes in plastid and mitochondria (Baud and Lepiniec, 2010; Bates et al., 2013). The fatty acid ACP are further converted to fatty acids via ACP thioesterase (FATA

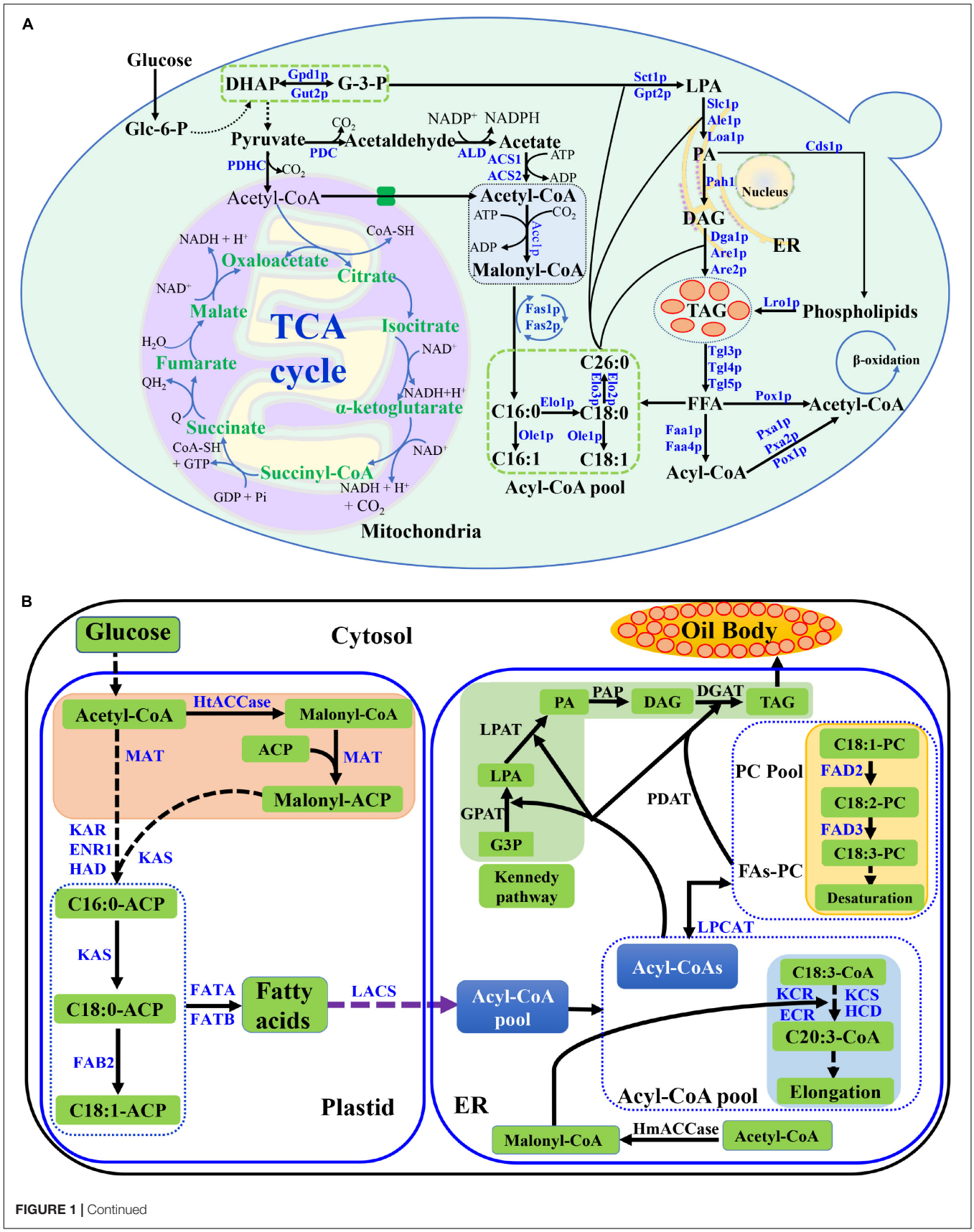

FIGURE 1 | Continued

FIGURE 1 | Lipid metabolic pathway in *S. cerevisiae* and lipid biosynthetic pathway in plant cell. **(A)** Lipid metabolic pathway in *S. cerevisiae*, including the biosynthesis and degradation, was described. The key enzymes were listed. Abbreviations: Glc-6-p, glucose 6 phosphate; DHAP, dihydroxyacetone phosphate; G-3-P, glyceraldehyde 3-phosphate; LPA, lysophophatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; TAG, triacylglycerols; FFA, free fatty acid; TCA cycle, tricarboxylic acid cycle; PDHC, pyruvate dehydrogenase complex; PDC, pyruvate decarboxylase; ALD, acetaldehyde dehydrogenase; ACS, acetyl-CoA synthetase; ER, endoplasmic reticulum; **(B)** Lipid biosynthetic pathway in plant cell. Abbreviations: HtACCase, heteromeric acetyl-CoA carboxylase; ACP, acyl carrier protein; MAT, plastidial malonyl-CoA-ACP malonyltransferase; KAS, ketoacyl-ACP synthase; KAR, plastidial ketoacyl-ACP reductase; HAD, plastidial hydroxyacyl-ACP dehydrase; ENR1, plastidial enoyl-ACP reductase; FAB2, stearoyl-ACP desaturase; FATA, acyl-ACP thioesterase; FATB, acyl-ACP thioesterase; LACS, long-chain acyl-CoA synthetase; FAD2, ER oleate desaturase; FAD3, ER linoleate desaturase; KCS, β-Ketoacyl-CoA synthase; KCR, ketoacyl-CoA reductase; ECR, enoyl-CoA reductase; HmACCase, homomeric acetyl-CoA carboxylase; LPCAT, lysophosphatidylcholine acyltransferase; PDAT, phospholipid:diacylglycerol acyltransferase; GPAT, glycerol-3-phosphate acyltransferase; LPAT, lysophosphatidic acid acyltransferase; PAP, phosphatidic acid phosphatase; DGAT, acyl-CoA:diacylglycerol acyltransferase; G3P, glycerol-3-phosphate. Dashed arrows indicate multiple steps, and solid lines indicate a single step in the metabolic pathway.

and FATB) (Baud and Lepiniec, 2010; Argout et al., 2011). The long-chain acyl-CoA synthetase catalyzes fatty acids to acyl-CoA in ER. The acyl-CoA are further elongated or desaturated with several enzymes. The final TAG products are formed via Kennedy pathway, and the main enzymes are GPAT, LPAT, PAP, and DGAT in ER (**Figure 1B**). The TAGs are stored in plant oil body.

ENGINEERING OF SOS PRODUCTION IN YEAST

The C18 content are lower than C16 content in *S. cerevisiae* (Ejsing et al., 2009). SOS is composed with one glycerol backbone, two steric acids (C18:0), and one oleic acid (C18:1). In order to increase CBE production in *S. cerevisiae*, the current efforts focused on: (1) increasing fatty acids especially C18 compositions via directing metabolic flux toward lipid synthesis; (2) overexpression of acyl-CoA transferases specially for CBE synthesis.

The Acetyl-CoA carboxylase Acc1p can convert acetyl-CoA to malonyl-CoA, and the *ACC1* expression level affects fatty acid composition in *S. cerevisiae*. By overexpression of *ACC1*, *FAS1*, and *FAS2*, TAG production increased four-fold over the control strain (Runguphan and Keasling, 2014). In another study, *ACC1* variant $ACC1^{S659A\ S1157A}$ (*ACC1***) can abolish *snf1* regulation and increased fatty acid production (Shi et al., 2014). Expression of *ACC1*** can increase TAG production, especially increased C18 composition and decreased C16:1 composition (Bergenholm et al., 2018). The Elo1p is responsible for the elongation of C16 and C18 fatty acids, while Elo2p and Elo3p are responsible for very long chain fatty acids biosynthesis. The overexpression of *ELO1* increase C18:1 titer. However the TAG content didn't change. Combination of engineering *ACC1***, *OLE1* and *ELO1* significantly increases TAG content and SOS content 5.8-fold and 48-fold, respectively (Bergenholm et al., 2018).

Several yeast fatty acid production platforms for oleochemics have been established (**Supplementary Table S1**; Li et al., 2014; Runguphan and Keasling, 2014; Leber et al., 2015; Zhou et al., 2016a,b; Dai et al., 2018; Ferreira et al., 2018a). Strengthening fatty acid biosynthetic pathway and weakening the degradation pathway increased C18 compositions and decreased C16:1 composition, and the final FFA titer reached 10.4 g/L (Zhou et al., 2016b). Further relieving the side-pathway competition by harnessing yeast peroxisomes increased the production of fatty-acid-derived chemical of fatty alcohols, alkanes and olefins up to seven-fold (Zhou et al., 2016a). By simplifying the lipid metabolic network with the redirection of fatty acid metabolism and the reduction of feedback regulation, a strain with 129 mg·g DCW^{-1} free fatty acid production was achieved (Ferreira et al., 2018b). Moreover, by global rewiring of cellular metabolism and adaptive laboratory evolution, the Crabtree effect of *S. cerevisiae* can be abolished, which is help for the acetyl-CoA derived product accumulation (Dai et al., 2018). The *S. cerevisiae* strain was reprogrammed from alcoholic fermentation to lipogenesis, and the final FFA titer reached 33.4 g/L (Yu et al., 2018). Overexpression of *ACC1***, *PAH1* and DGA1, and disruption of *TGL3*, *TGL4*, *TGL5*, *ARE1*, POX1, and PXA1, lead to the final TAG accumulation of 254 mg·g DCW^{-1}, reaching 27.4% of the maximum theoretical yield in *S. Cerevisiae*, which is the highest TAG titer reported in *S. cerevisiae* (Ferreira et al., 2018a).

GPAT, LPAT, and DGAT are the key enzymes in CBE/SOS production (Maraschin et al., 2019). The identification of plant SOS biosynthetic genes and their expression in *S. cerevisiae* can enhance SOS production (Wei et al., 2017a, 2018). There are two GPAT (Gat1p and Gat2p) and one DHAPAT in *S. cerevisiae*. The double deletions of both *GAT1* (also known as *GPT2*) and *GAT2* (also known as *SCT1*) leads to yeast lethality, showing that Gat1p and Gat2p are essential in TAG synthesis (Zheng and Zou, 2001). The acyl-specificity of Gat1p is diverse, as it can use a broad range of fatty acids as substrates; while the Gat2p displayed preference toward C16 fatty acids, suggesting that Gat1p and Gat2p can't be used to synthesize large amount of SOS directly in *S. cerevisiae* (Zheng and Zou, 2001). Plants usually contains three different types of GPAT genes, which are located in the plastid, mitochondria or cytoplasm (Yang et al., 2012). Among the 10 GPAT genes of *Arabidopsis thailiana*, GPAT-4, -6 and -8 genes strongly preferred C16:0 and C18:1 ω-oxidized acyl-CoAs over other substrates, providing hints that these GPAT genes might be responsible for SOS production in plants (Yang et al., 2012).

LPAT (EC 2.3.1.51) is believed to have the highest substrate specificity. In *S. cerevisiae,* LPATs were identified to acylate LPA with a range of different acyl-CoAs, including C18:1, C22:1, and C24:0-CoA. Introducing LPAT genes from *S. cerevisiae* into *Arabidopsis* resulted in 8% to 48% increasement of very-long-chain fatty in TAGs, showing that yeast LPAT genes would not be suitable for CBE production (Zou et al., 1997). Diverse LPATs that acylate the sn-2 of LPA to form PA had been identified, and eight LPAT genes were found in cocoa genome, which can

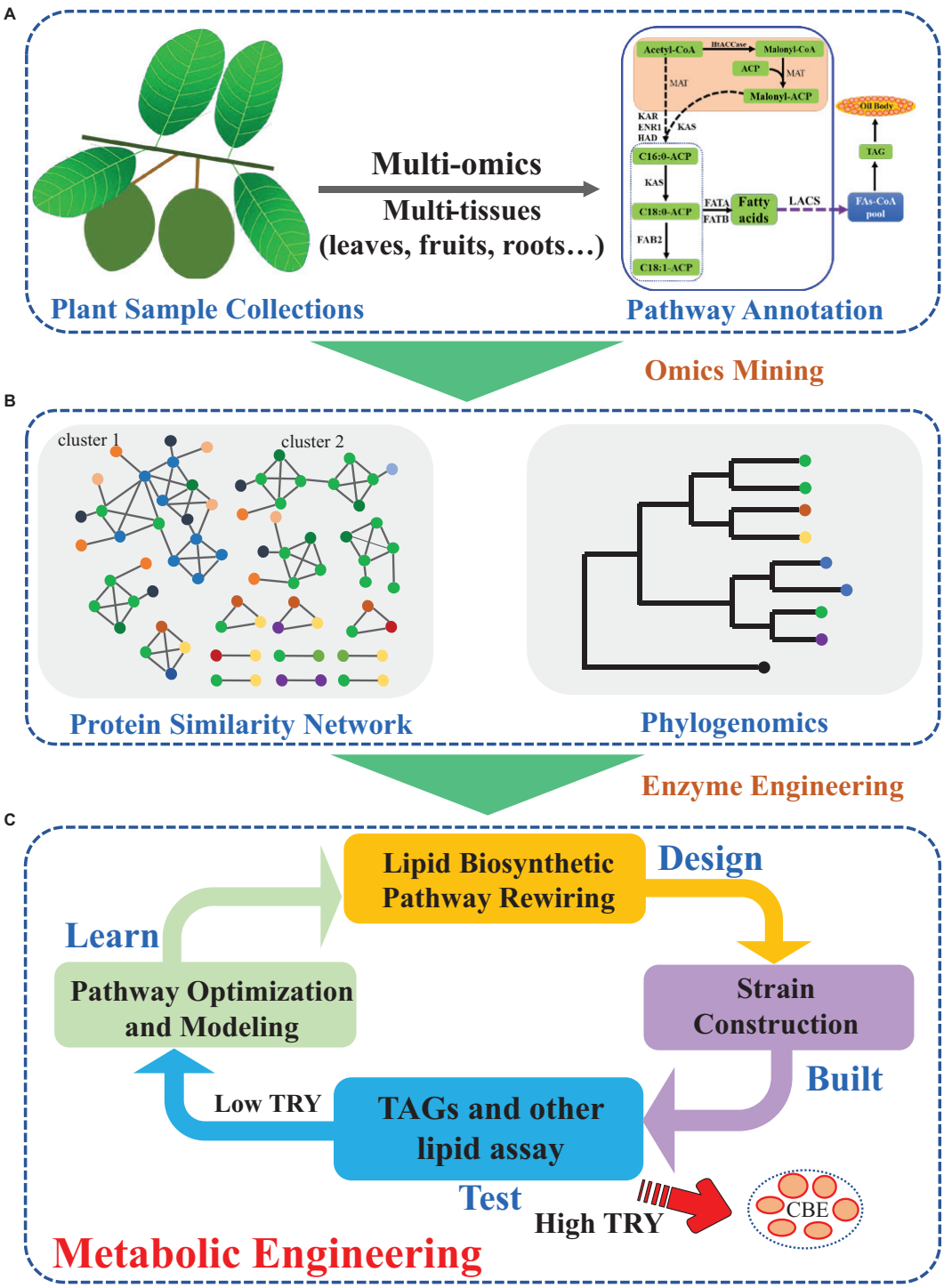

FIGURE 2 | Strategies for plant TAG biosynthetic enzyme mining and metabolic engineering for increased CBE production. **(A)** Plant sample collection and multi-omics integration analyses identified potential genes in plant lipid biosynthetic pathway; **(B)** Phylogenetic and protein similarity network analyses led to efficient enzyme selection; **(C)** Classic design-build-test-learn cycle enhanced CBE production in *S. cerevisiae* by introduction of efficient plant lipid biosynthetic enzymes.

be classified into three different clusters based on amino acid identities (Argout et al., 2011). Expression of some LPAT genes can significantly increase TAG production in *S. cerevisiae* (Wei et al., 2017a, 2018).

As previously described, two main pathways are responsible for TAG production from DAG in *S. cerevisiae*. Phospholiipid:diacylglycerol acyltransferase (PDAT, EC 2.3.1.158) uses PLs as acyl donors, and it distributes in

yeast and plants (Liu et al., 2012). DGAT catalyzes the last step of TAG biosynthesis from DAG and acyl-CoA. DGAT is an acyl-CoA dependent enzyme, which catalyzes the final and only committed step of the Kennedy pathway. DGAT is essential for TAG biosynthesis in seed. Overexpression of the yeast diacylglycerol acyltransferase (DGA1) can lead to TAG production increasement in the Δ*snf2* disruptant of *S. cerevisiae* (Kamisaka et al., 2007). Two different families of DGAT1 and DGAT2 are available in yeast, plants and animals, and DGATs from different species show diversal substrate preferences (Yen et al., 2008). For example, in *A. thaliana*, DGAT1 displays preference to C16:0, while DGAT2 displays preference to C16:1 (Aymé et al., 2014). The Overexpression of four DGAT1 genes from *Brassica napus* in *S. cerevisiae* increased TAG biosynthesis (Greer et al., 2015). Moreover, expression of *Arabidopsis* DGAT gene in yeast increased 200–600 folds of yeast DGAT activity, which can lead to 3–9 folds TAG increasement, showing DGAT might be a useful target for CBE accumulation in *S. cerevisiae* (Bouvier-Navé et al., 2000).

Global genomic and transcriptomic analyses identified thirteen potential GPATs, eight potential LPATs and two DGATs in *T. cacao* (Wei et al., 2017a, 2018). The overexpression of single or multiple cocoa genes in *S. cerevisiae* increased CBE production. The combinational expression of GPAT, LPAT, and DGAT genes showed 134-fold production of TAG over the control strain, showing these genes from cocoa have great potential for CB production (**Supplementary Table S1**; Wei et al., 2018). Shea butter contains high-level SOS, and their GPATs, LPATs, and DGATs had been identified through the transcriptomics analyses and the functional heterologous expression in *S. cerevisiae* (Wei et al., 2019). In the future, mining more specific and efficient enzymes for CBE production would help to increase CBE composition in *S. cerevisiae*.

PLANT GENE MINING AND THEIR APPLICATIONS FOR INCREASED CBE PRODUCTION

With the development of sequencing technologies, huge amounts of plant genomic or transcriptomic data had been generated (Leebens-Mack et al., 2019). The strategy to identify novel key CBE or other natural product biosynthetic genes had been extensively developed and applied (Wang et al., 2015). In general, plant samples were collected, and genome and/or transcriptome were obtained by next-generation or third generation sequencing technologies. The genome or transcriptome were assembled and annotated (**Figure 2A**). Plants usually harbor several enzymes for one biochemical reaction, therefore, quick identification of key enzymes for CBE production are essential. For CBE production, the potential genes involved in TAG biosynthesis can be identified based on the similarity or phylogeny (Wei et al., 2017a). Sequence similarity network, which clustered sequences based on pairwise similarity can be applied to classify sequences into subgroups. While phylogenetic analysis can give more direct insights into the relationships between newly identified genes and characterized genes. Based on phylogeny, potential CBE production genes can be identified via their neighboring characterized genes (**Figure 2B**). Several GPATs, LPATs, and DGATs had been identified from multi-omics data with such strategy (Wei et al., 2017a, 2018). In previous studies, cocoa GPAT, LPAT, and DGAT genes that are similar to the characterized genes or different from the known enzymes with C16 as preferred substrates were selected for experimental measurement in yeast (Wei et al., 2017a). The identified efficient/potential genes can then be used for further metabolic engineering or synthetic biology modification of *S. cerevisiae*. During the typical metabolic engineering cycle of Design-Build-Test-Learn (DBTL), lipid biosynthetic pathway can be redesigned and rewired (Nielsen and Keasling, 2016; Ko et al., 2020; **Figure 2C**). The designed pathway can be further built with advanced synthetic biology and systems biology tools to generate strains with high-level CBE production. When the TAG/CBE titer, rate and yield (TRY) of the engineered strains are high enough, they will be used for further large-scale fermentation; if the TRY are low, novel engineering strategies should be used for next round strain optimization until high TRY are obtained (Nielsen and Keasling, 2016; Ko et al., 2020).

CONCLUSION AND PERSPECTIVE

Based on the high-fatty acid production *S. cerevisiae* platform strains (Ferreira et al., 2018a; Yu et al., 2018), metabolic engineering to increase specific TAG productivity by introducing efficient enzymes of GPAT, LPAT, and DGAT would benefit CBE production. With the development of DNA sequencing technology, the whole-genome sequencing and transcriptomics of numerous plants have enabled systematic analysis of TAG and lipid production pathways in different plant species, which has provided the basis for future screening of efficient GPAT/LPAT/DGAT candidates (Cheng et al., 2018). By integrating the transcriptomics and lipidomics data, it will help to identify specific enzymes engaged in the production of targeted TAGs. Furthermore, in terms of engineering yeast, the enhancement of acetyl-CoA and malonyl-CoA pool by disruption of the PDC genes or engineering key fatty acid biosynthetic genes (*ACC1*, *FAA1*, *FAA4*, *FAS*, et al.), enhancing/balancing cofactor of NADPH supply, down-regulating completing pathways, harnessing yeast sub-organelle metabolism will increase CBE production in engineered *S. cerevisiae* (Shi et al., 2014; Zhou et al., 2016a,b; Dai et al., 2018; Yan and Pfleger, 2020). The TAG lipases encoded by *TGL3* and *TGL4* genes had been confirmed to involve in the TAG degradation (Dulermo et al., 2013). The blockage of TAG entering into the degradation pathways could also be an efficient strategy. Moreover, appropriate low-cost substrates (such as xylose and other lignocellulose components) can also be used to reduce microbial CBE production cost in the future (Hou et al., 2017). The adaptive laboratory evolution might help to increase strain adaptation to recalcitrant substrates, in order to produce high-level fatty acid-derived chemicals from recalcitrant substrates or one carbon source of CO_2/CH_4, which might be

applied in the future CBE production (Pereira et al., 2019; Cao et al., 2020; Liu et al., 2020; Zhu et al., 2020). Considering oleaginous yeasts can produce high-level lipids naturally (Wei et al., 2017b), metabolic engineering of selected oleaginous yeasts can be an alternative choice in the future (Kamineni and Shaw, 2020; Wang et al., 2020).

Biosynthesis of CBE using engineered *S. cerevisiae* is one promising way to satisfy growing CB demand. Efficient genes of GPATs, LPATs, and DGATs encoding for SOS production from oil crops can be screened using both computational and experimental approaches. The expression of these lipid biosynthetic genes in *S. cerevisiae* chasis with high-level C18:0- and C18:1-production ability would strongly increase the CBE production. Metabolic engineering and rewiring have enabled turning *S. cerevisiae* from alcoholic fermentation to lipogenesis, and further systems biology, synthetic biology, evolutionary engineering and other advanced systems metabolic engineering strategies might further increase CBE production in *S. cerevisiae*.

AUTHOR CONTRIBUTIONS

JN and YW conceived the study. MW, BJ, and YW drafted the manuscript. JN revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2020.594081/full#supplementary-material

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Metabolic Engineering for Glycyrrhetinic Acid Production in *Saccharomyces cerevisiae*

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Glycyrrhetinic acid (GA) is one of the main bioactive components of licorice, and it is widely used in traditional Chinese medicine due to its hepatoprotective, immunomodulatory, anti-inflammatory and anti-viral functions. Currently, GA is mainly extracted from the roots of cultivated licorice. However, licorice only contains low amounts of GA, and the amount of licorice that can be planted is limited. GA supplies are therefore limited and cannot meet the demands of growing markets. GA has a complex chemical structure, and its chemical synthesis is difficult, therefore, new strategies to produce large amounts of GA are needed. The development of metabolic engineering and emerging synthetic biology provide the opportunity to produce GA using microbial cell factories. In this review, current advances in the metabolic engineering of *Saccharomyces cerevisiae* for GA biosynthesis and various metabolic engineering strategies that can improve GA production are summarized. Furthermore, the advances and challenges of yeast GA production are also discussed. In summary, GA biosynthesis using metabolically engineered *S. cerevisiae* serves as one possible strategy for sustainable GA supply and reasonable use of traditional Chinese medical plants.

Keywords: glycyrrhetinic acid, *Saccharomyces cerevisiae*, metabolic engineering, microbial cell factories, natural product production

INTRODUCTION

Licorice refers to the legume plant of *Glycyrrhiza uralensis* Fisch., *Glycyrrhiza flata* Bat. or *Glycyrrhiza glabra* L., and it is widely used in traditional herbal medicine (Asl and Hosseinzadeh, 2008). Glycyrrhetinic acid (GA) is one of the main bioactive components of licorice plants and it has two optical isomers, 18 α-GA and 18 β-GA (Zhang and Ye, 2009; Li et al., 2014). Between the two isomers, the 18 α-GA isomer is present at low levels. The 18 β-GA is found at higher levels, and it is the main bioactive licorice component. 18 β-GA has a remarkably broad spectrum of biological activities, including as an anti-inflammatory agent (Kowalska and Kalinowska-Lis, 2019), an anti-tumor agent (Wang et al., 2017; Zhou et al., 2019), an immunomodulatory agent (Ayeka et al., 2016), a hepatoprotective

agent (Mahmoud and Dera, 2015), and other functional medical effects (Chen et al., 2013; Ding et al., 2020).

Currently, GA is mainly produced by the hydrolysis of glycyrrhizic acid, which is extracted from the dried root and rhizome of licorice (Mukhopadhyay and Panja, 2008; Wang C. et al., 2019). Wild licorice, however, has been severely damaged and has nearly disappeared due to excessive mining (Shu et al., 2011). Moreover, the quality of artificially cultivated licorice is variable, and the GA content in natural licorice is low. The complex structure of GA makes the chemical synthesis of GA difficult (Wang et al., 2018). The demand for GA has increased and the price of GA is high, so exploring other sustainable sources of GA is of great interest.

Introducing the biosynthetic genes of natural products into microbial chassis cells and the construction of microbial cell factories to obtain high levels of targeted natural products have become one of the most essential ways to produce plant natural products (Pyne et al., 2019). The development of synthetic biology and metabolic engineering makes the microbial biosynthesis of GA possible (Wang C. et al., 2019), and microbial cell factories provide a practical and effective way to solve the problem of sustainable production of medical plant resources (Xu et al., 2020).

Microorganisms can utilize a relatively cheap carbon source to balance cell growth and natural product synthesis (Jeandet et al., 2013; Xu et al., 2020). Normally, microorganisms have known genetic background, and there are many ways to genetically manipulate them, making them suitable for use in large-scale fermentation (Xu et al., 2020). At present, there are a variety of natural products, such as artemisinin (Paddon et al., 2013), protopanaxadiol (Dai et al., 2013), ginsenosides (Yan et al., 2014; Wang et al., 2015; Wei et al., 2015), tanshinones (Guo et al., 2016), cocoa butter (Wei et al., 2017a; Bergenholm et al., 2018; Wei et al., 2018), and cannabidiol acid (Luo et al., 2019), that have been successfully synthesized using metabolically engineered *Saccharomyces. cerevisiae*.

In this review, we summarize the current progress for GA production in *S. cerevisiae* and provide some future prospects for the field.

CHEMICAL STRUCTURE OF GA

GA is an oleanane-type triterpenoid, and it is soluble in ethanol, methanol, chloroform, ethyl acetate, pyridine and acetic acid, but is insoluble in water (Wang et al., 2018; Kowalska and Kalinowska-Lis, 2019). The chemical structure of GA is complex, hence its chemical synthesis is difficult (Wang C. et al., 2019). The hydroxy group at position C-3 and the carboxyl group at position C-30 are the main functional groups. Currently, chemical strategies are mainly used for modification of GA (Yang et al., 2020). Introduction of protected or de-protected amino acids have been shown to increase GA activity (Schwarz et al., 2014). By esterification of the C-3 and C-30 positions in GA, different GA groups can be introduced and other derivatives can be formed, and these derivatives have been shown to improve the activities of the GA-derived components (Maitraie et al., 2009; Schwarz and Csuk, 2010; Yang et al., 2020). Nowadays, the cost of chemical synthesis of GA is high, while the yield is low (Wang C. et al., 2019). Additionally, large amounts of wastes are generated during chemical synthesis of GA.

GA BIOSYNTHETIC PATHWAY

The production of a plant natural product in microorganisms requires an understanding of its biosynthetic pathways (Wei et al., 2019). Traditional pathway mining strategies are typically carried out through selection of mutant libraries, which is time-consuming and laborious. With the development of bioinformatics and computational biology, it has been possible to identify all potential biosynthetic pathways of the natural products in one species by establishing a multi-layered omics technology platform (Mochida and Shinozaki, 2011). The platform includes omics data analyses of transcripts, genomes, proteomes, metabolomes, the prediction of gene function, and the verification of key genes in the biosynthetic pathways of natural products (Nett et al., 2020).

Transcriptomic and genomic sequencing data are available for licorice (Li et al., 2010; Ramilowski et al., 2013; Mochida et al., 2017; Gao et al., 2020), and have contributed to a better understanding of natural product biosynthetic pathways in licorice plants. The GA biosynthetic pathway in licorice plants has been defined (Seki et al., 2011). GA belongs to the oleanane-type triterpenoid group, and shares the same precursor synthetic pathway with other terpenes. Both methylerythritol phosphate (MEP) and mevalonate (MVA) pathways are involved in GA precursor synthesis, but GA is mainly synthesized through the MVA pathway which can be divided into three stages (**Figure 1**).

In the first stage, acetyl-CoA is catalyzed by acetyl-CoA C-acetyltransferase (AACT), 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS), and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) to generate MVA, followed by multi-step reactions to synthesize isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP). HMGR is the first key rate-limiting enzyme in the MVA metabolic pathway, in which HMGR can catalyze the formation of MVA from HMG-CoA, which is an irreversible reaction (Friesen and Rodwell, 2004). IPP and DMAPP have been shown to be the important intermediate products of this process, and they are the precursors for terpenoids synthesis in plant cells (Wang Q. et al., 2019).

Secondly, following the catalysis of geranyl diphosphate synthase (GPPS), farnesyl diphosphate synthase (FPPS), squalene synthase (SQS) and squalene epoxidase (SQE), IPP is used to synthesize 2,3-oxidosqualе. 2,3-oxidosqualе is a common precursor for some natural product biosynthesis, such as sterols and triterpenes. SQS synthesize squalene using farnesyl diphosphate (FPP) as substrate, and it has been identified to be a key enzyme which can direct carbon flow to triterpene biosynthesis (Seo et al., 2005).

2,3-oxidosqualе is catalyzed by β-amyrin synthease (bAS) to generate β-amyrin, which is an important reaction step in GA biosynthesis. Following this, the licorice enzyme CYP88D6 catalyzes the hydroxylation of the 11-position of β-amyrin,

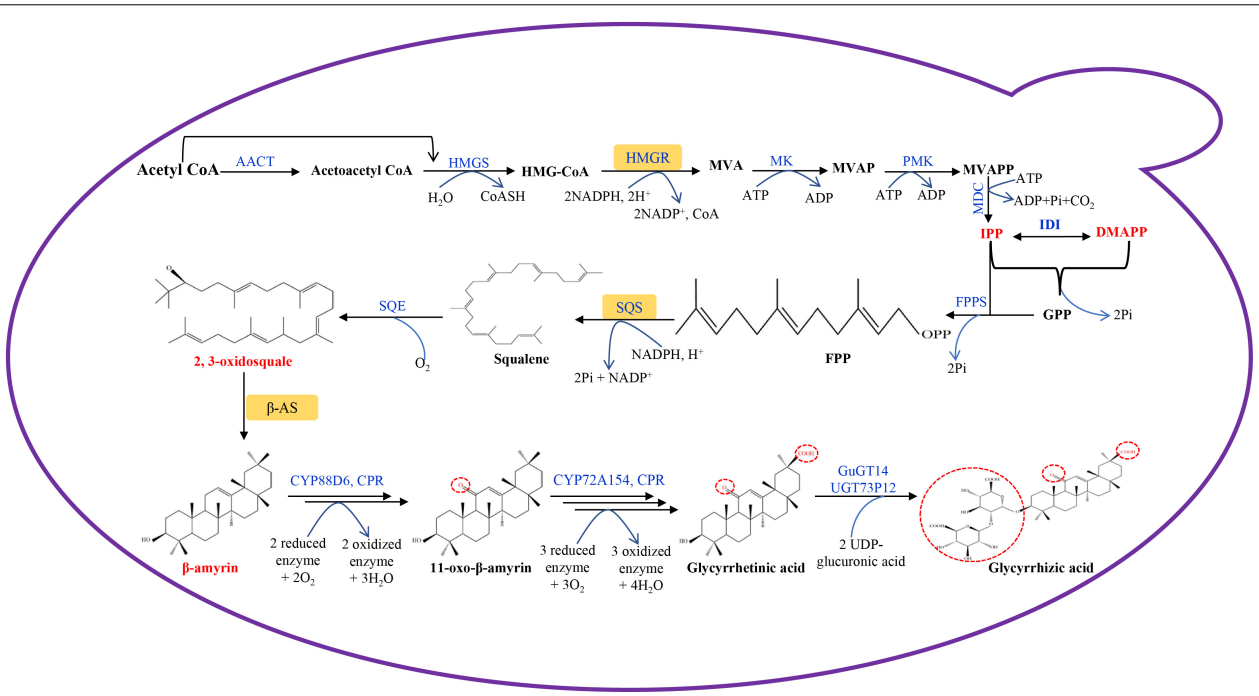

FIGURE 1 | Glycyrrhetinic acid biosynthetic pathway designed in engineered *S. cerevisiae*. AACT, acetyl-CoA C-acetyltransferase; HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; MK, mevalonate kinase; PMK, phosphomevalonate kinase; MDC, mevalonate diphosphate decarboxylase; GPP, geranyl diphosphate; GPPS, geranyl diphosphate synthase; IDI, isopentenyl-pyrophosphate delta isomerase; FPP, farnesyl diphosphate; FPPS, farnesyl diphosphate synthase; SQS, squalene synthase; SQE, squalene epoxidase; β-AS, β-amyrin synthase; CPR, cytochrome P450 reductase; GuGUAT, *Glycyrrhiza uralensis* UDP-dependent glucuronosyltransferases; MVA, mevalonic acid; MVAP, mevalonate phosphate; MVAPP, mevalonate diphosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate.

followed by further oxidation to 11-oxo-β-amyrin (Seki et al., 2008). Licorice CYP72A154 further catalyzes a three-step reaction. The C-30 position of 11-oxo-β-amyrin is firstly hydroxylated, then the product has been shown to be oxidized to form an aldehyde, and finally it is again oxidized to form the carboxyl group of GA (Seki et al., 2011). During this process, NADPH-cytochrome P450 reductase (CPR) is the main electron donor to cytochrome P450 (CYP450). The electrons are transferred to CYP450, followed by a redox reaction with the substrate (Zhu et al., 2018). The electron transfer reaction between CPR and CYP450 is the rate-limiting step of the CYP450 redox reaction, which affects GA synthetic efficiency (Zhu et al., 2018). GA is converted to glycyrrhizic acid by the UDP-dependent glucuronosyltransferases of GuGT14 and UGT73P12 (Chen et al., 2019; Nomura et al., 2019).

METABOLIC ENGINEERING OF *S. cerevisiae* FOR GA PRODUCTION

S. cerevisiae is one of the most commonly used model chassis, in particular grows rapidly on low-cost substrates and genetic manipulation is easy. Besides, the MVA pathway in *S. cerevisiae* can provide precursors like IPP and DMAPP for terpene synthesis. Many natural products have been successfully synthesized in *S. cerevisiae*. After a series genetic modification to enhance the MVA pathway in *S. cerevisiae*, the production of artemisinin can reach 300 mg/L. Further optimization of the metabolic network and fermentation conditions increased the titer to 25 g/L (Paddon et al., 2013). The annual artemisinin output in a 100 m^3 fermentation bioreactor is 35 tons, which is equivalent to the output of artemisinin of 50,000 mu in *Artemisia annua*, showing that yeast cell factories can provide large amounts of natural products in a short time and with limited space.

By introducing more than 20 genes from plants, bacteria or rodents into *S. cerevisiae*, an artificial *S. cerevisiae* cell factory that can convert sugars into opioid alkaloids has been successfully constructed, representing a breakthrough in the biosynthesis and manufacturing of opioid alkaloids (Galanie et al., 2015). Additionally, the complete microbial synthesis of major cannabinoids and their derivatives, including cannabigerol acid (CBGA), tetrahydrocannabinolic acid (THCA) and cannabidiol acid (CBDA), in *S. cerevisiae* cell factories has been achieved (Luo et al., 2019). The synthesis of GA requires the MVA pathway to provides precursor substances. *S. cerevisiae* can be used as an advantageous chassis cell to supply substrates for GA synthesis, and various metabolic engineering strategies had been used for GA production in *S. cerevisiae*.

β-amyrin serves as the oleanane-type triterpenoid precursor to various downstream products, and increasing β-amyrin synthesis in *S. cerevisiae* is essential for GA synthesis. The *AaBAS* gene

cloned from *A. annua* was introduced into *S. cerevisiae*, which altered the gene expression level of HMGR and lanosterol synthase gene (ERG7), increasing β-amyrin production in *S. cerevisiae* by 50% to 6 mg/L (**Supplementary Table 1**; Kirby et al., 2008). This result shows that triterpenes can be produced in *S. cerevisiae*, and a heterologous gene from another plant could increase β-amyrin production. Subsequently, Dai et al. (2015) integrated two different *bAS* genes derived from *Glycyrrhiza glabra* and *Panax ginseng*, and another copy of native yeast SQS and SQE genes in yeast. Following incubation in YPD medium with 2% glucose for 7 days, Gas chromatography–mass spectrometry (GC/MS) analysis showed that the β-amyrin production yield was 107 mg/L with a yield of 9.3 mg/g dry cell weight (DCW) (**Supplementary Table 1**; Dai et al., 2015). These studies indicated that overexpression of key genes in the GA biosynthetic pathway could increase precursor production.

A combination of stepwise rational refactoring and directed flux regulation strategies can improve β-amyrin production. Introduction of the *bAS* gene from *Glycyrrhiza glabra* and the heterologous squalene monooxygenase genes from *Candida albicans* into *S. cerevisiae*, combined with overexpression of isopentenyl pyrophosphate isomerase (IPI), FPPS and SQS genes, increased squalene production. UPC2 is a global transcription factor regulating sterol synthesis in *S. cerevisiae*, and modifying the UPC2 binding site directed metabolic flux for β-amyrin biosynthesis. After a series of genetic modifications and suitable ethanol fed-batch fermentation, the final titer of β-amyrin in *S. cerevisiae* reached 138.80 mg/L (Zhang et al., 2015). This suggested that pathway optimization and flux refactoring can improve natural product synthesis in engineered yeast. Liu et al. introduced an optimal acetyl-CoA pathway and deleted an acetyl-CoA competing pathway in *S. cerevisiae*, balancing various factors that greatly reduced energy consumption and glucose utilization. The final β-amyrin production was found to be 279.0 ± 13.0 mg/L, which is the highest β-amyrin production ever reported (**Supplementary Table 1**; Liu et al., 2019). This study suggested that a global analysis between precursor supply and product formation, rather than analysis of one key gene, could efficiently improve natural product biosynthesis in engineered microorganisms. In the GA biosynthesis pathway, β-amyrin is further catalyzed by CYP88D6 and CYP72A154 to synthesize GA (**Supplementary Table 1**; Seki et al., 2008, 2011).

Seki et al. (2011) also confirmed the key roles played by CYP450 genes in the GA biosynthesis pathway by introducing bAS, CYP88DE, CYP72A154, and CPR into wild-type *S. cerevisiae*, thus redirecting the metabolic flow to GA synthesis. This eventually led to 11-oxo-β-amyrin production levels of 76 μg/L and GA production amounts of 15 μg/L (**Supplementary Table 1**; Seki et al., 2011). These results indicated the feasibility of GA synthesis in yeast using synthetic biology. It is possible to improve GA production by enhancing the catalytic step in which CYP72A154 converts 11-oxo-β-amyrin to GA. Zhu et al. further balanced oxidation and reduction systems by introducing two novel CYP450 genes, Uni25647 and CYP72A63, and pairing the new cytochrome P450 reductases GuCPR1 from *Glycyrrhiza uralensis*. With the optimized oxidation and reduction modules, as well as scale-up fed-batch fermentation, 11-oxo-β-amyrin and GA synthesis reached 108.1 ± 4.6 mg/L and 18.9 ± 2.0 mg/L, respectively (Zhu et al., 2018). These are the highest titers ever reported for GA and 11-oxo-β-amyrin synthesized from *S. cerevisiae*. This work further showed that redox balance mediated by the CYP450 and CPR genes was important for GA synthesis. Wang et al. directed the upstream carbon flux flow from acetyl-CoA to 2,3-oxidosqualene by introducing a newly discovered gene, cytochrome b5, from *Glycyrrhiza uralensis* (GuCYB5) and overexpressing 10 known MVA pathway genes (*ERG20*, *ERG9*, *ERG1*, *tHMG1*, *ERG10*, *ERG8*, *ERG13*, *ERG12*, *ERG19*, *IDI1*) of *S. cerevisiae*. The shaking flask titer of 11-oxo-β-amyrin and GA increased to 80 and 8.78 mg/L, respectively (**Supplementary Table 1**; Wang C. et al., 2019).

Numerous studies have recently shown the feasibility of GA synthesis in *S. cerevisiae* (Zhu et al., 2018; Wang C. et al., 2019). According to published results, the microbial synthesis of GA

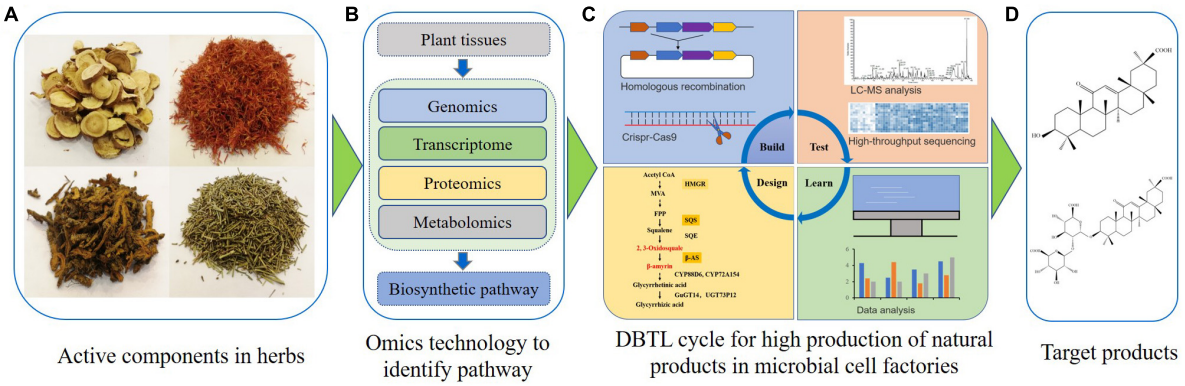

FIGURE 2 | Engineering microbial cell factories for synthesis of glycyrrhetinic acid or other plant natural products. **(A)** The active components are identified in traditional Chinese herbs or other candidate plants. **(B)** The fresh samples are collected, and multi-omics technologies are used to discover the biosynthetic pathways of natural products. **(C)** The Design-Build-Test-Learn cycle is used for construction and optimization of microbial cell factories for natural product biosynthesis. **(D)** Separation and purification of targeted natural products.

and other natural products might be achieved by several key procedures (**Figure 2**). First, plants harboring GA or other plant bioactive components are identified. Secondly, transcriptomics, genomics, proteomics and metabolomics analyses are used to recover pathways and key enzymes related to the synthesis of GA or other natural products (Chen et al., 2020; Gao et al., 2020; Nett et al., 2020; Srinivasan and Smolke, 2020). After that, appropriate chassis cells which provide precursors for natural product synthesis are selected for construction and optimization of associated microbial cell factories. Some synthetic biology tools, such as CRISPR-Cas9 technology, can efficiently introduce the biosynthetic pathway for GA or other natural products into chassis cells. These chassis cells may then be used as microbial cell factories. The artificially constructed cell factories need to be continuously optimized through the Design-Build-Test-Learn (DBTL) cycle (Nielsen and Keasling, 2016). The DBTL cycle includes new enzyme discovery, heterologous gene expression, promoter engineering, metabolic flux balance, pathway optimization, oxidation and reduction system balance, genome-scale metabolic models and other metabolic engineering strategies (Jiang et al., 2020; Ko et al., 2020; Tang et al., 2020; Wang M. et al., 2020). Finally, a high-yield GA or other natural product microbial cell factory can be obtained. As GA is insoluble in water, strategies utilizing the harnessing of lipid metabolism or other sub-organelle metabolism could lead to a high-yield production of GA (Cao et al., 2020; Ma et al., 2019). Besides *S. cerevisiae*, other yeasts could also be optimized for natural product biosynthesis in the future (Wei et al., 2017b; Wang J. et al., 2020).

CONCLUSION AND FUTURE PROSPECTIVES

GA is widely used in clinical practices, but the supply of GA is limited through traditional planting methods. Metabolic engineering and synthetic biology tools provide possible strategies for efficiently producing GA in *S. cerevisiae*. The successful production of high β-amyrin and GA using engineered *S. cerevisiae* shows that yeasts are suitable microbial chasses for GA production. The DBTL and other engineering strategies would further increase GA production, possibly providing one sustainable GA supply. In the future, other natural products derived from traditional Chinese herbs or other plants could also be synthesized using engineered *S. cerevisiae*.

AUTHOR CONTRIBUTIONS

YW and RG conceived this study. YW, RG, MW, and ZG wrote the manuscript. WL, C-YJ, and X-JJ revised the manuscript. All authors contributed to the article and approved the submitted version.

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Recent Advances on Feasible Strategies for Monoterpenoid Production in *Saccharomyces cerevisiae*

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Terpenoids are a large diverse group of natural products which play important roles in plant metabolic activities. Monoterpenoids are the main components of plant essential oils and the active components of some traditional Chinese medicinal herbs. Some monoterpenoids are widely used in medicine, cosmetics and other industries, and they are mainly obtained by plant biomass extraction methods. These plant extraction methods have some problems, such as low efficiency, unstable quality, and high cost. Moreover, the monoterpenoid production from plant cannot satisfy the growing monoterpenoids demand. The development of metabolic engineering, protein engineering and synthetic biology provides an opportunity to produce large amounts of monoterpenoids eco-friendly using microbial cell factories. This mini-review covers current monoterpenoids production using *Saccharomyces cerevisiae*. The monoterpenoids biosynthetic pathways, engineering of key monoterpenoids biosynthetic enzymes, and current monoterpenoids production using *S. cerevisiae* were summarized. In the future, metabolically engineered *S. cerevisiae* may provide one possible green and sustainable strategy for monoterpenoids supply.

Keywords: monoterpenoids, biosynthetic pathway, *Saccharomyces cerevisiae*, protein engineering, structure biology, synthetic biology

INTRODUCTION

Terpenoids are the largest and most structurally diverse class of natural products widely distributed in plants, microorganisms, and insects. More than 55,000 terpenoids have been identified so far (Christianson, 2008). Terpenoids have diverse biological activities, and they can function as chemical defense agents against predation and anti-pathogenic agents (Hijaz et al., 2016; Mahizan et al., 2019). Many terpenoids play critical roles in the interactions of plant-plant and plant-environment (Abbas et al., 2017). Among them, monoterpenoids are a kind of terpenoids composed with two isoprene units, which are widely distributed in plant and used in both pharmacy and medicine (Wojtunik-Kulesza et al., 2019). Recently, sabinene, one kind of monoterpene, has the

great potential to be used as advanced biofuel (Zhang et al., 2014). Nowadays, monoterpenoids are mainly extracted from plants using chemical methods. The monoterpenoid contents in natural or engineered plants are low, and the extraction processes are high-cost. Moreover, many medicinal plants grow slowly, and the planting area is limited due to the climate and other environmental conditions (Wang J. et al., 2015). The structure of most monoterpenoids is complex, thus, chemical synthesis is difficult and high-cost (Fu et al., 2018). Therefore, other sustainable supply of monoterpenoids is of great interest.

The development of synthetic biology and metabolic engineering provides opportunities for the microbial biosynthesis of monoterpenoids (Chandran et al., 2011). Industrial-scale production of some terpenoids using engineered microorganisms have been realized, such as artemisinin acid and ginsenosides (Paddon et al., 2013; Yan et al., 2014; Wang P. et al., 2015; Wei et al., 2015; Yang et al., 2020). Recovery of the monoterpenoid biosynthetic pathways and introduction of essential key enzymes into proper microbial host can lead to the production of targeted monoterpenoids (Zebec et al., 2016; Ignea et al., 2019). These provide an alternative way to sustainably supply of monoterpenoids and other Chinese herb-derived natural product (Guan et al., 2020; Wang et al., 2020).

The Function of Plant-Derived Terpenoids

Plant-derived terpenoids (also known as isoprenoids) are secondary metabolites, and they are widely used in the cosmetic, pharmaceutical, fragrance, and flavor industries (Goto et al., 2010; Caputi and Aprea, 2011). The basic skeleton of plant-derived terpenoids are the five-carbon unit of isoprene. According to the number of isoprene unit, terpenoids are classified into monoterpenoids, sesquiterpenoids, diterpenoids, triterpenoids, etc. (Ashour et al., 2010). Plant-derived terpenoids have diverse biological properties, for example, artemisinin (sesquiterpenoid) has anti-malarial effects (Talman et al., 2019); Taxol (diterpenoid) is used to treat ovarian cancer and breast cancer (Weaver, 2014); Glycyrrhetinic acid (triterpenoid) has antiviral and antimicrobial function (Kowalska and Kalinowska-Lis, 2019); Lycopene (tetraterpenoid) has antioxidant effects (Costa-Rodrigues et al., 2018) and helps reduce the risk of osteoporosis (Oliveira et al., 2019).

Many medicinal plants contain volatile monoterpenoids (Zhu et al., 2020). Among them, menthone and pulegone have anti-inflammatory and antiviral pharmacological effects (Božović and Ragno, 2017); (+)-Menthol and other monoterpenoids have antibacterial effects on *Staphylococcus aureus* and *Escherichia coli* (Trombetta et al., 2005). Monoterpenoids have been used as ingredients of soap, perfume and food, and some monocyclic monoterpenes can be used as insecticides (Rajput et al., 2018).

Terpenoid Production Strategies

Currently, three possible terpenoid production strategies have been applied, including plant extraction, chemical synthesis, and microbial biosynthesis (Li et al., 2020). Extraction from biomass of herb plants is the traditional terpenoid production method. The monoterpenoids of menthone and pulegone are the effective medicinal ingredients of *Nepeta cataria* (Wang et al., 2017). The volatile components of *N. cataria* are mainly extracted by the physical methods of distillation and extraction, including reflux method, and temperature-programmed microwave extraction method (Li et al., 2012). However, the volatile oil contents in *Nepeta* are low, and extraction of monoterpenoids from *Nepeta* biomass is difficult (Wen et al., 2019). The chemical structures of terpenoids are relatively complex. Chemical synthesis of terpenoids needs many steps, and the purity and yield of the products are not high enough, making chemical synthesis of terpenoids is difficult and high-cost. With the development of synthetic biology and metabolic engineering, many terpenoids have been successfully synthesized by engineered microorganisms (Sun et al., 2017; Guan et al., 2020). The most successful example is the synthesis of artemisinic acid using metabolically engineered yeasts (Paddon et al., 2013; Kai et al., 2018). In China, the most successful large-scale production of terpenoids is the synthesis of rare ginsenosides with engineered *S. cerevisiae* (Yan et al., 2014; Wang P. et al., 2015; Wei et al., 2015). Due to the complex structures of monoterpenoids, microbial production of monoterpenoids is of great interest.

Recovery of Monoterpenoid Biosynthetic Pathway

The recovery of monoterpenoid biosynthetic pathway is essential for microbial monoterpenoid biosynthesis. The carbon skeleton of monoterpenoid is formed by the condensation of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (Wang, 2013). There are two distinct biochemical pathways for the synthesis of IPP and DMAPP, the 2C-methyl-D-Erythritol-4-phosphate (MEP) pathway and the mevalonic acid (MVA) pathway (**Figure 1** and **Supplementary Figure 1**). In the plastids of plant cells, protists and most microorganisms, MEP pathway is used to synthesize IPP and DMAPP; While in the cytoplasm of higher eukaryotes, the MVA pathway is used to synthesize IPP and DMAPP (Laule et al., 2003; Vranová et al., 2013).

There are several metabolic rate-limiting enzymes in MVA and MEP pathways. In MVA pathway, 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMGR) is a rate-limiting enzyme, which catalyzes HMG-CoA to form MVA (Asadollahi et al., 2010); In MEP pathway, 1-deoxy-D-xyulose-5-phosphate synthase (DXS) is the rate-limiting enzyme, which catalyzes the formation of branched MEP using 1-deoxy-D-xyulose-5-phosphate (DXP) (Bergman et al., 2019). IPP and DMAPP are generated by the MEP pathway or MVA pathway, then they are catalyzed by GPPS (geranyl diphosphate synthase) to form geranyl diphosphate (GPP) with C10 backbone. Diverse terpene synthases (TPSs) catalyze the production of different monoterpenoids using the acyclic monoterpene precursor of GPP (Liao et al., 2016).

Some monoterpenoids exhibit significant toxicity to bacteria by interfering with bacterial cell membranes. Besides, the accumulation of terpenoids precursors (such as IPP) has a negative impact on bacterial growth (Paramasivan and Muttur, 2017). Production of monoterpenoids often needs to express

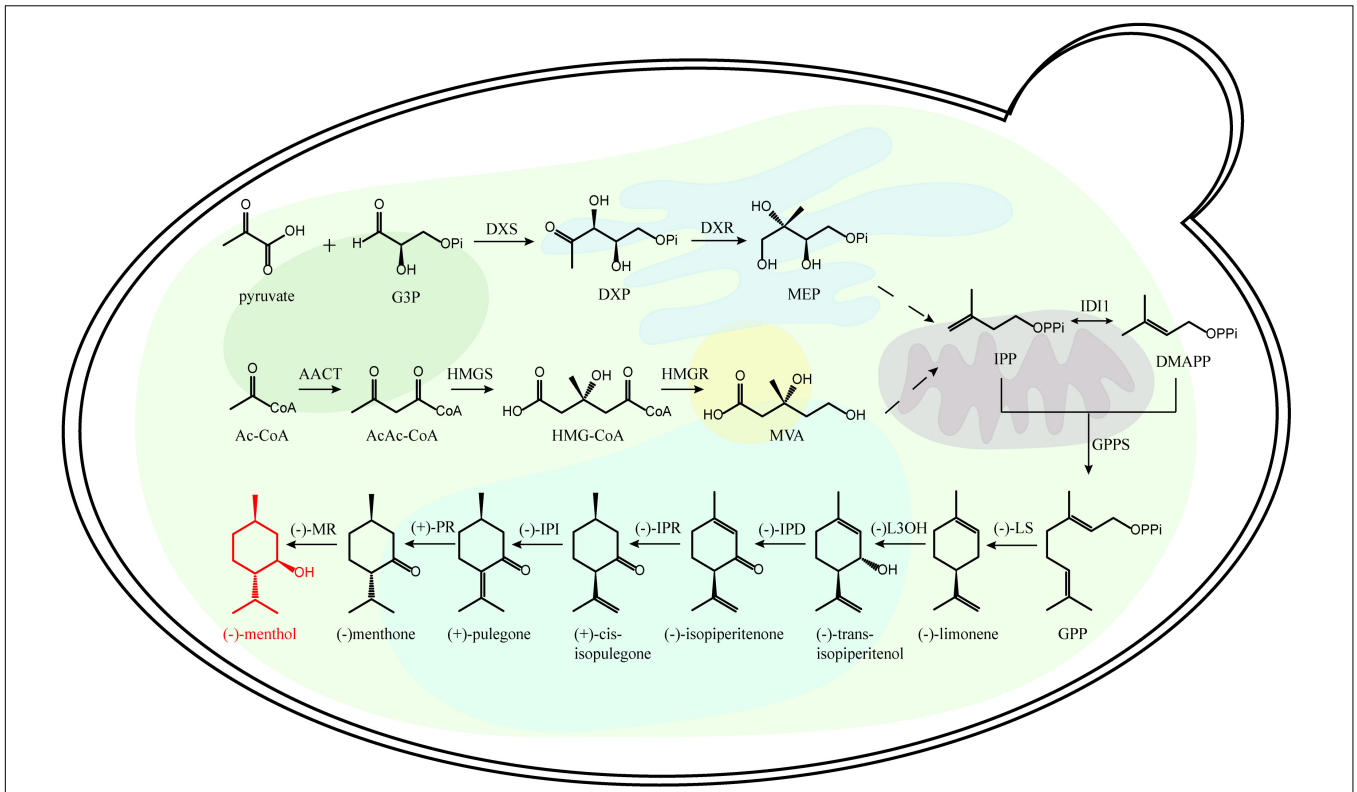

FIGURE 1 | Representative monoterpenoid (menthone) biosynthetic pathway. Ac-CoA, acetyl-CoA; DMAPP dimethylallyl diphosphate; DXP, 1-deoxy-D-xyulose-5-phosphate; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; G3P, glyceraldehyde 3-phosphate; GPP, geranyl diphosphate; GPPS, GPP synthase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; IDI1, isopentenyl diphosphate isomerase; IPD, *trans*-isopiperitenol dehydrogenase; IPI, *cis*-isopulegone isomerase; IPR, isopiperitenone reductase; L3OH, limonene-3-hydroxylase; LS, limonene synthase; MEP, 2C-methyl-D-erythritol 4-phosphate; MR, menthone reductase; MVA, mevalonate; PR, pulegone reductase.

cytochrome P450 genes, which is not easy to achieve in bacteria. In view of these, *S. cerevisiae* is becoming an attractive host for monoterpenoid bio-production, due to its robustness, well-studied genetic background, applicability to industrial bioprocesses, and the possibility for terpene scaffold decoration by the functional expression of cytochrome P450 enzymes (Zhang et al., 2008). In particular, *S. cerevisiae* is classified as generally regarded as safe (GRAS) by the U.S. Food and Drug Administration (FDA) (Nevoigt, 2008). However, compared with plant cells, *S. cerevisiae* lacks monoterpene synthetase, so it has no ability to synthesize monoterpenoids directly.

In order to realize the heterologous expression of target monoterpenoids in *S. cerevisiae*, exogenous plant monoterpene synthetases need to be introduced (**Figure 1**). Dudareva et al. (1996) identified one plant monoterpene synthetase gene responsible for the synthesis of terpene alcohol synthase. Chen et al. (2010) isolated and identified two monoterpene synthetases of AaLS1p and ApLS1p, from *Actinidia argute* and *Actinidia polygama*, respectively, which can synthesize (*S*)-linalool. Oswald et al. (2007) co-expressed linalool synthase from *Clarkia breweri* and geraniol synthetase from *Ocimum basilicum* in *S. cerevisiae*, and these enzymes improved the biosynthetic ability of monoterpene linalool and geraniol in *S. cerevisiae*. Herrero et al. (2008) successfully introduced linalool biosynthetic pathway to *S. cerevisiae*, and linalool was effectively produced in *S. cerevisiae*. These studies showed that *S. cerevisiae* has great potential to act as a cell factory for monoterpenoids synthesis.

Recently, transcriptomic analyses of the mint adenoids successfully identified the key enzymes of menthol biosynthetic pathway, further cloning and expression verified the biological functions of these key enzymes in the GPP to (−)-menthol pathway (**Figure 1** and **Supplementary Figure 1**; Vining et al., 2017). Different chiral terpenoids often have different biological activities (Hajagos-Tóth et al., 2015), and the key enzymes involved in different terpenoid biosynthetic pathway need to be identified. Therefore, global omics analyses of different monoterpenoid producing plants will give insights into key enzymes for diverse monoterpenoid biosynthesis using engineered microbial cell factories (Wei et al., 2019).

Applications of Protein Engineering, Metabolic Engineering and Structure Biology for Monoterpenoid Production in *S. cerevisiae*

Although *S. cerevisiae* has been proven to be able to synthesize monoterpenoids efficiently, the metabolic flux of MVA pathway in *S. cerevisiae* is still low (Ignea et al., 2019). The metabolic

regulation mechanism of the MVA pathway is complex, which greatly affects the final production of monoterpenoids. In order to increase the metabolic flux of the MVA pathway, several important metabolic rate-limiting/key enzymes have been identified and optimized.

3-hydroxy-3-methyl glutaryl coenzyme A reductase is a rate-limiting enzyme in monoterpenoid synthesis, which catalyzes HMG-CoA to generate MVA irreversibly (**Figure 1**). To relieve the intermediate product inhibition and accumulate of end products, the N-terminal membrane of HMGR was anchored and the C-terminal of HMGR separately (tHMGR) was expressed in *S. cerevisiae*, which increased the final production of target monoterpenoid (Rico et al., 2010). Overexpression of *tHMGR* and other genes in *S. cerevisiae* lead to 22.49 mg/L limonene (Wu et al., 2018). HMGR2 is the predominant isoenzyme that catalyzes HMG-CoA reduction under hypoxia condition (Mantzouridou and Tsimidou, 2010), an engineered stabilized mutant HMGR2 (K6R) successfully improved the production of terpenes (**Supplementary Figure 2**; Ignea et al., 2012, 2014). These protein engineering studies suggested that HMGR had a significant effect on target monoterpenoids production.

Isoprene pyrophosphate isomerase (IDI1) controls the first step of the monoterpenoid biosynthesis in the conversion process from IPP to DMAPP, which can adjust the proportion of IPP/DMAPP (**Figure 1**; Cao et al., 2016). Regulating the expression of *IDI1* successfully increased the yield of monoterpenoid. Further overexpression of *IDI1* gene in engineered *S. cerevisiae* increased sabinene production by 300% (Ignea et al., 2014). These examples indicate that overexpression of *IDI1* in *S. cerevisiae* could increase the production of desirable monoterpenoid.

The committed step in monoterpenoid biosynthesis involves the conversion of the acyclic isoprenoid diphosphate precursor to cyclic hydrocarbon product catalyzed by limonene synthase (LS) (**Figure 1** and **Supplementary Figure 1**). Limonene is well known for its olfactory characteristics and antibacterial activity, which is often added to soaps or detergents (Jongedijk et al., 2016). Limonene has an unsaturated ring structure, which can be oxidized to menthol, perillyl alcohol and other natural products (Alonso-Gutierrez et al., 2013). Expression of a (−)- limonene synthase from *Perilla frutescens* and a (+)-limonene synthase from *Citrus sinensis* resulted in the production of 0.028 mg/l (+)-limonene and 0.060 mg/l (−)-limonene in the engineered yeast cell factory (Jongedijk et al., 2014). The crystal structure of (−)-limonene synthase [(−)-LS] from *Mentha spicata* was determined at 2.7 Å resolution. The structural and biochemical results show that (−)-LS shares many hallmark features and catalytic characteristic with plant monoterpene synthases, including an all-α-helical domain secondary structure, a two-domain architecture with a catalytic C-terminal domain, an N-terminal domain of unknown function, and conserved divalent metal ion binding residues in the active site (**Figure 2A**; Hyatt et al., 2007).

(+)-Limonene is an abundant monoterpene in the essential oils of most citrus fruit, which is commonly used as an industrial and household solvent. In particular, the citrus smell enables its use in the fragrance and flavoring industries. The (+)-LS from *Citrus sinensis* was purified, and its crystal structure was further determined. The structural comparison of (+)-LS and (−)-LS showed that the conformation of short α1 helix played critical roles in determining (+)-LS or (−)-LS enantiomer. The structural and catalytic mechanism studies of (+)-LS and (−)-LS provided theoretical basis and new options for designing and achieving (+) or (−)-limonene rationally (**Figure 2B**; Morehouse et al., 2017; Ren et al., 2020). Based on the LS crystal structures and the proposed catalytic mechanism, a triple mutant (S454G, C457V, M458I) of *Mentha spicata* (−)-LS was successfully generated, which showed a new catalytic activity of producing more complex bicyclic monoterpenes (**Supplementary Figure 2**; Leferink et al., 2018).

Farnesyl pyrophosphate synthase (FPPS) condenses one molecule of DMAPP and one molecule of IPP to form GPP, then condenses GPP with one molecule of IPP to form FPP in *S. cerevisiae* (**Figure 1**). It is reported that FPPS of *S. cerevisiae* is encoded by the gene *ERG20*, which is responsible for the biosynthesis of GPP and FPP. GPP is usually closely bound to the catalytic pocket of FPPS and results in the low content of free GPP (Dai et al., 2013). Therefore, the precursor GPP for monoterpenoid synthesis was insufficient, which greatly restricted the biosynthesis of monoterpenoids. In addition, *S. cerevisiae* also lacks specific and efficient GPP synthase (Herrero et al., 2008). Sequence alignment of FPPS from different organisms showed that there were two ASP rich sequences in the conserved region of this enzyme. The first ASP rich sequence is DDXXD or DDXXXXD, which could bind DMAPP, GPP, and FPP in catalytic reaction. The second ASP rich sequence is DDXXD, which is mainly responsible for binding IPP. Previous studies showed that site directed mutagenesis in the conservative region of FPPS can cause GPP released from the binding site of FPPS, which increased the intracellular free GPP content (**Supplementary Figure 2**). By mutating the catalytic residue K197 of yeast farnesyl diphosphate synthase erg20p and expressing heterologous geraniol synthetase gene in engineered *S. cerevisiae*, the yield of geraniol increased significantly, suggesting that the regulation of *ERG20* gene can increase the GPP amount and balance GPP and FPP ratio for geraniol production (Fischer et al., 2011). Sabinene is seen as a potential component for the next generation of aircraft fuels (Rude and Schirmer, 2009). Ignea et al. (2014) identified that erg20p is an rate-limiting enzyme for monoterpene production, and integrating erg20p with a geranyl diphosphate synthase increased sabinene titer (17.5 mg/L). Compared with the canonical GPP-based monoterpenoid biosynthetic pathway, a synthetic orthogonal monoterpenoid pathway using neryl diphosphate as substrate was designed, and further dynamic regulation of some engineered enzymes in the synthetic monoterpenoid improved monoterpenoid production by seven-fold (Ignea et al., 2019).

The protein engineering and structural studies of key enzymes involved in monoterpenoid biosynthetic pathway provided new options for efficient monoterpenoid

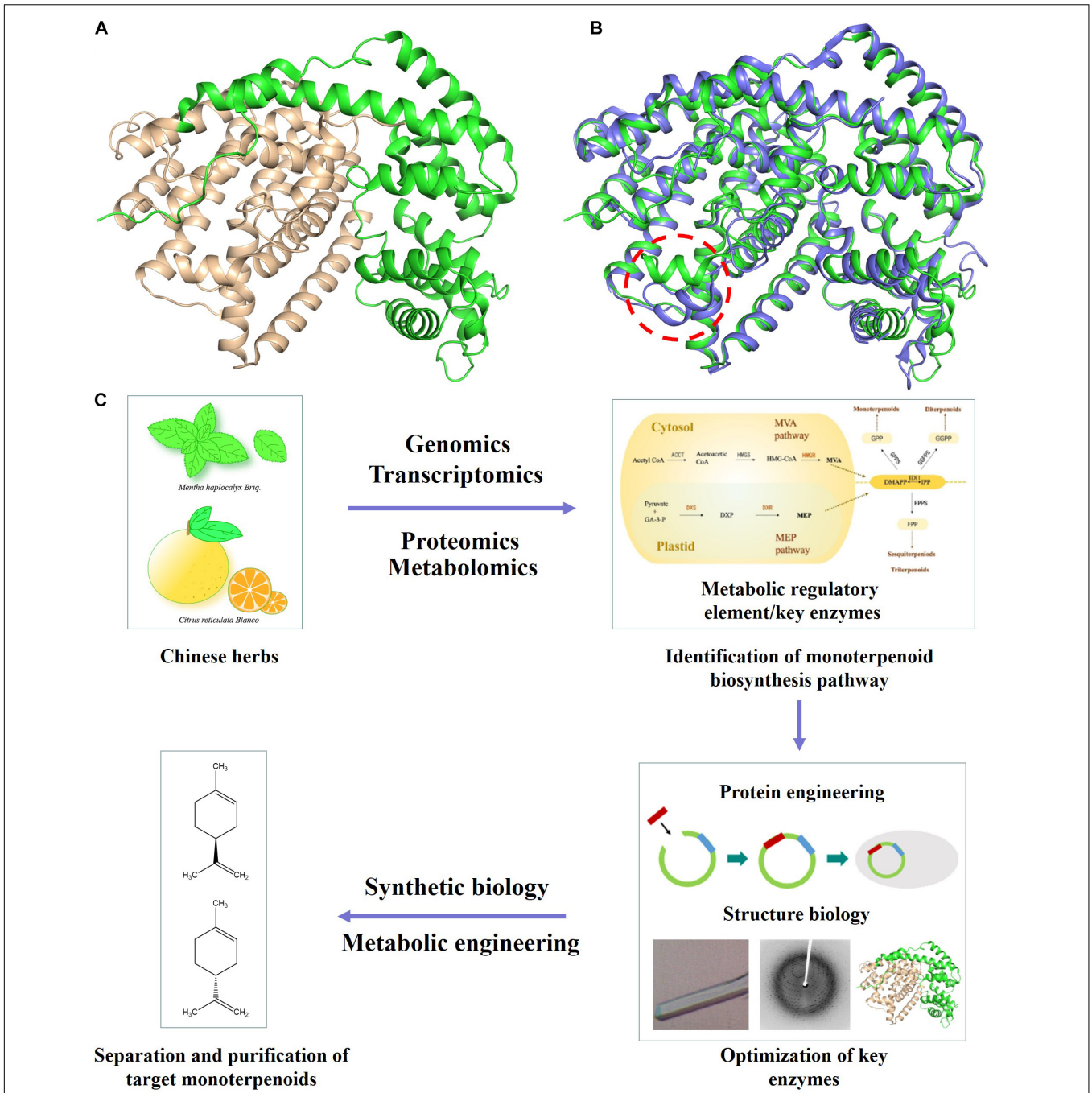

FIGURE 2 | Application of protein engineering and structural biology based approaches in yeast monoterpenoid production. **(A)** Structure of (–)-LS (PDB ID 2ONG). N-terminal and C-terminal domain are colored in green and wheat, respectively. **(B)** Comparison of the structure of (–)-LS (colored in green) and (+)-LS (colored in blue). **(C)** Increasing the production of target monoterpenoid through optimizing important metabolic nodes/key enzymes by enzyme engineering or structure biology.

biosynthesis in *S. cerevisiae* (**Figure 2C** and **Supplementary Figure 2**).

Conclusion and Future Perspective

Plant-derived monoterpenoids are widely used in daily life, and the demand is increasing. Microbial biosynthesis of monoterpenoid provides a new feasible strategy for the monoterpenoid production. *S. cerevisiae* might be an ideal microbial cell factory for monoterpenoid biosynthesis. With the help of omics technologies, systems biology, structural biology and protein engineering, it will further lead to the identification of highly efficient monoterpenoid biosynthetic enzymes, which can help to enable high yield of monoterpenoid in *S. cerevisiae*.

AUTHOR CONTRIBUTIONS

QG, LW, and MZ contributed to figures and manuscript draft preparations. YW and WL wrote and revised the manuscript. WL conceived the project. All authors approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2020.609800/full#supplementary-material

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REVIEW
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Yeast-Based Biosynthesis of Natural Products From Xylose

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Xylose is the second most abundant sugar in lignocellulosic hydrolysates. Transformation of xylose into valuable chemicals, such as plant natural products, is a feasible and sustainable route to industrializing biorefinery of biomass materials. Yeast strains, including *Saccharomyces cerevisiae*, *Scheffersomyces stipitis*, and *Yarrowia lipolytica*, display some paramount advantages in expressing heterologous enzymes and pathways from various sources and have been engineered extensively to produce natural products. In this review, we summarize the advances in the development of metabolically engineered yeasts to produce natural products from xylose, including aromatics, terpenoids, and flavonoids. The state-of-the-art metabolic engineering strategies and representative examples are reviewed. Future challenges and perspectives are also discussed on yeast engineering for commercial production of natural products using xylose as feedstocks.

Keywords: xylose, yeast, natural product, *Saccharomyces cerevisiae*, *Pichia stipitis*, *Yarrowia lipolytica*

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INTRODUCTION

Biomass hydrolysates are frequently used as a feedstock in biomass-based biorefinery. The conversion of these hydrolysates to useful compounds, such as natural products, is usually carried out by yeasts due to their advantageous properties such as tolerance of toxic inhibitors in hydrolysates, functional expression of eukaryote-derived heterologous pathways, and resistance to osmotic stress and harmful fermentation stimuli (Kwak et al., 2019; Li et al., 2019). A major challenge in this process is a waste of resources and high process cost partly due to inefficient utilization of xylose, which is the second most abundant saccharide in biomass hydrolysates (Zha et al., 2012, 2014). Xylose cannot be naturally metabolized by the commonly used yeast chassis such as *Saccharomyces cerevisiae* and *Yarrowia lipolytica* with the exception of *Scheffersomyces stipitis* (Jagtap and Rao, 2018). To overcome this difficulty, there have been many attempts over the past few decades on yeast engineering for xylose fermentation, with *Sa. cerevisiae* as the main focus (Hou et al., 2017).

The early endeavor of yeast engineering largely aimed at bioethanol production using *Sa. cerevisiae* and *Sc. stipitis* due to their intrinsic capability of synthesizing ethanol from various carbon sources. However, the complicated production process, including pretreatment, saccharification, and fermentation, results in high production cost and limited market competitiveness, and the relevant technologies are far from industrialization on a large scale (Kwak et al., 2019). In consequence, researchers started to explore yeast-based conversion of xylose, together with glucose, into value-added chemicals that are not readily available through extraction or chemical synthesis (Kwak and Jin, 2017; Kwak et al., 2019).

Although glucose is a preferred carbon source for many microbes, it is not always better than xylose when particular metabolic requirements need to be met. Xylose can induce respiratory effects on central carbon metabolism even under anaerobic conditions, and the metabolic flux of the TCA cycle, the pentose phosphate pathway (PPP), and acetyl-CoA biosynthesis is much higher on xylose than on glucose (Kwak et al., 2019). Compared with glucose, xylose induces a different metabolic flux distribution and enhances the generation of some key intermediate metabolites such as acetyl-CoA, malonyl-CoA, and erythrose-4-phosphate (E-4-P; Kwak and Jin, 2017). The elevated supply of these precursors is beneficial for the production of several classes of natural compounds such as terpenoids and aromatics.

In this review, we will summarize the recent progress on genetic modification of yeast strains for the biosynthesis of natural products using xylose as the carbon source, with a focus on three yeasts including the natural xylose-fermenting yeast *Sc. stipitis* and recombinant xylose-fermenting yeasts *Sa. cerevisiae* and *Y. lipolytica*. For each of these organisms, the description will be mainly centered on four aspects, which include general physiology related to metabolic properties, tools, and strategies available for genetic manipulation, metabolism of xylose, and the biosynthesis of typical natural products from xylose.

XYLOSE METABOLIC PATHWAYS

Three xylose catabolic pathways have been discovered so far in natural xylose-utilizing microorganisms (**Figure 1**). The first pathway is the XR-XDH pathway widely present in natural xylose-utilizing yeasts, such as *Sc. stipitis* and *Candida shehatae*,

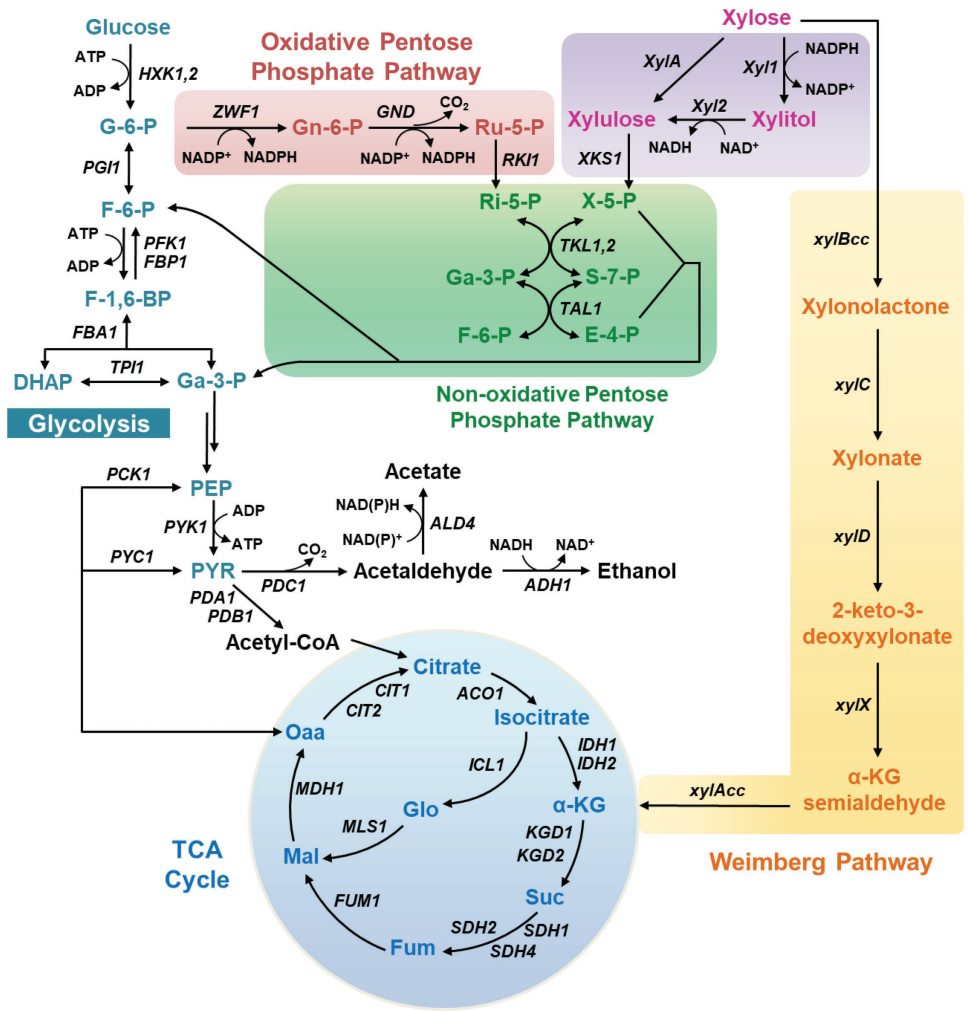

FIGURE 1 | The metabolic pathways for xylose metabolism in yeasts. X-5-P, xylulose-5-phosphate; Ri-5-P, ribose-5-phosphate; Ru-5-P, ribulose-5-phosphate; Ga-3-P, glyceraldehyde-3-phosphate; S-7-P, sedoheptulose-7-phosphate; F-6-P, fructose-6-phosphate; E-4-P, erythrose-4-phosphate; G-6-P, glucose-6-phosphate; Gn-6-P, 6-phosphogluconate; F-1,6-BP, fructose-1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; Gly-1,3-P, 1,3-bisphosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; Oaa, oxaloacetate; Mal, malate; Fum, fumarate; Suc, succinate; α-KG, α-ketoglutarate.

in which xylose is reduced to xylitol by xylose reductase (XR) and then oxidized to xylulose by xylitol dehydrogenase (XDH; **Figure 1**).

A more direct pathway is the XI pathway that converts xylose into xylulose in a single step using xylose isomerase (XI; **Figure 1**), after which xylulose can be channeled into glycolysis *via* phosphorylation and multiple biochemical reactions in the non-oxidative PPP. The XI pathway is intrinsic in bacteria and a few fungi.

The third xylose metabolic pathway is the Weimberg pathway, in which xylose is oxidized by xylose dehydrogenase (XylB) to xylono-γ-lactone and converted to xylonate by xylono-γ-lactone lactonase (XylC; **Figure 1**). Xylonate then undergoes two successive dehydration reactions by xylonate dehydratase (XylD) and 2-keto-3-deoxy-xylonate dehydratase (XylX) to form α-ketoglutarate semialdehyde, which is further oxidized to α-ketoglutarate by α-ketoglutarate semialdehyde dehydrogenase (XylA) and enters the TCA cycle.

Among these pathways, the XR-XDH route is the mostly explored in recombinant yeasts owing to its ease of expression and high metabolic flux, and this route has been successfully expressed in non-xylose-fermenting yeasts including *Sa. cerevisiae* and *Y. lipolytica* (Kim et al., 2013; Wu et al., 2019). The functional expression of bacterial XI pathway in non-xylose-fermenting yeasts is a challenge, whereas the XI pathway derived from *Piromyces* and other organisms operates well in *Sa. cerevisiae* (Kuyper et al., 2003). In comparison, the introduction of the Weimberg pathway is much more challenging, which has only been achieved recently in *Sa. cerevisiae* with albeit low efficiency of xylose metabolism (Borgström et al., 2019). In this work, the gene *xylA* was replaced with its ortholog *KsaD* from *Corynebacterium glutamicum*, and gene expression in the lower Weimberg pathway (*XylD*, *XylX*, and *KsaD*) was enhanced. Through further deletion of the iron regulon repressor *FRA2* and serial adaptive evolution, the engineered strain was capable of metabolizing up to 57% of the carbon from assimilated xylose into biomass and carbon dioxide in the mixture of glucose and xylose. The growth using xylose as the sole carbon source has not been reported, possibly due to the weak activity of the introduced Weimberg pathway.

BIOSYNTHESIS OF NATURAL PRODUCTS FROM XYLOSE BY *SCHEFFERSOMYCES STIPITIS*

Scheffersomyces stipitis, previously known as *Pichia stipitis*, is a facultatively anaerobic yeast that exists mostly in haploid form (Jeffries et al., 2007). This microbe can metabolize many lignocellulose-derived saccharides including hexoses, pentoses, and cellobiose attributed to various hydrolases encoded by its genome such as β-glucosidases, endoglucanases, xylanase, mannanase, and chitinase. Such a high diversity of carbon sources for this yeast may be a result of long adaptation to its natural habitat, as several known *Sc. stipitis* strains, such as CBS 6054, naturally dwell in insects that feed on lignocellulose. This unique capability makes *Sc. stipitis*, a promising strain to utilize lignocellulosic biomass as feedstocks, to produce biofuels and chemicals (Gao et al., 2017).

Scheffersomyces stipitis is a Crabtree negative yeast with greater respiratory capacity than *Sa. cerevisiae* due to the presence of an alternative respiration system beyond cytochrome system that is sensitive to salicylhydroxamic acid, the so-called SHAM-sensitive respiratory pathway (Jeppsson et al., 1995; Jeffries et al., 2007). This pathway branches out of the cytochrome pathway at ubiquinone, donating electrons directly to O_2 to form water (Jeppsson et al., 1995). *Scheffersomyces stipitis* cells growing without a functional cytochrome pathway can still metabolize xylose although the growth rate is reduced by half (Shi et al., 1999). In addition, this yeast contains NADH dehydrogenase complex I, which is absent in *Sa. cerevisiae*, for ATP generation through oxidative phosphorylation (Jeffries et al., 2007). *Scheffersomyces stipitis* is more stable than *Sa. cerevisiae* in terms of metabolite accumulation in response to oxygen supply, and its metabolic flux distribution is greatly affected by oxygen levels, which is different from *Sa. cerevisiae* that significantly relies on glucose concentrations for metabolic regulation.

Scheffersomyces stipitis is a natural xylose utilizer that harbors the XR/XDH pathway. XR uses either NADPH or NADH as cofactor, and excess NADH is produced when cells are grown on xylose, allowing for oxygen-independent utilization of xylose (Bruinenberg et al., 1984; Does and Bisson, 1989; Jeffries et al., 2007). The *Sc. stipitis* genome encodes multiple enzymes to participate in NAD(P)H oxidoreductase reactions for balancing of cofactors and redox potential (Jeffries et al., 2007). This organism has the highest native capability of xylose fermentation among all the known microbes, and its rate of xylose uptake and cell growth, when xylose is used as the sole carbon source, is one order of magnitude higher than that of the recombinant *Sa. cerevisiae* harboring the XR/XDH pathway (Jeffries et al., 2007; Feng and Zhao, 2013). In *Sc. stipitis*, xylose uptake is a rate-limiting step in xylose metabolism under aerobic conditions, whereas xylulose formation from xylitol is rate-limiting under anaerobic conditions (Ligthelm et al., 1988). There are two types of xylose transporters termed the high- and low-affinity transport systems, both of which are proton symports and can be inhibited by dinitrophenol, indicating active transport (Does and Bisson, 1989). The uptake of xylose can be inhibited by glucose competitively and non-competitively in the low-affinity and high-affinity transport systems, respectively (Kilian and van Uden, 1988; Jeffries et al., 2007).

Scheffersomyces stipitis uses CUG to encode serine instead of leucine, which makes codon optimization extremely important when heterologous genes contain leucine-encoding CTG. In addition, nonhomologous end joining (NHEJ) dominates DNA repair of double strand breaks, leading to very low efficiency (<10%) of genome editing (Cao et al., 2018). NHEJ in this organism relies on the dimeric protein complex Ku consisting of Ku70 and Ku80 subunits that binds to ends of DNA double strand breaks. Deletion of the Ku complex increases the efficiency of homologous recombination to ~70%. Using such a Ku^- background, CRISPR-Cas9 and CRISPR-dCas9 systems have been developed in *Sc. stipitis*, which has greatly facilitated the basic genetic engineering. Moreover, a wide spectrum of native

promoters and terminators has been reported, and a centromeric DNA sequence has been identified to stabilize episomal plasmids for stable expression of heterologous genes (Cao et al., 2017). These findings and tools make genetic engineering precise and facile in *Sc. stipitis*.

So far, *Sc. stipitis* is not as a popular host as *Sa. cerevisiae* in the production of natural products. This is partly due to the limited genetic tools such as expression plasmids and gene knock-out tools. On the other hand, the physiology and genetics of *Sc. stipitis* are less defined compared with *Sa. cerevisiae*. Nevertheless, *Sc. stipitis* has high metabolic flux toward PPP and abundant supply of E-4-P (Jeffries et al., 2007), which is the precursor of aromatics, therefore, this yeast is suitable for the biosynthesis of aromatics (Gao et al., 2017; Cao et al., 2018). Recently, *Sc. stipitis* was used to synthesize shikimate from xylose (Gao et al., 2017). In this strain, overexpression was performed on $aro4_{K220L}$, *tkt1*, and $aro1_{D900A}$, which encode a feedback insensitive DAHP synthase, a transketolase, and a pentafunctional enzyme converting DAHP to 5-enolpyruvyl-3-shikimate phosphate, respectively. Additionally, promoters and terminators were optimized to ensure strong constitutive expression of the pathway genes. These strategies allowed for the production of 3.11 g/L shikimate from xylose under aerobic conditions, which was 7-fold higher than the highest reported titer ever achieved in *Sa. cerevisiae*. This work opens the door to the biosynthesis of aromatic compounds in *Sc. stipitis*.

USING *SACCHAROMYCES CEREVISIAE* AS THE HOST TO PRODUCE NATURAL PRODUCTS FROM XYLOSE

Saccharomyces cerevisiae is one of the mostly used model hosts in metabolic engineering. It is a single-celled GRAS (generally regarded as safe) fungus that proliferates through budding or fission. *Saccharomyces cerevisiae* has high tolerance to environmental stress, such as low pH, high osmotic pressure, and phage infection, making it advantageous in industrial fermentation. Moreover, the well-defined physiological information and sophisticated metabolic engineering tools of this yeast greatly facilitate its engineering and applications.

Natural *Sa. cerevisiae* contains native xylose metabolic pathway genes, such as aldose reductase-encoding *GRE3*, sorbitol dehydrogenase-encoding *SOR1* and xylulose kinase (XKS) gene *XKS1*, the counterpart of *XYL1*, *XYL2*, and *XYL3* from *Sc. stipitis* (Konishi et al., 2015). However, these genes are not expressed sufficiently to support significant growth on xylose. Metabolic engineering efforts have created excellent xylose-utilizing recombinant *Sa. cerevisiae* through various approaches including the introduction of efficient heterologous xylose metabolic pathway, activation of endogenous genes involved in xylose utilization, enhancement of the PPP, balance of cofactors, evolutionary engineering using xylose as the sole carbon source, and so on (Hou et al., 2017). These attempts have potentiated *Sa. cerevisiae* as a robust chassis organism in generating a wide variety of useful compounds using xylose alone or together with glucose as the carbon source.

Unlike *Sc. stipitis*, *Sa. cerevisiae* is a Crabtree-positive strain. The metabolic pattern on xylose is quite different from that on glucose. The efficiency of xylose assimilation and metabolism in recombinant *Sa. cerevisiae* is generally much lower than glucose although xylose uptake and utilization has been engineered extensively and improved dramatically (Qi et al., 2015; Hou et al., 2017). Inefficient xylose assimilation leads to carbon starvation-like metabolomic patterns of glycolysis, indicated by the observation of low pools of glycolytic intermediates except for the accumulation of phosphoenolpyruvate, which is required for the production of aromatic compounds through the shikimate pathway (Mert et al., 2017). Growth on glucose induces the expression of hexokinase 2 (Hxk2 encoded by *HXK2*), activates ethanol-producing metabolism, and represses mitochondrial activities (Moreno and Herrero, 2002). In contrast, this glucose-dependent repression on the respiratory energy metabolism can be dysregulated by xylose (Jin et al., 2004). The mitochondrial genes and the TCA cycle are very active when *Sa. cerevisiae* are grown on xylose. In addition, xylose can upregulate the glyoxylate pathway and activate cytosolic acetyl-CoA metabolism with enhanced expression of genes encoding aldehyde dehydrogenases and acetyl-CoA synthetase 1 (*ALD2*, *ALD3*, *ALD6*, and *ACS1*). Meantime, the gene encoding ethanol reoxidizing alcohol dehydrogenase (ADH2) is also highly induced upon xylose utilization (Matsushika et al., 2014). Besides, the non-oxidative PPP is activated when recombinant *Sa. cerevisiae* is grown on xylose, leading to the accumulation of some important intermediates such as E-4-P, which is one of the precursors for the synthesis of aromatic compounds (**Figure 2**). All of these metabolic properties make *Sa. cerevisiae* beneficial for the biosynthesis of many classes of natural products, such as terpenoids, flavonoids, and other polyphenols.

To direct the production of natural compounds from xylose, the relative pathways need to be constructed and introduced into xylose-utilizing *Sa. cerevisiae*. Currently, production of vitamin A, protopanaxadiol, *p*-coumaric acid, carotenoid, and other natural products has been achieved in *Sa. cerevisiae* by fermentation on xylose (**Table 1**; Kwak et al., 2017; Borja et al., 2019; Su et al., 2020). For instance, a lycopene biosynthetic pathway consisting of *CrtE*, *CrtB*, and *CrtI* was introduced into xylose-fermenting *Sa. cerevisiae* overexpressing native *XK* and *Sc. stipitis*-derived *XYL1* and *XYL2*. The PK pathway consisting of xylulose-5-phosphate phosphoketolase (xPK) and phosphotransacetylase (PTA) was further introduced to directly convert xylulose-5-phosphate into acetyl-CoA. The resultant strain produced 1.6-fold more lycopene using the mixture of glucose and xylose than using glucose alone (Su et al., 2020). In another case, squalene-producing recombinant *Sa. cerevisiae* showed 8-fold higher production on xylose than on glucose (Kwak et al., 2017). A recombinant strain of xylose-metabolizing *Sa. cerevisiae* was engineered to carry the pathway for *p*-coumaric acid production through the expression of tyrosine ammonia lyase (TAL) and overexpression of some tyrosine biosynthetic pathway genes (Borja et al., 2019). This strain produced 242 mg/L of *p*-coumaric acid from xylose while the titer was only 5.35 mg/L on glucose. Moreover, a xylose-fermenting strain

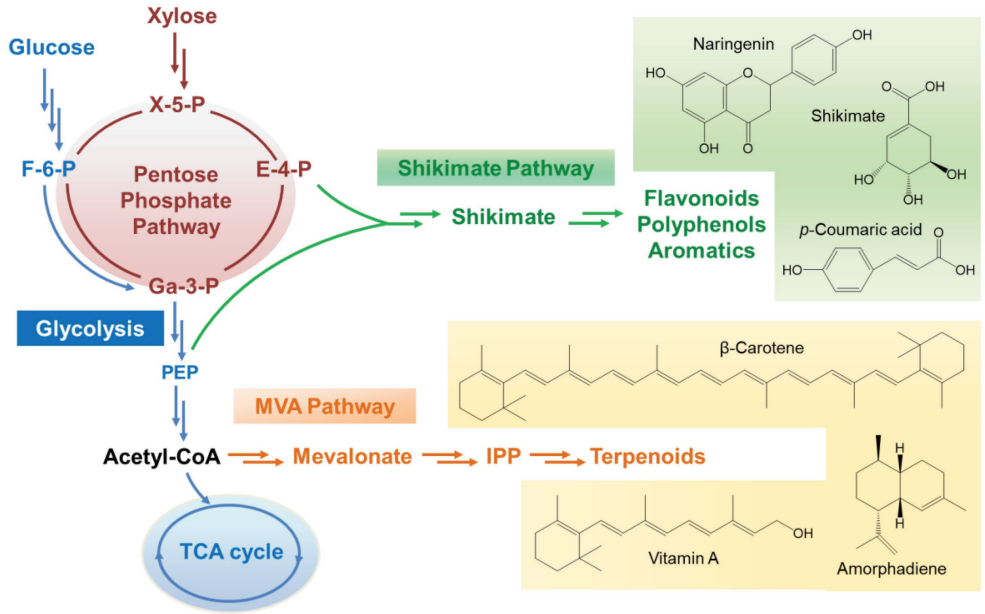

FIGURE 2 | The metabolic flux channel to direct the production of natural products through the shikimate pathway and mevalonate pathway when xylose is employed as the carbon source. X-5-P, xylulose-5-phosphate; E-4-P, erythrose-4-phosphate; F-6-P, fructose-6-phosphate; Ga-3-P, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; IPP, isopentenyl diphosphate.

expressing the biosynthetic pathway of shinorine, a natural sunscreen material, produced a trace amount of shinorine in glucose, whereas the titer was dramatically increased by adding xylose in the medium (Park et al., 2019). This interesting result was related to enhanced PPP flux triggered by xylose and abundant supply of sedoheptulose-7-phosphate, which is the preliminary precursor for the synthesis of shinorine.

BIOSYNTHESIS OF NATURAL PRODUCTS FROM XYLOSE BY *YARROWIA LIPOLYTICA*

Yarrowia lipolytica is an obligate aerobe that has a high flux of the TCA cycle and high translational efficiency of mitochondrial genes involved in aerobic respiration (Man and Pilpel, 2007; Christen and Sauer, 2011; Zhu and Jackson, 2015; Abdel-Mawgoud et al., 2018; Shi et al., 2018; Ma et al., 2019). This yeast grows at temperatures below 34°C and over a wide pH range, with metabolic performances varying with cultivation conditions (Egermeier et al., 2017; Abdel-Mawgoud et al., 2018). It is a GRAS microbe due to its low tendency of growing at human body temperature and the low probability of causing only mild infections in immunocompromised people (Groenewald et al., 2014). Phylogenetically dissimilar to other members in the yeast family, *Y. lipolytica* is considered to be nonconventional (Dujon et al., 2004). Its genome is naturally in haploid form facilitating genetic manipulation, although diploids are occasionally observed (Knutsen et al., 2007; Abdel-Mawgoud et al., 2018). The gene density in this microbe is much lower compared with *Sa. cerevisiae*, with the large and abundant intergenic regions suitable for gene integration (Dujon et al., 2004; Abdel-Mawgoud et al., 2018; Holkenbrink et al., 2018). The genes in *Y. lipolytica* are rich in introns (Stajich et al., 2007; Mekouar et al., 2010), the presence of which can positively affect expression levels of the relative exons (Le Hir et al., 2003; Hong et al., 2012; Tai and Stephanopoulos, 2013; Shaul, 2017). In addition, this strain has a broad spectrum of carbon sources, including sugars, acetate, fatty acids, alcohols, waste cooking oil, and so on (Abdel-Mawgoud et al., 2018).

Genetic engineering of *Y. lipolytica* is generally challenging compared with *Sa. cerevisiae*. First, it is not easy to precisely integrate gene fragments into the genome of *Y. lipolytica* at specified loci because this organism prefers NHEJ (Richard et al., 2005), whereas homologous recombination usually occurs only when long homologous arms (>1 kb) are used (Verbeke et al., 2013). This is attributed to the Ku70/Ku80 protein heterodimer that repairs breaks in DNA double strands (Lustig, 1999). Deletion of the relevant genes results in much higher frequency in the occurrence of homologous recombination with short (50 bp) homologous arms (Verbeke et al., 2013), and repression of both *KU70* and *KU80* by CRISPR-dCas9 greatly improves the efficiency of homologous recombination (>90%; Schwartz et al., 2017a). Second, there are not many engineering tools available for *Y. lipolytica* given that this yeast has only been used in metabolic engineering for less than two decades. The emergence of *Y. lipolytica*-based CRISPR systems (Gao et al., 2016; Schwartz et al., 2016, 2017b, 2018; Morse et al., 2018; Zhang et al., 2018), transposon systems (Casaregola et al., 2000; Patterson et al., 2018; Wagner et al., 2018; Yu et al., 2018)

TABLE 1 | Summary of the biosynthesis of natural products from xylose using yeast strains.

Chassis	Engineering strategy	Product	Titer (mg/L)	References
Scheffersomyces stipitis	↑DAHP synthase variant (aro4K220L), ↑ aro1(D900A), and ↑ Tkt1	Shikimate	3,110	Gao et al., 2017
Saccharomyces cerevisiae	↑ SsXYL1, ↑SsXYL2, ↑SsXYL3, ΔARO10, ΔPDC5, ↑shikimate kinase II (aroL) from *E. coli*, ↑tyrosine ammonia-lyase (TAL) from *Flavobacterium johnsoniae*, ↑DAHP synthase mutant, ↑chorismate mutase mutant	*p*-coumaric acid	242	Borja et al., 2019
Saccharomyces cerevisiae	↑ SsXYL1(K271N), ↑ SsXY2, ↑XKS,↑Gal2(N376F), ↑xPk,↑PTA, ↑tHMG1, ↑CrtEBI, Δpho13, and ΔAld6	β-Carotene	903	Su et al., 2020
Saccharomyces cerevisiae	↑ SsXYL1, ↑SsXYL2, ↑SsXYL3, ↑TKL1, ↑NpR5600, ↑NpR5599, ↑NpR5598, ↑NpR5597, and ΔTAL1	Shinorine	31	Park et al., 2019
Saccharomyces cerevisiae	↑ SsXYL1, ↑SsXYL2, ↑SsXYL3, ↑TKL1, ↑ERG10, ↑tHMG1, ↑TAL1, ΔTAL1, Δpho13, and ΔAld6	Squalene	532	Kwak et al., 2017
Saccharomyces cerevisiae	↑ SsXYL1, ↑SsXYL2, ↑SsXYL3, ↑TKL1, ↑ERG10, ↑tHMG1, ↑TAL1, ↑ADS, ΔTAL1, Δpho13, and ΔAld6	Amorphadinene	254	Kwak et al., 2017
Saccharomyces cerevisiae	↑ SsXYL1, ↑SsXYL2, ↑SsXYL3, ↑CrtE/I/YB, ↑Blh, Δpho13, and ΔAld6	Vitamin A	3,350	Sun et al., 2019
Yarrowia lipolytica	↑ XYL1, ↑XYL2, ↑XKS, ↑TAL (tyrosine ammonia lyase), ↑4CL, ↑CHS, ↑CHI, and ↑XT (xylose transporter, YALI0B00396)	Naringenin	715	Wei et al., 2020b

and artificial genomes (Guo et al., 2020) has greatly facilitated genetic modifications, although delicate design is always required. A detailed description of all the genetic tools and strategies suitable for *Y. lipolytica* engineering can be found in a very recent review (Ma et al., 2020).

It had long been thought that *Y. lipolytica* could not naturally utilize xylose as the only carbon source (Blazeck et al., 2014; Zhao et al., 2015), and early attempts enabling xylose metabolism relied on introduction of heterologous pathways from S. stipitis, although such a phenotype tends to be unstable and needs to be strengthened through further adaptation for higher expression levels of XR-encoding genes (Ledesma-Amaro et al., 2016; Wu et al., 2019). However, recent studies have discovered a functional endogenous xylose-metabolizing pathway, which can be highly efficient after facile engineering. Wildtype strain PO1f (ATCC MYA-2613) carries genes encoding active XR, XDH, and XKS at low expression levels, which can be upregulated when xylose is used as the sole carbon source (Ryu et al., 2015; Rodriguez et al., 2016), although it is reported that the two XR-encoding genes are constitutively expressed at stable levels irrespective of the growth stage or the carbon source used for cell cultivation (Rodriguez et al., 2016). Overexpression of XDH- or XKS-encoding gene alone or in combination considerably improves xylose assimilation and conversion (Ryu et al., 2015; Rodriguez et al., 2016), whereas simultaneous overexpression of XR and XKS does not enable cells to grow on xylose (Wu et al., 2019), suggesting expression of XDH and XKS is the bottleneck in natural xylose metabolism (Ryu et al., 2015; Rodriguez et al., 2016). In addition, strain PO1f contains five putative xylose-specific transporters (Ryu et al., 2015), and overexpression of the transporter YALI0B00396 (a co-transporter for xylose and cellobiose) improves xylose uptake (Ryu and Trinh, 2018; Wu et al., 2019; Wei et al., 2020b). Interestingly, xylose metabolism in strain PO1f is not repressed by the presence of glucose (Ryu et al., 2015) as long as glucose concentration is below 2 g/L (Rodriguez et al., 2016), which is distinct from another natural xylose-utilizing strain PO1g whose xylose metabolism is mildly repressed by glucose (Tsigie et al., 2011). These studies have opened up an avenue to xylose utilization in *Y. lipolytica* without the need of complicated genetic engineering, despite the fact that xylose-based cell growth is, in many cases, slower compared with cell growth on glucose.

Yarrowia lipolytica has excellent capability of accumulating acetyl-CoA and malonyl-CoA, and is hence a theoretically preferred host for the production of fatty acids, terpenoids, flavonoids, and other compounds that use acetyl/malonyl-CoA as a precursor (Abdel-Mawgoud et al., 2018). In the past few years, the potential of this host has been greatly explored in the biosynthesis of useful compounds such as lipids and biofuels from various carbon sources (Zhu and Jackson, 2015;

Du et al., 2016; Ledesma-Amaro et al., 2016; Lv et al., 2019; Ma et al., 2019; Palmer et al., 2020; Shang et al., 2020); however, xylose-based biosynthesis of natural products has been scarcely investigated despite attempts to understand and improve xylose metabolism in *Y. lipolytica* (Tsigie et al., 2011; Ryu et al., 2015; Rodriguez et al., 2016; Wei et al., 2020b). On one hand, this is attributed to the lower growth rate and biomass accumulation when cells are cultivated in xylose than in glucose (Ledesma-Amaro et al., 2016); on the other hand, the functional and highly efficient expression of heterologous metabolic pathways relevant to natural product biosynthesis depends on sophisticated tools for genetic manipulation, which are still very limited. Recently, strain ATCC 201249 was engineered to produce the isoprenoid compound protopanaxadiol from xylose (Wu et al., 2019). Overexpression of XR (with K270R/N272D mutations to convert cofactor preference from NADPH to NADH) and XDH from *Sc. stipitis* and endogenous XKS, followed by xylose adaptation and overexpression of xylose transporter YALI0B00396, enabled the strain to consume 20 g/L of xylose in 72 h, reaching an OD_{600} of 32 in shake flasks. Introduction of the biosynthetic pathway of protopanaxadiol together with fusion expression of pathway enzymes and overexpression of genes involved in precursor supply resulted in a titer of 300 mg/L using fed-batch fermentation. In this process, xylose was preferred to glucose as the carbon source, due to the fact that glucose supported fast cell growth and led to rapid generation and accumulation of acetyl-CoA, which was then channeled for the generation of metabolites other than precursors (Wu et al., 2019). In the latest study, a xylose-inducible machinery was designed to couple endogenous xylose utilization and naringenin biosynthesis, producing 511 mg/L of naringenin in 120 h from 20 g/L glucose and 20 g/L xylose in shake flasks without externally added precursors; the titer was further increased to 715 mg/L in 144 h with an elevated xylose concentration (60 g/L; Wei et al., 2020a,b).

Recently, *Y. lipolytica* has been engineered to produce triglycerides, which can form oil droplets inside cells. These droplets are capable of trapping and storing certain heterologously generated compounds, whose overproduction imposes a threat to cell growth. Thus, *Y. lipolytica* can be used as a good host to produce hydrophobic natural products, including terpenoids and aromatics, which are prone to formation of crystals in the cytosol that are harmful to the host. Concomitant generation of triglycerides allows hydrophobic natural compounds to be stored in oil droplets, and hence protects the host and guarantees continuous production of the target compounds.

CONCLUDING REMARKS

Biorefinery of lignocellulosic hydrolysates as feedstocks to produce advanced fuels and chemicals holds great promise to develop sustainable bioeconomy. As the second most abundant monosaccharide in lignocellulosic hydrolysates, xylose has been attracting much attention for its efficient utilization and conversion into valuable compounds beyond ethanol. Metabolic engineering of yeasts to convert xylose into natural products is a promising option to implement the biotransformation due to some special characteristics of certain yeast strains, such as moderate tolerance to toxic inhibitors in lignocellulosic hydrolysates, and moderate compatibility of heterologous pathway genes derived from both prokaryotes and eukaryotes. Recently, metabolic engineering of *Sc. stipitis*, *Sa. cerevisiae* and *Y. lipolytica* has been accomplished for the biosynthesis of natural products, mainly terpenoids, flavonoids, and aromatics. Some of these microbial production processes function better on xylose than on glucose, as xylose induces reprograming of central metabolism and enhances the supply of key precursors, such as acetyl-CoA.

Currently, most of the studies are centered on *Sa. cerevisiae* due to the availability of facile genetic engineering tools and methods. However, this yeast relies on heterologous pathways for xylose utilization, which are not always highly efficient due to cofactor imbalance. *Sc. stipitis* is a superior xylose-utilizer and, when grown on xylose, generates many intermediate metabolites that are critical for the formation of natural compounds. Nevertheless, the lack of physiological information and genetic engineering tools limits its applications mainly to ethanol production. It appears that *Y. lipolytica* has the merits of both *Sa. cerevisiae* and *Sc. stipitis*. This organism contains native xylose-utilizing genes, and can be turned into an efficient xylose-utilizing factory upon overexpression of these native genes. In addition, the gene arrangement in its genome as well as the established gene manipulation strategies allows for the construction of *Y. lipolytica* workhorse for the generation of natural compounds from xylose. However, the growth rate of this yeast is generally much lower on xylose than on glucose, making the whole bioconversion inefficient. Extensive engineering work is needed to overcome these difficulties.

Another challenge associated with the utilization of lignocellulosic hydrolysates is the presence of toxic compounds, such as furfural and phenolics, generated in the physiochemical pretreatment of biomass. These compounds greatly suppress the growth of fermentation microorganisms and inhibit the biosynthesis of the target products. Thus, development of highly tolerant yeast strains is of critical significance. Strain adaptation or engineering should be considered for the construction of highly efficient yeast cell factories capable of utilizing lignocellulosic hydrolysates for the generation of natural products.

AUTHOR CONTRIBUTIONS

JZ, WQ, and XW conceived the project. JZ, MY, and XW wrote the manuscript. All the authors read and approved the manuscript.

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Transcription Factor-Based Biosensor for Dynamic Control in Yeast for Natural Product Synthesis

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The synthesis of natural products in yeast has gained remarkable achievements with intensive metabolic engineering efforts. In particular, transcription factor (TF)-based biosensors for dynamic control of gene circuits could facilitate strain evaluation, high-throughput screening (HTS), and adaptive laboratory evolution (ALE) for natural product synthesis. In this review, we summarized recent developments of several TF-based biosensors for core intermediates in natural product synthesis through three important pathways, i.e., fatty acid synthesis pathway, shikimate pathway, and methylerythritol-4-phosphate (MEP)/mevalonate (MVA) pathway. Moreover, we have shown how these biosensors are implemented in synthetic circuits for dynamic control of natural product synthesis and also discussed the design/evaluation principles for improved biosensor performance.

Keywords: yeast, biosensor, natural products, fatty acids, shikimate pathway, MEP/MVA pathway, transcription factor

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INTRODUCTION

Microbial synthesis of natural products not only ensures sustainable production but also enables synthesis of novel derivatives of interest. Yeast has been exploited as microbial cell factories to produce natural products that belong to several families, including fatty acids, isoprenoids, flavonoids, and alkaloids (Meadows et al., 2016; Rodriguez et al., 2017; Yu et al., 2018; Srinivasan and Smolke, 2020). With good knowledge of cell metabolism and well-developed synthetic biology technology and tools, much progress has been made through intensive metabolic engineering efforts to maximize flux toward natural products and improve titers, rates, and yields (Cravens et al., 2019; Liu et al., 2019, 2020). However, efficient biosynthesis is still challenging, and metabolic imbalance might be a key issue accounting for low yields and titers. Recently, dynamic control strategies have been developed to address the imbalance issue, as well-reviewed in Shen et al. (2019); Hartline et al. (2020), and Marsafari et al. (2020). Among these strategies, transcription factor (TF)-based biosensors could regulate the expression of gene circuits in response of specific intracellular metabolites and have been applied in natural product synthesis (Hossain et al., 2020). Besides, biosensors could accelerate metabolite quantification and have been used for strain evaluation, high-throughput screening (HTS), and adaptive laboratory evolution (ALE) (Williams et al., 2016; Qiu et al., 2019; Hossain et al., 2020).

In a TF-based biosensor, TF undergoes an allosteric conformational change induced by metabolite binding, which affects its binding at its operator and thereof regulates gene transcription (Zhang and Keasling, 2011; Zhang et al., 2015). Among a wide range of natural TFs that have

been utilized in yeast, prokaryotic TFs have attracted much attention due to its relatively simple transcriptional regulation mechanism compared to eukaryotic TFs. A prokaryotic TF can be translated into a functional yeast biosensor with its operator inserted into a well-characterized promoter or a synthetic promoter (Qiu et al., 2019; Wan et al., 2019). Besides, endogenous TFs could potentially be utilized in yeast biosensor, as demonstrated in several studies (Zhang et al., 2016; Leavitt et al., 2017). For better performance in dynamic control of gene circuits, a TF-based sensor is required to be orthogonal and tunable in its dynamic range, operational range, specificity, and sensitivity, considered as common design and evaluation principles (Hossain et al., 2020).

Here we reviewed several TF-based biosensors developed in yeast and recent applications for production of natural products, including biosensors for malonyl-CoA, fatty acyl-CoA, isopentenyl pyrophosphate (IPP), naringenin, and aromatic amino acids (AAAs), which are core metabolites for synthesis of several families (**Figure 1**). Through these studies on the biosensors, design and evaluation principles were also reviewed and discussed, as well as engineering strategies for improved performance.

SENSORS DEVELOPED FOR DYNAMIC CONTROL OF THE FATTY ACID SYNTHESIS PATHWAY

The fatty acid synthesis pathway from malonyl-CoA via a cyclic elongation manner could be engineered and utilized for production of natural products like cocoa butter equivalents and jojoba-like wax esters (Bergenholm et al., 2018; Wenning et al., 2019; **Figure 1**). Biosensors for two essential intermediates malonyl-CoA and fatty acyl-CoA have been developed and discussed as below.

Malonyl-CoA Biosensor

Malonyl-CoA serves as a basic unit for fatty acids, flavonoids, and non-ribosomal polyketides. The fatty acid and phospholipid regulator FapR and its operator *fapO* from *Bacillus subtilis* have been extensively studied and exploited for malonyl-CoA sensors in *E. coli*, *S. cerevisiae*, and mammalian cells (Ellis and Wolfgang, 2012; Xu et al., 2014a; Li et al., 2015; Johnson et al., 2017). FapR binds to *fapO* and functions as a repressor to inhibit gene transcription, and the repression can be relieved by malonyl-CoA, which induces a conformational shift in FapR and releases it from *fapO* (Schujman et al., 2006; Xu et al., 2014b; **Figure 2A**).

In yeast, FapR was usually expressed under a strong constitutive promoter to regulate reporter gene expression under a *fapO*-hybrid promoter, in which *fapO* was inserted in a well-characterized promoter. Using fluorescence proteins as the reporters, different designs were evaluated for sensitivity, dynamic range, and operational range (Li et al., 2015; David et al., 2016; Chen et al., 2018), including varying FapR/*fapO* ratio or *fapO* position in hybrid promoters and introducing nucleus localization sequences (NLS).

With a dual plasmid sensor system, one harboring FapR expressed under a *TEF1* promoter and the other harboring tdTomato regulated under a hybrid *fapO-GPM1* promoter, Li et al. (2015) found that a strong SV40 NLS significantly enhanced FapR nuclear import and subsequent repression. The biosensor with multicopy FapR and single *fapO* was identified as a better design with a broad dynamic range, up to 4-fold fluorescence increase in response to 8 mg/L cerulenin, which was supplemented as a fatty acid synthase inhibitor to increase the cellular malonyl-CoA level (Li et al., 2015). In another study, David et al. (2016) established a malonyl-CoA sensor with FapR expressed under a *TEF1* promoter and a green fluorescent protein (GFP) under a hybrid *fapO-TEF1* promoter. The optimized sensor with three *fapO* sites could respond to 13.5 μM cerulenin with up to 1.9-fold increase in the GFP fluorescence. The biosensor was then utilized for dynamic control for 3-hydroxypropionic acid (3-HP) production with fatty acid synthase expressed under a glucose sensitive *HXT1* promoter to control the malonyl-CoA availability. The hierarchical dynamic control improved 3-HP titer from 0.4 to 1.0 g/L, which could be applied for production of other malonyl-CoA-derived natural products. Ferreira et al. (2019) also combined the sensor with dCas9-based regulation for fine-tuned metabolism to increase acetyl-CoA and malonyl-CoA availability, which facilitated HTS and significantly improved 3-HP production. In a recent study, a malonyl-CoA sensor was constructed with FapR expressed under a *TEF1* promoter and yeGFP (yeast-enhanced GFP) under a hybrid *TEF1-fapO-GAL1* promoter and showed a better sensitivity, up to 8-fold yeGFP increase in response to 10 μM cerulenin (Chen et al., 2018). The sensor was then used to screen phosphorylation site mutations of acetyl-CoA carboxylase Acc1 and identified that $Acc1^{S686AS659AS1157A}$ could benefit malonyl-CoA and 3-HP production.

Interestingly, when FapR was fused with transcriptional activation domain (AD) of an activator, it activated gene transcription under a *fapO*-hybrid promoter (Qiu et al., 2020; **Figure 2B**) instead of repressing. Qiu et al. (2020) compared several activators, including Gal4 AD, herpes simplex virus VP16, yeast transcriptional mediator Med2, and hybrid activators Med2-Gal4 and VP64-p65-Rta (VPR), and identified Med2 as the best candidate, which activated transcription by more than 40-fold, compared with the control without FapR-AD. With an optimized hybrid promoter, the *LEU2* promoter with the 1 *fapO* site inserted, the malonyl-CoA sensor increased GFP intensity by 53-fold compared to the control without FapR-AD. The final sensor showed a dose-dependent repressive response to cellular malonyl-CoA in an operational range of 0–20 μ M cerulenin.

Fatty Acyl-CoA Biosensor

Fatty acyl-CoAs serve as direct precursors for fatty acid-derived bioproducts (Marchetti et al., 2018). Transcription factor FadR and its operator *fadO* from *E. coli* have been evaluated for fatty acyl-CoA sensors in yeast (Teo et al., 2013; Teo and Chang, 2014; Dabirian et al., 2019). In *E. coli*, FadR regulates fatty acid metabolism with dual functions, which represses essential genes of fatty acid degradation and activates those of fatty acid synthesis

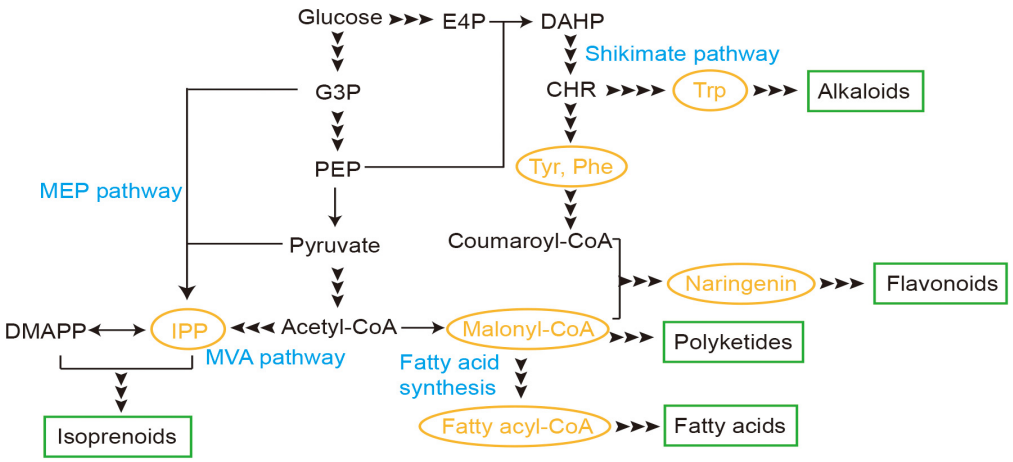

FIGURE 1 | Transcription factor (TF)-based biosensors involved in synthesis pathways of natural products in yeast. E4P, erythrose-4-phosphate; G3P, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; DAHP, 3-deoxy-D-arabino-2-heptulosonic acid 7-phosphate; CHR, chorismic acid; MEP, methylerythritol-4-phosphate; MVA, mevalonate; Trp, tryptophan; Tyr, tyrosine; Phe, phenylalanine.

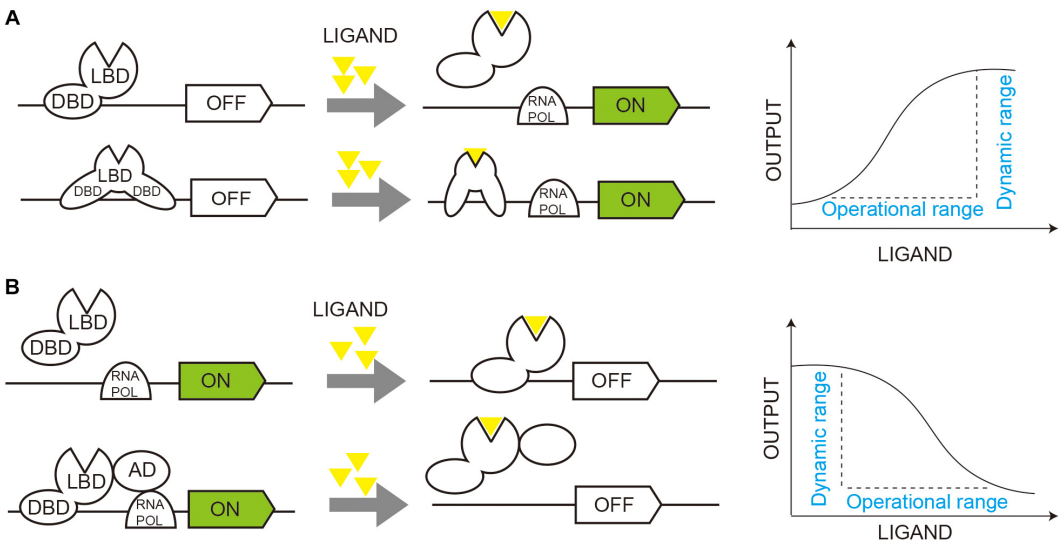

FIGURE 2 | Dynamic regulation of gene circuits through TF-based biosensors. **(A)** Activated regulation of gene circuits. Transcription factor (TF) functions as a repressor to inhibit gene transcription (OFF), and ligand binding induces a conformational shift in TF and activates gene transcription (ON). **(B)** Repressive regulation of gene circuits. TF functions as an activator of gene transcription (ON), and ligand binding induces a conformational shift in TF and inhibit gene transcription (OFF). DBD, DNA binding domain; LBD, ligand binding domain; RNA Pol, RNA polymerase.

by binding different regions of corresponding promoters (van Aalten et al., 2001; Iram and Cronan, 2005).

Teo et al. (2013) first established a FadR-*fadO* sensor in yeast with FadR expressed under a *TEF1*/*CYC1* promoter and yEGFP under a hybrid *fadO*-*GAL1* promoter, in which FadR functioned as a repressor. Biosensor optimization with varied FadR/*fadO* ratios identified that a combination of a *TEF1* promoter and three operators resulted in a sensor with a broad dynamic range, which could respond to 1 mM extracellular myristic acid with 1.4-fold induced transcription (Teo et al., 2013). Dabirian et al. (2019) also tuned FadR expression and modified hybrid *fadO*-*TEF1* promoters to optimize the biosensors. The optimized biosensor was then utilized in HTS of a gene overexpression library to increase the fatty acyl-CoA pool. The enriched genes were then evaluated for fatty acid and fatty alcohol production and identified three genes that could increase fatty alcohol levels by 1.8-fold (Dabirian et al., 2019). In another study, Teo and Chang (2014) combined the FadR-*fadO* biosensor with inducible promoters to construct AND-gate dynamic controllers, in which enhancer sequences of inducible promoters (*CUP1*, *PHO5*) were fused to a synthetic *GAL1* core promoter containing three *fadO* sites. In the AND-gate controllers, both fatty acids and

copper presence/phosphate starvation were required to switch the AND-gate ON (Teo and Chang, 2014). The successful combination of logic gates with a biosensor would represent more flexible dynamic control on gene circuits for bioproduct synthesis in yeast.

By fusing AD to FadR, the FadR repressor could be translated to a transcriptional activator in the presence of the effector molecule acyl-CoA (Qiu et al., 2020). When FadR was fused with VPR and Med2, the biosensor resulted in transcriptional activation by 23.9- and 28.4-fold, respectively. The engineered biosensor with FadR-Med2 and one *fadO* downregulated gene expression to 42.8% as 2.0 mM oleic acid was supplemented.

SENSORS DEVELOPED FOR DYNAMIC CONTROL OF THE MVA/MEP PATHWAY

Isoprenoids were synthesized via carbon chain elongation by repeated addition of IPP or dimethylallyl pyrophosphate, which could be produced either from acetyl-CoA via the MVA pathway or from pyruvate and glyceraldehyde-3-phosphate through the MEP pathway (**Figure 1**). IPP biosensors have been developed and engineered for its potential application in isoprenoid synthesis.

Isopentenyl pyrophosphate (IPP) is an essential intermediate for isoprenoid synthesis, and its supply may be limited to its high toxicity. Therefore, dynamic control through the IPP node will benefit synthesis pathways for isoprenoids. However, no natural TF has been identified responsive to IPP. In yeast, a synthetic TF was repurposed for sensing IPP by fusing IPP isomerase Idi to the *GAL4* AD and DNA binding domain (DBD), respectively. With Idi-DBD bound to the *GAL10* promoter, Idi dimerization triggered by IPP could bring Idi-AD close enough to activate transcription of a fluorescent protein yEcitrine (Chou and Keasling, 2013). The IPP biosensor was introduced into a reported isoprenoid-producing strain MO219 (Ro et al., 2006) and showed 1.5-fold increase in fluorescence when its MVA pathway overexpression was induced to increase IPP supply. The authors additionally replaced Idi with another two enzymes utilizing IPP as a substrate (Idi1 and Erg20), and the two sensors showed higher fluorescence levels upon galactose induction. The strategy to construct IPP sensors with synthetic TFs was demonstrated applicable in *E. coli* as well and was used in a feedback-regulated evolution to improve lycopene production by nearly 6.8-fold. Therefore, synthetic TFs alleviated the need to rely on preexisting biological components.

SENSORS DEVELOPED FOR DYNAMIC CONTROL OF THE SHIKIMATE PATHWAY

The shikimate pathway for biosynthesis of AAAs has been studied and engineered for *de novo* biosynthesis of various aromatic products, like flavonoids and alkaloids (**Figure 1**). Here, we focused on biosensors developed for core intermediates, including naringenin and AAAs.

Naringenin Biosensor

Naringenin can be synthesized from malonyl-CoA and coumaroyl-CoA derived from intermediates of the shikimate pathway and is a key intermediate for synthesis of other flavonoids used as nutritional supplementary and pharmaceuticals (**Figure 1**). Transcription factor FdeR from *Herbaspirillum seropedicae* and its operator *FdeO* were repurposed as naringenin biosensors in both *E. coli*, *S. cerevisiae* and *Yarrowia lipolytica* (Skjoedt et al., 2016; Lv et al., 2020).

In *S. cerevisiae*, a naringenin biosensor was constructed via expressing FdeR under a *TDH3* promoter and GFP under a hybrid *FdeO-CYC1* promoter (Skjoedt et al., 2016), in which FdeR functioned as a co-inducer of naringenin. The sensor, which could induce 1.7-fold GFP increase in response to 0.2 mM naringenin, was then transformed into engineered strains to monitor naringenin production. Strong linear correlations at 24 h (r = 0.87) and 48 h (r = 0.96) between GFP intensities and metabolite concentrations (Skjoedt et al., 2016) made the sensor applicable for HTS of naringenin production strains. In a recent study, by varying NLS locations and expression levels, a naringenin sensor with NLS-FdeR expressed under a *TDH3* promoter and mCherry under a hybrid *FdeO-GPM1* promoter in a high-copy number plasmid showed a relatively broad dynamic range and sensitivity, a 3-fold fluorescence increase in response to 0.2 mM naringenin. The sensor was then to screen a modular assembled naringenin biosynthetic library with 972 combinations and obtained a strain with a titer of 52.0 mg/L naringenin (Wang et al., 2019).

In *Y. lipolytica*, a naringenin biosensor with FdeR expressed under a *TEF* promoter and the *Nluc* luciferase under a hybrid *Fdeo-TEF* promoter was constructed, and the sensor showed an operational range of 0–50 mg/L naringenin (Lv et al., 2020). By expressing FdeR under a weak promoter and Leu2 under a hybrid *Fdeo-TEF* promoter, the sensor enabled naringenin inducible growth in a leucine auxotrophic strain. The modified sensor was found to selectively enrich the naringenin-producing population and maintain strain stability (Lv et al., 2020).

Aromatic Amino Acid Biosensor

Aromatic amino acids (AAAs) including tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe) are synthesized from the intermediates of the shikimate pathway (**Figure 1**). Endogenous regulation induced by AAAs in yeast has been studied and considered as attractive and potent inducible for metabolic engineering. Expression of *ARO9*, encoding AAA transferase II protein, was found activated via transcription factor Aro80 in the presence of AAAs (Lee and Hahn, 2013), which could be exploited for development of AAA biosensors.

Leavitt et al. (2016) enlarged the minimally sufficient UAS_{aro} element dissected from the *Aro9* promoter with Aro80 binding site and fused it with a core promoter to form a hybrid promoter, which could induce the expression of yellow fluorescent protein (YFP) in response to exogenous tryptophan. With an Aro80 variant expressed under a strong *GAL1* promoter and five UAS_{aro} sites inserted in a minimal core promoter, a AAA biosensor was constructed, which could respond to 1 g/L tryptophan

and 1 g/L galactose with 6- and 12-fold induced expression, respectively. In the presence of tryptophan and galactose, the biosensor resulted in 14-fold induced GFP expression. This work demonstrated the potential of native transcription factors for biosensor construction.

A biosensor based on Aro80-UAS_{aro} was then used in ALE for production of AAAs and muconic acid (Leavitt et al., 2017). In the biosensor, *YFP* was replaced by the antibiotic gene *KanNeo* under a hybrid UAS_{aro}-*LEU* promoter to couple cell growth with AAA production. Based on the anti-metabolite selection with 4-fluorophenylalanine in ALE, the strains were evolved with up to 2-fold higher total AAA production, which were engineered to redirect increased flux to muconic acid production by expressing a truncated Aro1 and an aromatic decarboxylase. The final strain could produce 0.5 g/L muconic acid in shake flasks and 2.1 g/L in a fed-batch bioreactor.

Prokaryotic TF was also exploited for tryptophan biosensors in yeast. TrpR from *E. coli* functions as an aporepressor, and it binds at its operator *TrpO* in the presence of tryptophan to repress transcription (Gunsalus and Yanofsky, 1980; **Figure 2B**). In yeast, a tryptophan biosensor was developed based on TrpR-*TrpO* with *GFP* expressed under a hybrid *TrpO-TEF1* promoter (Zhang et al., 2020). The biosensor could repress GFP expression by up to 2.4-fold in an operational range of 2–200 mg/L tryptophan. The repression biosensor was then converted to an activation sensor by fusing Gal4 AD to TrpR ($Gal4_{AD}$-TrpR), which was expressed under a weak *REV1* promoter. With six copies of *TrpO* inserted into a *GAL1* core promoter, the constructed biosensor could sense 2–200 mg/L tryptophan with a 5-fold dynamic range. The biosensor was used for HTS of a combinatorial library based on a platform strain with increased AAA accumulation and facilitated mechanistic and machine learning models with the recommendations, which improved tryptophan titer and productivity by up to 74 and 43%, respectively (Zhang et al., 2020).

CONCLUSION AND PERSPECTIVES

Transcription factor (TF)-based biosensors have become powerful tools in strain evaluation, HTS, and ALE for yeast synthesis of natural products. With more natural TFs identified and characterized both experimentally and bioinformatically, TF-based biosensors for more metabolites could be established to facilitate natural product synthesis. However, one big challenge for TF biosensors might be for their applicability in natural product synthesis where a larger dynamic range is needed. The biosensor design may need to be adjusted or evolved as the titers become higher or be matched to the production kinetics of the individual strain or library of biocatalysts.

Biosensor engineering on TFs and the hybrid operator-promoters have enabled improved sensing sensitivity, dynamic range, operational range, and ligand specificity, as well as inversion of function between activated and repressive regulation (Skjoedt et al., 2016; Snoek et al., 2019; Ambri et al., 2020; Qiu et al., 2020). Evolution-guided engineering equipped with TF mutagenesis and user-defined FACS-based toggled selection could be a versatile and high-throughput method to generate user-defined biosensors (Snoek et al., 2019). Also, the variants of a TF could be targeted for new effectors with the help of protein engineering as well as computational design (de los Santos et al., 2016). Optimal biosensor reporter promoter scanning revealed that TF operator positions could be critical for improved biosensor performances, enabling a redesigned TF biosensor with a dynamic output range up to 26-fold (Ambri et al., 2020). The functional inversion of TF-based biosensors allows for two distinct regulatory responses to the ligand metabolites and could be utilized to construct metabolite switch, as demonstrated in *E. coli*, which resulted in a balanced metabolism between cell growth and product formation (Xu et al., 2014a).

It is also interesting to notice the construction of logic gates in TF-based biosensors (Teo and Chang, 2014; Leavitt et al., 2016), which could be possibly exploited for higher-order designs in reprogramming dynamic regulation of gene circuits for natural product synthesis. Besides, G-protein-coupled receptor (GPCR)-based biosensors have been engineered for sensing medium-chain fatty acids in yeast (Mukherjee et al., 2015), representing alternative genetic biosensors to dynamically control the synthesis of natural products in yeast (Marsafari et al., 2020). Additionally, the emerging optogenetics-based sensors can be applied with any change in chemicals (Marsafari et al., 2020), giving great advantages to advance the construction of future yeast cell factory to dynamically control cellular metabolism or dissect cellular network function.

AUTHOR CONTRIBUTIONS

YZ and SS outlined this manuscript. YZ drafted the manuscript. SS revised the manuscript. Both authors contributed to the article and approved the submitted version.

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Refining Metabolic Mass Transfer for Efficient Biosynthesis of Plant Natural Products in Yeast

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Plant natural products are important secondary metabolites with several special properties and pharmacological activities, which are widely used in pharmaceutical, food, perfume, cosmetic, and other fields. However, the production of these compounds mainly relies on phytoextraction from natural plants. Because of the low contents in plants, phytoextraction has disadvantages of low production efficiency and severe environmental and ecological problems, restricting its wide applications. Therefore, microbial cell factory, especially yeast cell factory, has become an alternative technology platform for heterologous synthesis of plant natural products. Many approaches and strategies have been developed to construct and engineer the yeast cells for efficient production of plant natural products. Meanwhile, metabolic mass transfer has been proven an important factor to improve the heterologous production. Mass transfer across plasma membrane (trans-plasma membrane mass transfer) and mass transfer within the cell (intracellular mass transfer) are two major forms of metabolic mass transfer in yeast, which can be modified and optimized to improve the production efficiency, reduce the consumption of intermediate, and eliminate the feedback inhibition. This review summarized different strategies of refining metabolic mass transfer process to enhance the production efficiency of yeast cell factory (**Figure 1**), providing approaches for further study on the synthesis of plant natural products in microbial cell factory.

Keywords: plant natural products, intracellular mass transfer, trans-plasma membrane mass transfer, metabolic engineering, yeast

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INTRODUCTION

Plant natural products are secondary metabolites with complex molecular structures and inherent bioactivities (Williams et al., 1989), including a variety of organic compounds, such as flavonoids, terpenes, saponins, alkaloids, and sterols, which are widely used in medicine, food, perfume, and cosmetic (Mizutani et al., 1994; Enserink, 2005; Peraltayahya et al., 2011; Xu et al., 2017). Although these plant natural compounds and their derivatives display a broad range of applications in many areas, the current production mode mainly relies on extraction from plants, which is costly and complex because of the low concentration and plentiful structural analogs in their native producers (Nour et al., 2009). The low concentration and complex purification process limit the scale-up production of plant natural compounds via phytoextraction or chemical synthesis, failing to meet the market demand.

Fortunately, synthetic biology and system biology provide alternative approaches to solve such problems, which has facilitated the heterologous synthesis of plant natural products by microbial cell factory in the passing two decades (Shan et al., 2005; Moses et al., 2014;

King et al., 2016). Compared with traditional extraction and chemical synthesis, the usage of fast-growing microorganisms dramatically shortens the production cycle, reduces environmental pollution, and simplifies the separation process for the synthesis of single structural compound in microbial cell factory (Sun et al., 2020). Many metabolic engineering strategies including overexpression of key enzymes, knocking out of competition pathway, and enzyme engineering have been employed to produce different kinds of plant natural products, such as amorphadiene (Reddingjohanson et al., 2011), farnesene (Su et al., 2015; Meadows et al., 2016; Yang et al., 2016), bisabolene (Kirby et al., 2014), miltiradiene (Hu et al., 2020), naringin (Park et al., 2009; Lv et al., 2019), kaempferol (Trantas et al., 2009), and coumarin (Ro and Douglas, 2004). Among these strategies, efforts to refining metabolic mass transfer of microorganisms (Noorman, 2001) by forming substrate channel or metabolic compartmentalization have been proven to play important roles on improving the production of plant natural products. For instance, the heterologous synthesis of tropane alkaloids was enhanced by refining metabolic mass transfer (Srinivasan and Smolke, 2020). Nowadays, many microorganisms have been used as chassis to produce plant natural products. Yeast, especially *Saccharomyces cerevisiae*, is one of the most favorite hosts for its inherent characteristics such as abundant precursors and complete endomembrane system (Li et al., 2018). As a GRAS (generally recognized as safe) strain with good robustness, yeast has been widely used in food and pharmaceutical production (Paddon et al., 2013; Khan et al., 2015). According to the space where transfer happens, the metabolism mass transfer in yeast can be divided into two types, which are the intracellular mass transfer (Lodish, 1988) and the trans-plasma membrane mass transfer (Whittam and Wheeler, 1970). In this article, recent advances about metabolic engineering strategies of plant natural compounds, especially optimization of metabolic mass transfer in yeast cell factory, were reviewed. Insights of engineering yeast as an efficient platform for product synthesis are also provided. Moreover, we illustrated the great potential for the synthesis of plant natural compound in yeast.

INTRACELLULAR MASS TRANSFER IN YEAST CELL FACTORY

Intracellular metabolic mass transfer is an essential part of cell metabolism, which mainly refers to the substance's delivery between enzymes and organelles. It is also one of the key factors to develop efficient microbial cell factory containing biosynthetic pathways with multienzyme reactions. The regulation of metabolic flux of the biosynthetic pathway and the spaces of different enzymes have been proven efficient ways for the refining of intracellular mass transfer. The production of plant natural products can be thus improved (**Figure 1**). In recent years, to boost the production of plant natural products in yeast cell factory, several strategies including protein fusion (Zhou et al., 2012) and artificial scaffolds (Wang and Yu, 2012) have been developed to strengthen the efficiency of mass transfer especially improve the catalytic efficiency of multi-enzyme reactions. In some cases, plant natural products and their intermediates are toxic to yeast cells (Valachovic et al., 2016), and some intermediates have feedback inhibitions to enzymes. To solve these issues, regulation strategies such as enzyme compartmentalization (Avalos et al., 2013) and suborganelles (Smith et al., 2000) were used to restrict intermediate distribution and redirect them toward the catalytic enzymes. As a result, the cytotoxicity was reduced, and the production was promoted. Here, several strategies such as optimizing the space location of enzymes and substances and enhancing the intracellular mass transfer in yeast cell factory have been summarized.

Fusion Expression of Enzymes Shortens the Distance of Metabolite Diffusion

The biosynthetic pathway of plant natural compounds usually contains multienzyme reactions and locates in different organelles, which leads to some challenges in mass transfer efficiency and the toxicity of intermediates in microorganisms. Fusion expression is an effective way to shorten the distance between two proteins. Linking different genes together to obtain fusion protein is the most common strategy to colocalize enzymes of long pathway. In this way, the spatial location of enzymes can be adjusted to reduce cytotoxicity of intermediates and then to increase the intermediates transfer efficiency between different enzymes. For example, the 4-coumarate CoA-ligase (4CL), stilbene synthase (STS), and tyrosine ammonia lyase (TAL) were necessary enzymes in the polyketide resveratrol biosynthetic pathway in yeast. When the 4CL and STS were expressed in a fusion protein, the yield of resveratrol was increased by 15-fold (Zhang et al., 2006). The titer of patchoulol was increased by two times when the fusion expression of patchoulol synthase and FPP synthase was employed in *S. cerevisiae* (Albertsen et al., 2011). By fusion expression of SmCPS and SmKSL, as well as fusing the GGPP synthase and farnesyl diphosphate synthase, the metabolic flux of miltiradiene synthetic pathway was enhanced, and the titer of miltiradiene reached 488 mg/L in a 15-L bioreactor (Zhou et al., 2012). To improve geraniol production, the synthesis of geranyl diphosphate (GPP), the precursor of geraniol, was regulated by controlling the expression of endogenous ERG20, coupled with up-regulation of the mevalonate pathway by co-overexpressing IDI1, tHMG1, and UPC2-1 (Zhao et al., 2016). Moreover, the fusion expression of the key enzymes was improved by optimizing the amino acid linker and the order of the proteins, resulting in a production of 293 mg/L geraniol in fed-batch cultivation (Zhao et al., 2016). The fusion expression of enzymes can adjust the intracellular metabolic mass transfer of synthetic pathways, which suggests that adjusting physical localization of key enzymes can shorten the distance of metabolite diffusion and thus facilitate the yield of production.

Utilization of Artificial Scaffolds Forms the Substrate Channeling

The utilization of fusion protein is a relatively simple and efficient way to regulate intracellular mass transfer. However, there are still some problems. For instance, in some cases, the fusion protein will form inclusion body without enzyme activities.

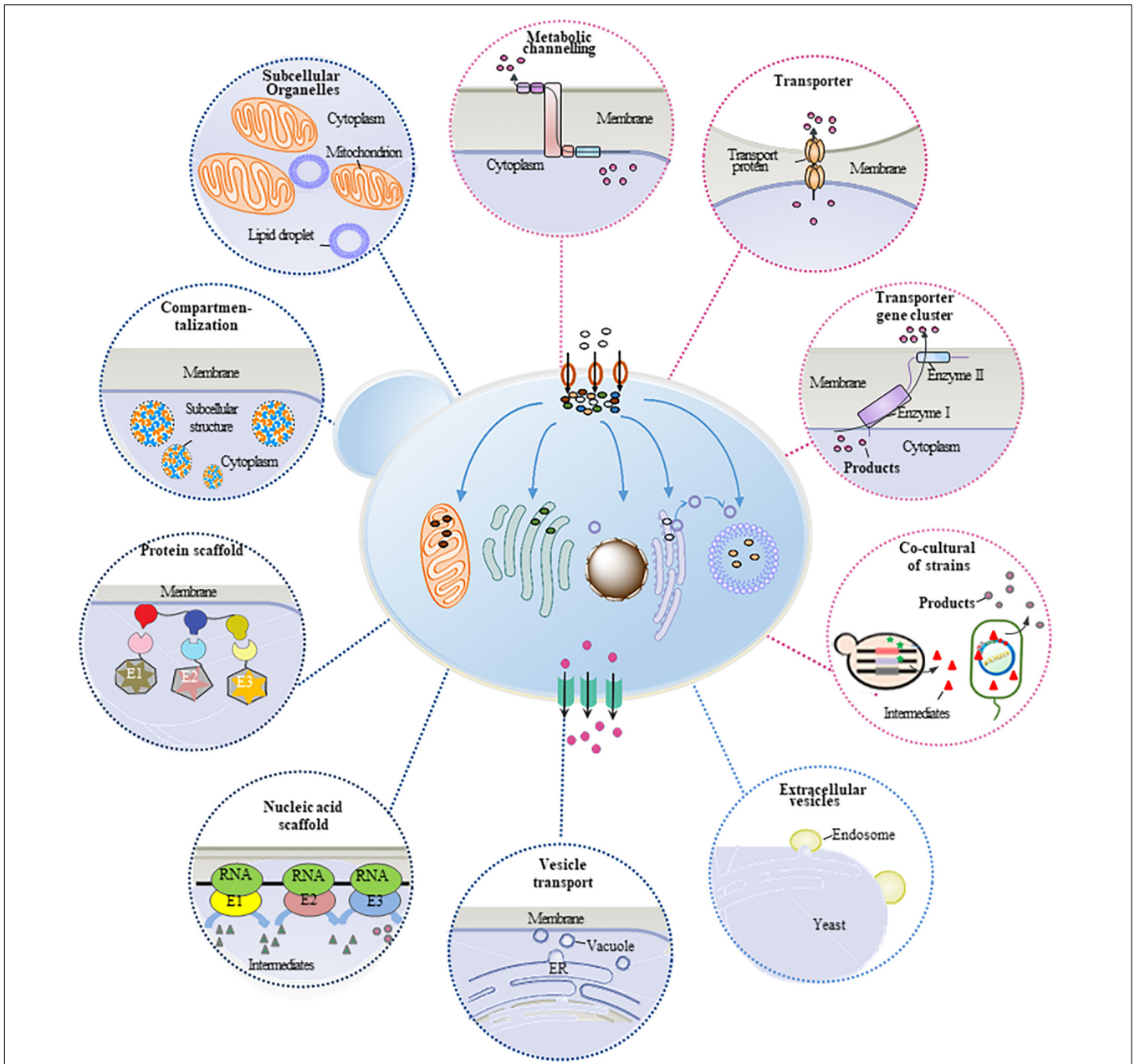

FIGURE 1 | Transfer process of engineered *S. cerevisiae* in the synthesis of plant natural products. The brown circle with black arrow indicates substrate transferred into the cells. The green square with black arrow indicates the product transferred out of the cell. The blue arrow indicates intermediates transferred in cell. The circles around the cell represent different strategies to refine the metabolic mass transfer in *S. cerevisiae*. Blue circles represent strategies to enhance intracellular mass transfer process. Pink circles represent strategies to enhance trans-plasma membrane mass transfer process.

More frequently, fusion expression may reduce the enzyme activity and destroy the structure of enzymes (Wheeldon et al., 2016; Poshyvailo et al., 2017). To solve these problems, some artificial scaffolds (including nucleic acid scaffolds and protein scaffolds) have been developed to form substrate channeling for the cascade enzymatic reactions, which can enhance the membrane free mass transfer process and improve the mass transfer efficiency. Han employed the retrotransposon element Ty1 as a scaffold to spatially organize enzymes involved in biosynthesis of farnesene and farnesol, forming the substrate channel and resulting in threefold and fourfold increased titers, respectively (Han et al., 2018). The key enzymes in resveratrol synthesis pathway including 4CL1 and STS were recruited to the protein scaffold to relocated in *S. cerevisiae*, and the yield of resveratrol was increased by five times (Wang and Yu, 2012). Through construction and optimization of RNA scaffolds, the two key enzymes in the pentadecane synthetic pathway were spatially colocated to form substrate channel, and the yield of

pentadecane was increased by 140% (Gairik et al., 2014). The DNA scaffold can also be used in the production of plant natural products. For example, Conrado constructed a stable and configurable scaffold based on plasmid DNA. Two enzymes 4-coumarate-CoA ligase and STS involved in the resveratrol biosynthesis were arranged and coexpressed via the Zif268 and PBSII ZF domains in the constructed DNA scaffold. As a result, the titer of resveratrol was increased by fivefold. This scaffold can also be used in improving 1,2-propanediol and mevalonate acid production (Conrado et al., 2012). In addition to nucleic acid scaffolds, protein scaffold is also a useful tool to reduce spatial distance by forming substrate channel of multienzyme reactions. The SH3 and PDZ are common ligands in constructing protein scaffold, which are often used in biosynthetic pathway of plant natural products to optimize the intracellular metabolic mass transfer process (Horn and Heinrich, 2015).

Construction of Metabolic Compartmentalization Enriches the Concentration of Precursors

Heterologous synthesis of plant natural products often brings enormous stress to host cells, such as the accumulation of cytotoxic intermediates and the competition of precursors coupled with the low mass transfer efficiency (Qiu et al., 2019). At the same time, the synthesis of plant natural products usually involves multiple enzymes, and the catalyzed reactions may occur in different locations within the cell, which are affected by the mass transfer efficiency. To avoid these problems and refine the mass transfer, many efficient strategies have been adopted. Among them, spatialized metabolic engineering, especially the metabolic compartmentalization engineering, has been considered as an efficient way. By this method, the whole pathway could be compartmentalized into several modules to enrich the concentration of precursors, and thus the production of plant natural products can be improved. Many organelles are used as metabolon in yeast cell factory, such as mitochondria, peroxisomes, endoplasmic reticulum, and vacuole, which can enhance the mass transfer and provide enough precursors and suitable environment for the synthesis of plant natural products. For example, the sesquiterpene synthetase was relocated in mitochondrial so that the precursors could be catalyzed directly in the same metabolon. Therefore, the production of sesquiterpenoids was increased significantly (Farhi et al., 2011). In a study on squalene production, peroxisomes were harnessed as subcellular compartments to produce the target product, leading to the production of squalene reaching 1,312.8 mg/L, which increased by 138-fold compared with the control strain (Liu et al., 2020). Amorpha-4,11-diene is one of the most important precursors in the artemisinin biosynthesis, starting from acetyl CoA. By colocalizing the FPP biosynthetic pathway (including eight genes) and the amorpha-4,11-diene synthase into mitochondria, the yield of amorpha-4,11-diene was increased significantly as more precursors such as acetyl CoA were provided and mass transfer was enhanced (Yuan and Ching, 2016). To produce isoprene efficiently, its biosynthetic pathway was assembled into mitochondria of yeast cells, and a dual regulation system based on GAL promoter was constructed to control the synthesis. The final isoprene production was 2,527 mg/L in fed-batch fermentation (Lv et al., 2016). Peroxisome is another organelle successfully used in the production of plant natural products. After introducing the farnesyl diphosphate synthetic pathway and α-humulene synthase into the peroxisome, the production of α-humulene was increased by 2.5-fold with a titer of 1,726.78 mg/L in fed-batch fermentation (Zhang et al., 2020). By adopting the modular pathway rewiring strategy, which involved relocalization of the engineered pathway and improving the precursor supply, the titer of L-ornithine reached 1,041 mg/L (Qin et al., 2015).

TRANS-PLASMA MEMBRANE MASS TRANSFER IN YEAST CELL FACTORY

In addition to the intracellular mass transfer, trans-plasma membrane mass transfer also plays a key role in cell metabolism. The intake of nutrients and export of toxic metabolic wastes, as well as secondary metabolites, are the most common transport processes across cell membrane. Moreover, trans-plasma membrane mass transfer is also necessary to synthesize plant natural products. Therefore, enhancing the process of transport is more beneficial for relieving feedback inhibition and toxicity of some products (Agapakis et al., 2012).

Enhancing Trans-Plasma Membrane Mass Transfer by Transporters

Enhancing the intake of nutrients can provide enough cofactors and precursors to the target pathways. Many uptake transporters have been used to the heterosynthesis of plant natural products. For example, the ATP-binding cassette (ABC) transporter of maltose can enhance the trans-plasma membrane of glucose and maltose. When it was expressed in engineered yeast, the production of ivermectin was improved by obtaining more precursors (Li et al., 2010). Transporter engineering and secretion strengthening of products are among the most effective strategies for improving the production of plant natural products, which not only release the feedback inhibition but also reduce the cytotoxicity. With the development of genome sequencing and metabonomic, the enzymes with new functions (Zhu et al., 2018) together with specific transporters can be mined for the plant natural products. For example, artemisinic acid is the most famous drug to treat malaria, which has been synthesized in *S. cerevisiae* successfully. But in the early development stage, the titer was very low, and many strategies were tried. The pleiotropic drug resistance proteins, which belong to the ABC transporter family, were induced by artemisinic acid. According to the results, it could be speculated that the use of ABC transporters may improve the production of natural products in the engineered yeast (Ro et al., 2008). In addition, the titer of avermectin was also increased two times by overexpression of the ABC transporter AvtAB to enhance the secretory capacity of products (Qiu et al., 2011). However, the research of transporters for plant natural product is still lacking, and the study on secretion of plant natural products in yeast cell factory remains to be further explored.

Enhancement of Non-specific Trans-Plasma Membrane Mass Transfer

Because of the lack of studies on transporters of plant natural products, some non-specific transport methods have been proposed, including the usage of vesicular, endocytosis, and exocytosis. Vesicle could be used to store tetraterpenes and involves their trans-plasma membrane transport in prokaryote. Regarding the biosynthesis of plant natural products in yeast, vesicle has been proven to be useful as a functional compartment and storage pool. For example, to identify the functional enzyme and improve the titer of tropane alkaloids, more than 20 proteins were integrated into yeast. Among them, the acyltransferase was located to the vacuole to improve the production of products in yeast (Srinivasan and Smolke, 2020). These researches make it possible to enhance trans-plasma membrane transport of plant natural products via vesicles from yeast. Furthermore, the high production of some plant natural products with long synthetic pathway is challenging in a single strain, because of the transmembrane of some intermediate metabolites and the heavy metabolic burden to the yeast cell (Zhang and Wang, 2016; Wang et al., 2020). To reduce the metabolic burden and provide stable environment, the biosynthetic pathway can be divided into several stages and segregated into different strains. Depending on the mass transfer between different cells, it is suitable to produce plant natural products especially terpenoids by constructing *Escherichia coli–S. cerevisiae* coculture system. The precursors can be synthesized in *E. coli* and secreted to the medium efficiently. Meanwhile, the secreted precursors are ingested by *S. cerevisiae*, which can provide membrane system and suitable environment for expressing cytochrome P450s (Zhou et al., 2015). For example, paclitaxel is a blockbuster anticancer drug with long and complex biosynthetic pathway. Its *de novo* synthesis in yeast is challenging as some steps of the pathway are not elucidated. Oxygenated taxane is an important intermediate of paclitaxel, whose heterologous biosynthesis has been achieved. In previous study, the *E. coli–S. cerevisiae* coculture system was used to increase the titer of oxygenated taxane. In this system, the multistep pathway was divided into several modules to reduce the metabolic burden and facilitate metabolic mass transfer. The engineered *E. coli* provided the important precursor taxadiene, which was then consumed by yeast with inherent membrane system expressing the heterologous P450 monooxygenases (taxadiene 5a-hydroxylase) to produce the oxygenated taxane (Zhou et al., 2015). The opiate is a famous alkaloid compound, whose biosynthetic pathway can be divided into four modules. Galanie constructed a coculture system using four engineered yeasts. The precursors can be synthesized in the first yeast host and transferred into the medium, which could be provided to the next host. Hence, mass transfer optimization by avoiding the degradation of intermediates was achieved, and the production of opiates increased by 300 folds (Galanie et al., 2015). Compared with single strain culturing, coculture of two engineered yeasts achieved higher production of polyketide drug monacolin J and lovastatin with the titer increased by 70% (Liu et al., 2018). Basically, the synthetic pathway of natural products could be divided into several modules. Different modules could be designed and functionalized in different hosts, and the final products can be obtained in the coculture system, which not only reduces the metabolic inhibition of some intermediates but also benefits the yield increase.

CONCLUSION AND PERSPECTIVE

Over the past 20 years, the rapid development of synthetic biology and system biology has provided a versatile technical platform for heterologous synthesis of plant natural products. Yeast is turned out to be an attractive host strain. Although various feasible metabolic engineering tools and useful strategies have been developed in yeast to improve the production of plant natural products, there are still many obstacles in the large-scale production. One notable obstacle is the unbalance of metabolic mass transfer process. In this regard, the regulation of metabolic mass transfer has attracted many interests in metabolic engineering applications. For example, regulation and optimization of intracellular and trans-plasma membrane mass transfer can enhance the transfer efficiency, eliminate the feedback inhibition, and reduce the cytotoxicity of products. However, most studies on the optimization of mass transfer process were under specific conditions and lacked of systematization and universality. More importantly, there are seldom successful studies on the mining of direct transporters for plant natural products, which is the main bottleneck for the export of plant natural products in yeast. Thanks to the repaid progress in synthetic biotechnology and sequencing technology, the candidate transporters of plant natural products can be mined by the omics data, bioinformatics analysis and machine learning. Another limitation in the exploring of mass transfer strategies is the lack of high-throughput screening method. As a result, the mining of transporter as well as the integrated platform for high-throughput screening would be future research directions.

In summary, the study of mass transfer has achieved initial success. With the comprehensive progress of the metabolic engineering as well as the genome editing method, the challenges in this field for the synthesis of plant natural products will be solved in the near future. Meanwhile, the mechanisms of metabolic mass transfer process will be revealed and may have a far-reaching significance for constructing a comprehensive and efficient yeast cell factory.

AUTHOR CONTRIBUTIONS

All authors contributed to the conception and design of the study. HX and WS participated in searching and analyzing literature for this review and wrote the manuscript. YW and CL edited and corrected the manuscript. All authors approved the submitted version.

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MINI REVIEW
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Recent Advances in Producing Sugar Alcohols and Functional Sugars by Engineering *Yarrowia lipolytica*

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The sugar alcohols and functional sugars have wide applications in food, pharmaceutical, and chemical industries. However, the smaller quantities of natural occurring sugar alcohols and functional sugars restricted their applications. The enzymatic and whole-cell catalyst production is emerging as the predominant alternatives. The properties of *Yarrowia lipolytica* make it a promising sugar alcohol and functional sugar producer. However, there are still some issues to be resolved. As there exist reviews about the chemical structures, physicochemical properties, biological functions, applications, and biosynthesis of sugar alcohols and/or functional sugars in *Y. lipolytica*, this mini review will not only update the recent advances in enzymatic and microbial production of sugar alcohols (erythritol, D-threitol, and xylitol) and functional sugars (isomaltulose, trehalose, fructo-oligosaccharides, and galacto-oligosaccharides) by using recombinant *Y. lipolytica* but also focus on the studies of gene discovery, pathway engineering, expanding substrate scope, bioprocess engineering, and novel breeding methods to resolve the aforementioned issues.

Keywords: sugar alcohols, functional sugars, *Yarrowia lipolytica*, gene discovery, substrate scope, strain breeding

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INTRODUCTION

Sugar alcohols or polyols are defined as a group of acyclic hydrogenated carbohydrates, but they are not sugars. Functional sugars generally refer to the sugars with unique structural and physiological characteristics, but occur in very low quantity in nature (Bilal et al., 2020). Sugar alcohols and functional sugars have various applications in pharmaceutical, agro-food, and chemical industries (Fickers et al., 2020). With the properties of same or better sweeting but less caloric value of sucrose, sugar alcohols and functional sugars are evolving as food ingredients (Park et al., 2016). Besides, they are increasingly used in pharmaceutical applications because of their excellent functional properties and health benefits. It was estimated that the global consumption of sugar alcohols will reach 1.9 million metric tons by 2022 (Grembecka, 2015). However, sugar alcohols and functional sugars are present in smaller quantities in nature (plants, fungi, and algae). *In planta*, sugar alcohols are temporarily accumulated

in leaves during light and are transported to other organs during dark. The low content makes plant extraction of sugar alcohols difficult. Chemical approaches to sugar alcohols and functional sugars suffer from strict reaction conditions, limited yield, expensive raw starting feedstocks, and safety risks (Huang et al., 2018). Engineering enzymes or whole-cell catalyst is emerging as the predominant alternative approach (Fickers et al., 2020).

The non-model organism *Yarrowia lipolytica* is an emerging host for the production of non-native chemicals and is expeditiously becoming a model and unique microorganism with strong industrial potentials (Sharma and Lee, 2020). *Y. lipolytica* was empowered by the recently developed genetic and synthetic biology tools and metabolic engineering methods (Madzak, 2018; Shi et al., 2018; Ganesan et al., 2019). *Y. lipolytica* has been harnessed for heterologous synthesis of many bioactive natural compounds such as polyketides, isoprenoids, α-santalene, limonene, (+)-nootkatone, protopanaxadiol, gensenoside K, astaxanthin, and flavonoids (Cao et al., 2016; Guo et al., 2018; Jia et al., 2019; Li et al., 2019; Lv et al., 2019; Tramontin et al., 2019; Wu et al., 2019; Luo et al., 2020; Palmer et al., 2020).

Promising results have been achieved by engineering *Y. lipolytica* into efficient producer of sugar alcohols and functional sugars due to its low nutritive requirements, feasibility of high cell density culture, and ease of genome editing (Fickers et al., 2020). However, there remain some issues (inefficiency of enzyme activity and microbial cell factory, high cost of substrates, suboptimal bioprocess, and strain unstability) to be resolved before making the bioprocesses commercially feasible. As the physicochemical properties, biological functions, applications, and biosynthesis of sugar alcohols and functional sugars have been discussed in multiple reviews (Grembecka, 2015; Carly and Fickers, 2018; Bilal et al., 2020; Fickers et al., 2020), this mini review will update the recent advances (2016–2020) in enzymatic and microbial production of sugar alcohols (erythritol, D-threitol, and xylitol) and functional sugars (isomaltulose, trehalose, fructo-oligosaccharides, and galacto-oligosaccharides) by using recombinant *Y. lipolytica* (**Table 1**) and focus on the studies of gene discovery, pathway engineering, expanding substrate scope, bioprocess engineering, and novel breeding methods to resolve the aforementioned issues.

PRODUCTION OF SUGAR ALCOHOLS AND FUNCTIONAL SUGARS IN *Yarrowia lipolytica*

Erythritol

Erythritol (1,2,3,4-butanetetrol) is a sugar alcohol with four-carbons. Due to its sweetening properties, it is employed as food additive in agro-food industries (Carlya et al., 2017). Erythritol is a safety sweetener for diabetics, because it does not affect the insulin level within the blood due to its chemical features (Janek et al., 2017). It is also used as chemical precursor for the synthesis of substances having phase transition behavior (Qiu et al., 2020). Yeast and bacteria produce erythritol in the form of osmoprotectant. The oleaginous yeast *Y. lipolytica* has been reported for the synthesis of erythritol since 1970s (Mironczuk et al., 2017). *Y. lipolytica* possesses the primary metabolic pathway for erythritol synthesis and can be engineered into erythritol over-producer from both glucose and glycerol by overexpressing erythrose-4-phosphate kinase and erythrose reductase (**Figure 1A**; Mironczuk et al., 2017). The glycerol is a renewable feedstock and is produced in the form of waste product on large scale in various industries (Mironczuk et al., 2017). Promising results have been achieved in engineering *Y. lipolytica* into erythritol over-producer and expanding its substrate scope, which will be discussed in detail in sections "Gene discovery and pathway engineering" and "Expanding substrate scope and bioprocess engineering" in this mini review.

D-Threitol

D-threitol is a diastereoisomer of erythritol. Few osmotolerant yeasts synthesize D-threitol as an osmoprotective agent. It is widely used in pharmaceutical, medicine, food, and green chemistry (Carly and Fickers, 2018). D-threitol can be synthesized from erythritol by xylitol dehydrogenase (XDH) with erythrulose as an intermediate, which is the most explored method (**Figure 1B**). Chi et al. (2019) purified an XDH from *Scheffersomyces stipitis* (Ss-XDH) and found that it has the potential to convert erythritol into erythrulose and threitol *in vitro*. By overexpressing Ss-XDH in an erythritol-producing *Y. lipolytica*, they achieved 112 g/L D-threitol with a yield of 0.37 from glucose (Chi et al., 2019). The upregulation of mannitol dehydrogenase encoding gene resulted in the accumulation of mannitol in the culture broth. They developed the *Candida parapsilosis* culture in order to remove the byproduct (mannitol) and coproduce erythritol to facilitate the subsequent purification of threitol, as *C. parapsilosis* has the ability to catabolize erythritol and mannitol but not threitol (Chi et al., 2019).

Xylitol

Xylitol is a sugar alcohol utilized as sweetener. Naturally, it is present in very small quantities. It has applications in dental and oral diseases. Due to its excellent anti-inflammatory potential, xylitol can be used to cure chronic inflammatory diseases effectively (Benahmed et al., 2020). It is also used to treat the respiratory diseases such as pneumonia and middle ear infections. In mammals, xylitol is responsible for the secretion of insulin in blood plasma. It also plays a role in the reduction of obesity and some other metabolic syndromes (Benahmed et al., 2020). In addition, it is also used in sugar free gum and is commonly not harmful to humans but can be highly toxic to dogs (Rajapaksha et al., 2019). The synthesis of xylitol through chemical process is costly and laborious. On the contrary, xylitol production through microbial cell factories facilitates the inexpensive and profitable alternative process (Prabhu et al., 2020). *Y. lipolytica* possesses the complete xylose utilizing pathway (**Figure 1C**). However, the expression of xylitol dehydrogenase (XDH) is strictly regulated, resulting in *Y. lipolytica* unable to grow on xylose (Rodriguez et al.,

TABLE 1 | Sugar alcohols and functional sugars produced by engineering *Y. lipolytica*.

Products	Carbon source	Genetic engineering strategy	Cultivation Strategy	Titer	References
	Glycerol	The overexpressed codon optimized bacterial hemoglobin from *Vitreoscilla stercoraria* was used	Bioreactor culture	55.75 g/L	Mironczuk et al., 2019
	Glycerol	Overexpressed TKL1 and GUT1, Disruption of EYK1	Bioreactor culture	80.6 g/L	Carly et al., 2017; Carlya et al., 2017
	Glycerol	Ultraviolet (UV) mutagenesis and optimal C:N ratio	Chemostat culture	113.1 g/L	Rakicka et al., 2017a
Erythritol	Glycerol	The overexpression of Erythrose reductase encoding gene YALI0F18590gwas used	Batch culture	44.44 g/L	Janek et al., 2017
	Glycerol	The four genes *ZWF1*, *TAL1*, *GND1*, and *TKL1* were functionally overexpressed	Shake flask experiment	51.09 g/L	Mironczuk et al., 2017
	Glycerol	The disruption of Gene encoding erythrulose kinase YALI0F01606g was used	Batch bioreactor	35.7 g/L	Carly et al., 2017; Carlya et al., 2017
	Glycerol	Span 20 surfactant was added	Fed-batch culture	142 g/L	Rakicka et al., 2017b
D-threitol	Glucose	The overexpression of xylitol dehydrogenase gene (*Ss-XDH*) from *Scheffersomyces stipitis*	Culture broth	112 g/L	Chi et al., 2019
Isomaltulose	Sucrose	Displaying sucrose isomerase encoding gene from *Pantoea dispersa* UQ68J (PdSIase) on the *Y. lipolytica* cell surface	Fermentor	465 g/L	Li et al., 2017
	Sucrose	gene encoding sucrose isomerase (Slase) from *Pantoea dispersa* UQ68J	Fermentor	572.1 g/L	Zhang et al., 2018
Trehalose	Maltose	Displaying encoding trehalose synthase gene (TreS) on the Y. lipolytica cell surface	Bioreactor	219 g/L	Li et al., 2016
Fructo-oligosaccharides	Sucrose	Erythritol producing *Y. lipolytica* cells containing displayed fructosyltransferase	Bioreactor	480 g/L	Zhang et al., 2016
	Inulin	The overexpression of optimized endo-inulinase gene from *Aspergillus niger* was used	Bioreactor	546.6 g/L	Han et al., 2017
Galacto-oligosaccharides	lactose	Displaying β-galactosidase on *Y. liplytica* cell surface	Bioreactor	160 g/L	An et al., 2016

2016). This characteristic has been substantially explored for the bioconversion of xylose into xylitol (Prabhu et al., 2020). For instance, Prabhu et al. established and optimized the bioprocess of converting xylose into xylitol. They found that glycerol is a better co-substrate for biomass accumulation and subsequent bioconversion compared with glucose. After condition optimization and scale up in bioreactor, they achieved 53.2 g/L xylitol from pure glycerol (PG) with a yield of 0.97 g/g. When they substituted PG with biodiesel-derived crude glycerol (CG), similar results (50.5 g/L xylitol with a yield of 0.92 g/g) were achieved. They also tried sugarcane bagasse hydrolysate as the feedstock and achieved 0.54 g/g xylitol yield. Finally, they successfully crystallized xylitol from CG/xylose and PG/xylose fermented broths with recovery of 35.3 and 39.5%, respectively. This study demonstrated the potential of *Y. lipolytica* as a microbial cell factory for the production of xylitol from low-cost feedstocks (Prabhu et al., 2020).

Isomaltulose

Isomaltulose (IM) is a disaccharide sugar usually present in nature. It consists of glucose and fructose monomers which are connected with each other through α-1,6 glycosidic linkage (Shyam et al., 2018). The well-known trade name of IM is Palatinose^{TM}. IM is found to be a stable but completely digestible sugar with low glycemic index (Maresch et al., 2017). IM garnered attentions as an alternative sweetener to sucrose. IM is also involved in management and prevention of chronic diseases like cancers and cardio-metabolic diseases (Shyam et al., 2018). IM is mostly produced from sucrose by sucrose isomerase (SIase) (Zhang et al., 2018). By overexpressing SIase from *Pantoea dispersa* UQ68J in *Y. lipolytica* and converting monosaccharide byproducts into intracellular lipids, Zhang et al. (2018) produced 572.1 g/L IM with 97.8% purity in a final fermented broth. To circumvent the obstacle of cell membranes, the Zixin Deng group displayed a SIase on the *Y. lipolytica* cell surface by

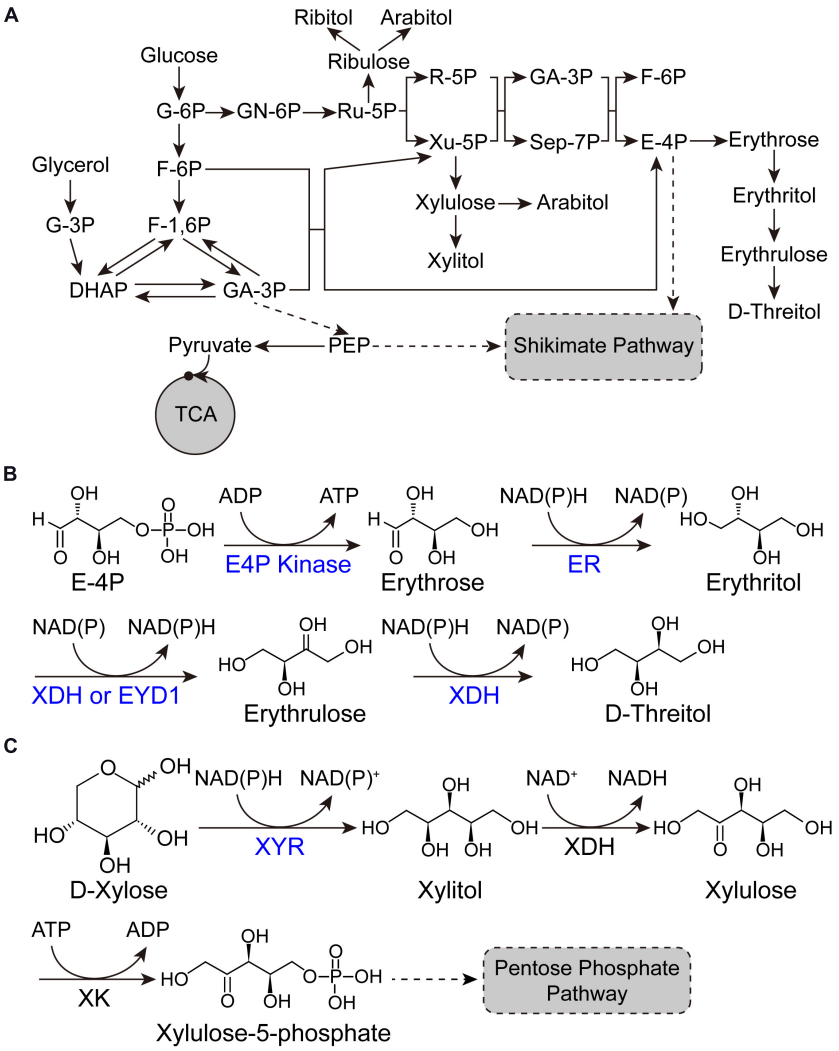

FIGURE 1 | Pathways for sugar alcohol biosynthesis. **(A)** Metabolic pathway of sugar alcohols in *Y. lipolytica*. **(B)** Biotransformation pathway from E-4P to D-threitol. **(C)** Biotransformation pathway from D-xylose to xylitol. G-6P, glucose-6-phosphate; F-6P, fructose-6-phosphate; F-1,6P, fructose-1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; G-3P, glycerol-3-phosphate; GA-3P, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvic acid; TCA, tricarboxylic acid cycle; GN-6P, gluconolactone-6-phosphate; Ru-5P, ribulose-5-phosphate; R-5P, ribose-5-phosphate; Xu-5P, xylulose-5-phosphate; GA-3P, glyceraldehyde-3-phosphate; Sep-7P, sedoheptulose-7-phosphate; E-4P, erythrose-4-phosphate; ER, erythrose reductase; XDH, xylitol dehydrogenase; EYD1, erythritol dehydrogenase; XYR, xylose reductase; XDH, xylitol dehydrogenase; XK, xylulose kinase.

using the cell wall protein (CWP) Pir1 and achieved 465 g/L IM production. The cell surface displayed that SIase was found to be stable under broad ranges of temperature (20–40°C) and pH values (4.5–7.0) and maintained more than 80% activity after 12 reaction cycles (Li et al., 2017). Zheng et al. (2019) also displayed a SIase on the *Y. lipolytica* cell surface by utilizing the glycosylphosphatidylinositol (GPI)-linked CWP anchor signal sequence. The cell surface display achieved the highest SIase activity of 2,910.3 U/g of dry cell weight. The surface displayed SIase showed the same optimal temperature (30°C) as the free SIase, but higher thermostability and longer half-life than the free SIase. In the subsequent IM production from low-cost cane molasses, the IM conversion rate maintained more than 85% after nine cycles of reaction. The high operational stability is a desired characteristic in industrial production (Zheng et al., 2019).

Trehalose

Trehalose is a non-reducing disaccharide composed of two glucose subunits connected by α,α-1,1-glycosidic linkage. Because of the outstanding osmoprotective activity, trehalose serves as one of the most efficient functional molecules to protect the cells from stresses like drought, salt, and heat (Li et al., 2009; Trevisol et al., 2011). Trehalose has also been used as a neuroprotective agent against neurodegenerative diseases like Huntington and Parkinson in animal models (Lee et al., 2018). In *S. cerevisiae*, trehalose synthesis involves two synthetic enzymes trehalose-phosphate synthase (Tps1p) and trehalose-6-phosphate

phosphatase (Tps2p), and two regulatory proteins Tps1p and Tps3p (Reilly and Doering, 2010). However, this pathway has not been explored for industrial scale synthesis. Trehalose is currently produced by two enzyme-based processes. The first one is a multi-enzyme process, which utilizes maltooligosaccharides or starch as substrate and involves maltooligosyltrehalose hydrolase, maltooligosyltrehalose synthase, α-amylase, and pullulanase. The second process utilizes trehalose synthase (TreS) to directly transform maltose into trehalose through intra-molecular transglycosylation. The TreS-catalyzed one-step transformation is the most explored process, because it is fast, simple, and less expensive (Zheng et al., 2015). The Zixin Deng group simplified the TreS-based process by integrating enzyme production, trehalose transformation, and ethanol removal in one pot (Li et al., 2016). They firstly displayed the *Picrophilus torridus* TreS on *Y. lipolytica* cell surface by fusing it with the *Y. lipolytica* cell wall anchoring protein YlPir1. The optimal pH and temperature were subsequently figured out, and 219 g/L trehalose was produced. The displayed enzymes were found more stable at optimal pH and temperature in comparison with free enzymes. *S. cerevisiae* was used to ferment the residual maltose and glucose into ethanol, which was subsequently removed by distillation. Finally, high-purity trehalose was easily obtained from the broth. This bioprocess represents an easier and low-cost access to trehalose (Li et al., 2016).

Fructo-Oligosaccharides

Fructo-oligosaccharides (FOSs) are oligosaccharides consisting of β-2,1 fructosyl-fructose glycosidic linkage (Chen et al., 2016). The FOSs have many applications including gastrointestinal improvement, modulation of immune system, colon cancer protection, reduction of obesity linked disease, and helping in mineral uptake (Chen et al., 2016). Fructosyltransferase (FTase) can be used to transform sucrose into FOSs. Multiple FTase-based biotransformation processes have been developed, including the use of whole cells harboring native FTase, purified FTase, immobilized whole cells, and immobilized FTase (Bali et al., 2015). Although the immobilized methods are effective, the carriers are expensive, and the manipulations are complicated. The Zixin Deng group developed an in-expensive and industrially attractive biotransformation process by utilizing the erythritol industry yeast pastes to produce FOSs. They engineered the erythritol-producing *Y. lipolytica* into FOS-producing whole cell catalyst by displaying an FTase from *Aspergillus oryzae* on the cell surface. Under the optimal conditions, the whole-cell catalyst produced 480 g/L FOS from 800 g/L sucrose, with a yield of 60% and productivity of 160 g/(L·h). The engineered yeast pastes from erythritol industry was stable in FOS production, with only 10% FTase activity lost after 10 recycling number (Zhang et al., 2016). FOSs can also be produced by directly hydrolyzing inulin by the endo-inulinase (EC 3.2.1.7) (Singh et al., 2016). Inulin is an abundant renewable natural resource. Han et al. (2017) developed a two-stage bioprocess using recombinant *Y. lipolytica* strain Enop56 to produce FOSs from inulin. *Y. lipolytica* Enop56 was constructed by overexpressing an optimized endo-inulinase encoding gene from *Aspergillus niger*. This method produced 546.6 g/L FOSs from 600 g/L inulin in a 10-L bioreactor, with a yield of 0.91 g/L and productivity of 15.18 g/(L·h). Moreover, FOSs were the main hydrolysis products, with only 4.97% of total amount of non-prebiotic saccharides in the fermented broth (Han et al., 2017). This will greatly simplify the downstream purification process. This method also showed promising industrialization potential.

Galacto-Oligosaccharides

Galacto-oligosaccharides (GOSs) are lactose-derived non-digestible prebiotics and are widely used as substitutes of human milk oligosaccharides in milk formulas of infants and newborn babies (Vera et al., 2016). GOSs have been produced from lactose by using free or immobilized β-galactosidase (EC 3.2.1.23), which shows both glycoside hydrolase and galactosyltransferase activities (Vera et al., 2016). However, these methods suffer from time-consuming, expensive carriers, enzyme diffusion, and/or loss of activity (Bilal et al., 2020). To resolve these issues, the Zixin Deng group displayed an *Aspergillus oryzae* β-galactosidase on the cell surface of the erythritol producing *Y. lipolytica*. The recombinant strain produced 160 g/L GOSs from 500 g/L lactose, with a yield of 51% of consumed lactose. The optimal temperature of the surface displayed β-galactosidase was found to be 20°C higher than that of the free enzyme. The surface displayed β-galactosidase showed substantial stability during the GOS production, in which GOS yield did not decrease significantly even after 10 rounds of reaction (An et al., 2016).

GENE DISCOVERY AND PATHWAY ENGINEERING

Yarrowia lipolytica has the potential to produce erythritol at high level from glycerol. However, it also consumes erythritol as carbon source, which has negative impact on erythritol production. By using insertional mutagenesis strategy, Carly et al. (2017) obtained a mutant without erythritol catabolism. Subsequent genome sequencing confirmed that the mutant phenotype is directly linked to the disruption of gene *YALI0F01606g*, which was suggested to rename as *EYK1*, encoding an erythrulose kinase. Their results also demonstrated that disrupting *EYK1* enhanced erythritol production from glycerol (Carly et al., 2017). In another study, they identified and characterized another erythritol metabolism gene *EYD1*, encoding the erythritol dehydrogenase in *Y. lipolytica*. They found that strains containing disrupted *EYD1* cannot utilize erythritol as carbon source. They further used EYD1 for the bioconversion of erythritol into erythrulose. By constitutively expressing *EYD1* in an *EYK1* disrupted *Y. lipolytica* chassis, erythrulose was produced at a rate and yield of 0.116 g/(g_{DCW}·h) and 0.64 g/g (Carly et al., 2018).

Recently, the erythritol metabolic pathway in *Y. lipolytica* was characterized by functionally overexpressing four genes involved in the pentose phosphate pathway. Among them, the *TKL1* (*YALI0E06479g*, encoding transketolase) was found to be a crucial gene for the erythritol synthesis. Overexpressing *TKL1* improved

erythritol titer by twofold in shaking flasks and 70% in 5-L bioreactor at low agitation. In addition, overexpressing *TKL1* permits efficient erythritol production at low dissolved oxygen level (Mironczuk et al., 2017).

The NAD(P)H dependent erythrose reductase (ER) catalyzes the final and crucial step of erythritol synthetic pathway in *Y. lipolytica*. Janek et al. validated a predicted native ER encoding gene (*YALI0F18590g*) by overexpressing and characterizing its influence on erythritol synthesis in *Y. lipolytica*. The results showed that overexpressing *YALI0F18590g* improved erythritol titer by 20%, and Zn^{2+} had a positive effect on the activity (Janek et al., 2017). Later, Cheng et al. identified another two novel erythrose reductases (ER25 and ER10) by characterizing the purified enzymes and overexpressing the respective encoding genes in *Y. lipolytica*. Finally, by overexpressing the newly isolated ER genes and engineering NADPH metabolism, they produced 190 g/L erythritol in baffled flasks, with the yield and productivity improved by 23.5 and 50%, respectively (Cheng et al., 2018).

Yarrowia lipolytica accumulates the osmoprotectant erythritol as a response to the hyperosmotic stress. The high osmolality glycerol (HOG) pathway, which has been well studied in *S. cerevisiae*, senses and responds to the stressful hyperosmotic signals. Rzechonek et al. (2018) identified a *Y. lipolytica* homolog of HOG1 (yl-Hog1, encoded by *YALI0E25135g*) and proved that it is responsible for the response to the hyperosmotic stress and induction of erythritol production.

EXPANDING SUBSTRATE SCOPE AND BIOPROCESS ENGINEERING

Expanding the substrate scope to inexpensive materials or industrial wastes is helpful to improve the economical and environmental effects of sugar alcohol and functional sugar production. Crude glycerol is a main byproduct of the biodiesel industry and is a cheap and renewable resource. Rakicka et al. (2016) developed a chemostat culture process and obtained very promising results when they replaced pure glycerol with crude glycerol (103 vs. 81.9 g/L) as carbon source. Several other studies has been published on the production of erythritol from crude glycerol in recent years (Mironczuk et al., 2016; Yang et al., 2016; Rakicka et al., 2017a,b; Da Silva et al., 2018; Rakicka-Pustulka et al., 2020).

Okara (soybean residue), oil crop wastes, and waste cooking oil (WCO) have also been used for erythritol production. Okara is thought to be an ideal substrate for its low-price and high-nutrient content. Liu et al. developed, optimized, and scaled up a pretreatment and fermentation process using okara as feedstock. No mineral or nitrogen supplementation was added during the erythritol producing process (Liu et al., 2017b). However, to make okara more accessible, hydrolysis by fungal in-house enzymes was needed before erythritol production. This will make the process more complicated and time-consuming (optimally 5-day pretreatment).

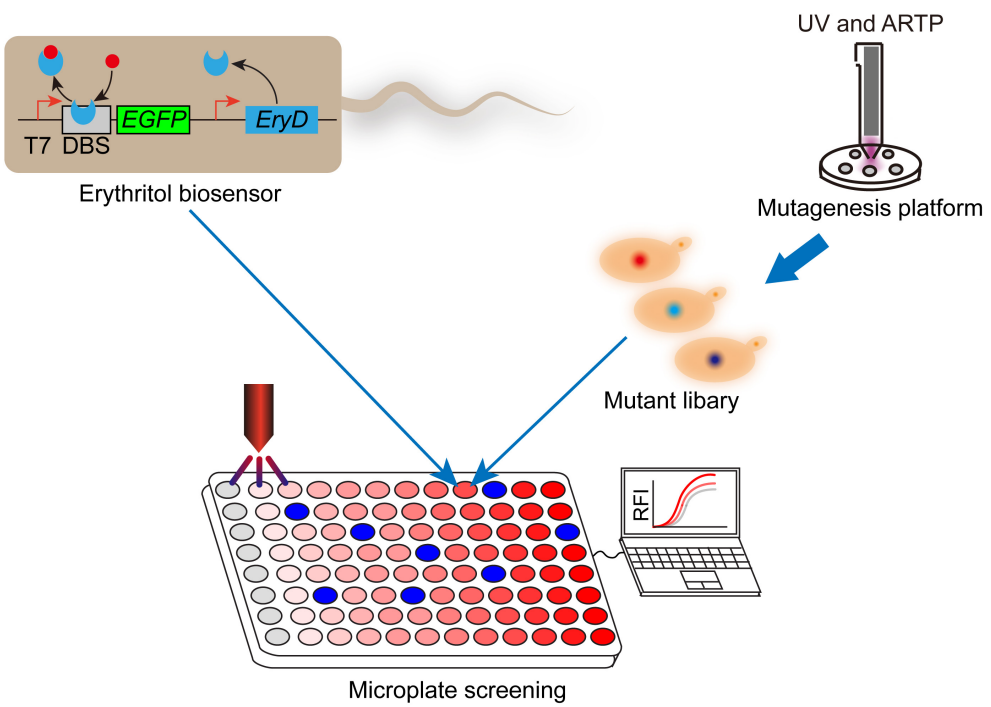

FIGURE 2 | Work flow of combining genetically encoded erythritol biosensor with high-throughput screening to improve erythritol production in *Y. lipolytica*. The erythritol biosensor was developed in *Escherichia coli* BL21 (DE3). Transcriptional repressor EryD and cognate DNA-binding site were used to control the expression of enhanced green fluorescence protein (GEFP). After mutagenesis, mutant library of erythritol producing *Y. lipolytica* was combined with biosensor and submitted for microplate-based high-throughput screening. High performers can be screened out according to the fluorescent signals. T7, T7 promoter; DBS, EryD DNA-binding site; RFI, relative fluorescence intensity.

Oil crop wastes are attractive and economical feedstocks for erythritol production in *Y. lipolytica*. However, undesirable excessive nitrogen content in oil crop wastes hampers the erythritol yield, whose synthesis is triggered only under nitrogen starvation condition. To resolve this issue, Liu et al. deleted the sucrose non-fermenting protein kinase gene (*SNF1*), which is involved in the nitrogen starvation-triggered process. The carbon source utilization and erythritol production were enhanced by the engineered strain under nitrogen-rich conditions (Liu et al., 2020). In the following research, they developed a one-step solid-state fermentation process, in which improved erythritol production was achieved from unrefined oil crop wastes using this *SNF1*-deficient *Y. lipolytica*. The developed process showed advantages of lower material cost and shorter fermentation period (Liu et al., 2019a). Their studies represent a new way for the development of cost-effective method for the effective synthesis of *Y. lipolytica* metabolites from nitrogen-rich wastes.

The WCO from catering services can no longer be reused because of the oxidized fatty acids and hazardous components. However, the high energy value and low-price make WCO an excellent alternative carbon source for the oleaginous yeast *Y. lipolytica*. Liu et al. investigated the crucial factors of osmotic pressure and pH in the erythritol and citric acid production from WCO. Their results showed that high osmotic pressure together with low pH promoted erythritol production and inhibited citric acid production, and vice versa (Liu et al., 2018). In another study, they used an agricultural waste loofah sponge (LS) as dispersant to improve the insolubility of WCO in the culture. The results showed that LS enhanced the WCO dispersion, utilization, *Y. lipolytica* growth, and erythritol production. LS was stable during cultivation process and can be easily recycled (Liu et al., 2019b).

BREEDING EFFICIENT STRAINS USING NOVEL MUTATION METHOD

In addition to genetic engineering and bioprocess engineering, breeding competitive strains are an important alternative approach to improving sugar alcohol production in *Y. lipolytica*. The traditional mutation processes are usually not environmentally friendly. The atmospheric and room temperature plasma (ARTP) is an emerging mutation platform, whose process is rapid, low cost, under low temperature, and environmentally friendly (Liu et al., 2017a). In this section, we will discuss the recent studies about breeding efficient sugar alcohol producing strains by using ARTP platform. Using ARTP platform, Liu et al. established a mutant library of marine fish gut isolated *Y. lipolytica*, which was found to be able to accumulate erythritol in their previous study. The best-performing mutant produced 169.3 g/L erythritol in 168 h in a 5-L fermentor in fed-batch fermentation, while low-level byproducts were detected in the broth (Liu et al., 2017a). Although the ARTP platform can produce large-scale mutant library conveniently, screening the best-performing mutant out from the library is laborious and time-consuming. Qiu et al. developed a genetically encoded biosensor-regular platform to rapidly screen and characterize the erythritol overproducers from the mutant library (**Figure 2**). The transcriptional repressor EryD and cognate DNA-binding sequence were utilized to control the expression of a fluorescence reporter (eGFP). In the absence of erythritol, the *EGFP* transcription was repressed; while in the presence of erythritol, the transcriptional repression was relieved. By coupling this biosensor-regulator system with microplate reader, they screened and characterized a library of more than 1,152 mutants derived from combined UV and ARTP mutagenesis, in a short period of time (1 week). The best-performing mutant produced 148 g/L erythritol in bench-top fermenter. By coupling genetically encoded biosensor with mutagenesis, this study provided a convenient high-throughput screening and characterization framework to improve the performance of industrial microbial producer (Qiu et al., 2020).

AUTHOR CONTRIBUTIONS

YL and JX conceived the topic. AA and YL drafted the manuscript. YL and JX revised the manuscript. JL, ZW, AZ, HY, LQ, MA, and WX gave suggestions. All authors contributed to the article and approved the submitted version.

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Advances in the Development of Microbial Double-Stranded RNA Production Systems for Application of RNA Interference in Agricultural Pest Control

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RNA interference (RNAi) is a valuable and revolutionary technology that has been widely applied in medicine and agriculture. The application of RNAi in various industries requires large amounts of low-cost double-stranded RNA (dsRNA). Chemical synthesis can only produce short dsRNAs; long dsRNAs need to be synthesized biologically. Several microbial chassis cells, such as *Escherichia coli*, *Saccharomyces cerevisiae*, and *Bacillus* species, have been used for dsRNA synthesis. However, the titer, rate of production, and yield of dsRNA obtained by these microorganism-based strategies is still low. In this review, we summarize advances in microbial dsRNA production, and analyze the merits and faults of different microbial dsRNA production systems. This review provides a guide for dsRNA production system selection. Future development of efficient microbial dsRNA production systems is also discussed.

Keywords: RNA interference, dsRNA, microbes, synthetic biology, production

INTRODUCTION

The large-scale use of chemical pesticides creates tremendous ecological pressure on soil, water, air, and the human living environment. After the long-term use of chemical pesticides, resistance, resurgence, and residue (3R) problems have become increasingly prominent (Tudi et al., 2021). The emergence of RNA interference (RNAi) technology has brought new hope of solving these problems. In this technology, double-stranded RNA (dsRNA) enters a host and triggers the RNAi effect—the expression of the complementary target gene is silenced, which affects the growth and development of the target organism, thus achieving pest control (Fire et al., 1998; Fletcher et al., 2020; Zhu and Palli, 2020). RNAi pesticides are considered novel, and ecofriendly, because RNAi technology uses precise targeting and the pesticide agent could be easily degradable.

However, several problems need to be solved before this technology can be widely applied, such as efficient, high-throughput target gene acquisition, dsRNA delivery strategies in different organisms (insects, plants, fungi, bacteria, and viruses), the stability of the dsRNA in field application, and construction of multi-species integrated control strategies in complex ecological environments (Zhang et al., 2013). Besides, large-scale, low-cost synthesis of dsRNA is crucial for applying RNAi technology in agriculture (Silver et al., 2021). Chemical synthesis of RNA is suitable for the synthesis

of short RNAs, such as small interfering RNAs (siRNAs), because the synthesis error rate increases and the yield decreases when the length of the target RNA product increases (Mu et al., 2018). *In vitro* synthesis strategies relying on T7/SP6 RNA polymerase and *in vivo* synthesis by engineered bacteria are often used for dsRNA synthesis. The *in vitro* synthesis strategy can produce high-purity dsRNA, but the cost is relatively high. Moreover, this method requires auxiliary materials, such as DNA templates, enzymes, and nucleotides (Mu et al., 2018). The *in vivo* synthesis strategy produces low-cost dsRNA in high yields, but this strategy requires later purification of the product and inactivation of the engineered microbial strain (Mendiola et al., 2020). Nevertheless, the *in vivo* synthesis strategy is more likely to reduce dsRNA production costs and increase yields in the future (Cooper et al., 2021). In this review, applications of microbe-mediated dsRNA expression systems are summarized, and the selection of efficient microbial dsRNA production systems is discussed.

Escherichia coli dsRNA Expression Systems

E. coli is a commonly used bacterium for dsRNA expression because of its clear genetic background and convenient genetic manipulation. *E. coli* strain HT115 (DE3), which is RNase III deficient, and L4440 vector with a pair of oppositely oriented T7 promoters (one on each side of the multiple cloning site) are widely used as an expression strain and vector for dsRNA production (Timmons et al., 2001; Voloudakis et al., 2015). After introduction of the L4440 vector ligated with the target fragment into strain HT115 (DE3), large amounts of T7 RNA polymerase can be synthesized on induction by isopropyl β-D-1-thiogalactopyranoside (IPTG); the T7 RNA polymerase binds to the T7 promoter in L4440, which mediates the transcription of downstream DNA sequences into RNA. As a result, two complementary RNAs are synthesized, which in turn form the target dsRNA (Voloudakis et al., 2015).

The production of dsRNA using engineered bacterial expression was first attempted by Timmons and Fire (1998), and the corresponding RNAi phenotype was verified after feeding to the nematode *Caenorhabditis elegans*, showing that dsRNA expressed by bacteria can induce RNAi effects (Timmons et al., 2001). Using this dsRNA generation strategy, RNAi effects were induced in a variety of insects, such as *Spodoptera exigua* (Tian et al., 2009), *Bactrocera dorsalis* (Li et al., 2011), *Chilo infuscatellus* (Zhang et al., 2012), *Spodoptera exigua* (Vatanparast and Kim, 2017), *Plagiodera versicolora* (Zhang et al., 2019), *Tuta absoluta* (Bento et al., 2020), *Harmonia axyridis* (Ma et al., 2020), *Spodoptera littoralis* (Caccia et al., 2020), *Agrilus planipennis* (Leelesh and Rieske, 2020). In addition, expressed virus dsRNA can protect a plant or animal against viral infection. For example, *E. coli* strain HT115 (DE3) was used to express dsRNA of the Chinese Sacbrood Virus (CSBV) VP1, which was fed to Chinese honeybees (*Apis cerana*) and effectively prevented the virus infecting the bees (Zhang et al., 2016). Treating *Nicotiana benthamiana* with dsRNAs of fragments of two major plant viruses, Pepper Mild Mottle Virus (PMMoV) and Plum Pox Virus (PPV), effectively reduced the infection of *N. benthamiana* by these two viruses (Tenllado et al., 2003). All these results show that engineered *E. coli* can synthesize dsRNAs, and the dsRNA produced can induce RNAi effects in the corresponding target organisms.

The yield of dsRNA synthesized in *E. coli* has been improved over time. An average of 4 μg of dsRNA was obtained per ml of *E. coli* culture in 2003 (Tenllado et al., 2003), and 45 μg hairpin dsRNA per ml of bacteria (optical density at 600 nm = 1) in 2013 (Posiri et al., 2013). The increase of dsRNA yield is due to the fermentation methods and operation parameters used (Thammasorn et al., 2015; Papic et al., 2018). dsRNA production using batch fermentation and fed-batch fermentation was compared in a 10 L fermenter, and the dsRNA titer in fed-batch fermentation (95.0 ± 21.5 μg/ml) was nearly 30-fold that found in batch fermentation (3.4 ± 0.5 μg/ml) (Thammasorn et al., 2015). The nutrition can also affect the final dsRNA yield, and the production of dsRNA using Terrific broth (TB) (6.2 ± 0.2 μg/ml) was higher than that using Luria-Bertani (LB) broth (2.6 ± 0.8 μg/ml). After further optimization, the yield was close to 0.06 g/g, the maximum production rate reached 11.1 mg L^{-1} h^{-1} by batch fermentation and 15.2 mg L^{-1} h^{-1} by fed-batch fermentation (Papic et al., 2018). Therefore, the dsRNA yield is related to bacterial growth, and fed-batch fermentation resulted in a higher dsRNA yield by sustainably supplying nutrition.

Modification of the expression vector and host strain can further improve the efficiency of dsRNA synthesis. dsRNA production using a new *E. coli* expression system, pET28-BL21 (DE3) RNase III- was thrice than that of L4440-HT115 (DE3) (Ma et al., 2020).

Moreover, extraction methods are closely linked to the yield of dsRNA. The titer of dsRNA extracted from *E. coli* by ultrasonic crushing and phenol extraction was 19.5 μg/ml, while sonication and heating before dsRNA extraction increased the titer of dsRNA by 2.5- to 5- fold (Ahn et al., 2019).

Nowadays, large-scale synthesis of dsRNA in *E. coli* has developed, but further increasing the titer, rate, and yield (TRY) of dsRNA production is essential for future applications.

Saccharomyces cerevisiae dsRNA Expression Systems

The model eukaryotic species *Saccharomyces cerevisiae* has also been used as a chassis for dsRNA production. *S. cerevisiae* has a clear genetic background, easy genetic engineering methods, and well-developed fermentation processes (Nandy and Srivastava, 2018). Besides, *S. cerevisiae* does not contain the core genes *Dicer-2* and *Argonaute-2* of the RNAi pathway (Drinnenberg et al., 2009), which allows efficient dsRNA synthesis in *S. cerevisiae* compared with *E. coli* and other bacterial species (Zhong et al., 2019). Similarly, plant chloroplast does not contain RNAi pathway, and dsRNA can be enriched to 0.4% of total RNA in plant chloroplast; expression of dsRNA in plant chloroplast can be used to protect plants from being fed by insects, which would be more efficient than expressing dsRNA form the plant leaves (Zhang et al., 2015).

Feeding the fruit fly *Drosophila suzukii* with recombinant yeast expressing insect dsRNA targeting *y-Tubulin* resulted in a significant reduction in larval survivorship, adult motility, and reproduction (Murphy et al., 2016). Moreover, feeding *D. suzukii* with genetically modified *S. cerevisiae* expressing dsRNA (targeting *y-tubulin23C*) resulted in a significant decrease in the fitness of *D. suzukii* in the environment (Abrieux and Chiu, 2016).

The expression of dsRNA in *S. cerevisiae* has also been validated in the mosquito *Aedes aegypti*. *Fez2* and *lrc* were selected as target genes in *A. aegypti*, and shRNAs of these genes were expressed in *S. cerevisiae*. When the genetically-modified *S. cerevisiae* was heated, dried, and fed to insects, this led to >95%mortality of *A. aegypti* (Hapairai et al., 2017). The same effects were observed with *Aedes albopictus*, *Anopheles gambiae*, and *Culex quinquefasciatus* (Mysore et al., 2017; Mysore et al., 2019a; Mysore et al., 2019b). In this way, biocontrol strategies for specific mosquito species can be developed, to effectively suppress human diseases transmitted by mosquitoes.

Many insects, livestock, aquaculture species and humans consume yeast. Therefore, developing efficient yeast expression systems might increase the possibility of applying yeast-derived dsRNA commercially (Duman-Scheel, 2019). The dsRNA produced by *S. cerevisiae* can also serve as a potential oral delivery system for shRNA to mammalian cells (mouse intestinal DCs) and be used in human disease therapeutics (Zhang et al., 2014; Duman-Scheel, 2019). Several companies have developed yeast dsRNA expression systems. In May 2019, Renaissance BioScience filed a patent application for the production and delivery of bioactive dsRNA ingredients using yeasts.

In the future, large-scale production of dsRNA in *S. cerevisiae* can be enhanced by improving the expression vectors (Crook et al., 2014), the promoters for the dsRNA transcription (Voineagu et al., 2008), the length of the hairpins (Yoshimatsu and Nagawa, 1989), and the sites of integration positions (Kim et al., 2015).

Bacillus dsRNA Expression Systems

Some *B. subtilis* strains are classified as probiotics for human and animal consumption (Rosales-Mendoza and Angulo, 2015). Therefore, this species has also been selected for dsRNA expression. The dsRNA (*daf-2*, *unc-62*) expression vector pBSR was introduced into *B. subtilis*, and feeding *C. elegans* this genetically-modified *B. subtilis* strain induced RNAi effects (Lezzerini et al., 2015). A *B. subtilis* strain with dsVP28 expression was able to effectively prevent shrimp infection with white spot syndrome virus (WSSV); the survival rate of shrimp treated with the *B. subtilis* strain was 91.67%, while that in the control group was only 28.57% (Saelim et al., 2020).

Bacillus thuringiensis (Bt) is an effective biopesticide production strain that has been widely used for control of lepidopteran pests. Bt has been used as an expression host for dsRNA production. In the vector pBTdsSBV-VP1, two spore-producing-dependent cyt1Aa promoters in opposite direction were linked to the *VP1* gene of Sacbrood virus (SBV), and a Shine-Dalgarno sequence (GAAAGGAGG) was added at specific positions, which increased the stability of the RNA. Transfer of pBTdsSBV-VP1 into Bt strain 4Q7 led to the expression of dsRNA. Feeding the total RNA extracted from this Bt strain to *Apis cerana* (honeybees) infected with SBV virus significantly reduced the viral infection of the insects (Park et al., 2020).

The Bt-based dsRNA production platform has some advantages compared with other platforms. The *cry* sporulation-dependent gene promotor was used for dsRNA expression, and the dsRNA could be produced during the sporulation phase of Bt. Moreover, other expression systems (like *E. coli*, *B. subtiis*, *S. cerevisiae* expression systems) require an inducer (IPTG or others) to induce dsRNA expression, but no inducer is needed for expression in Bt. Finally, Bt cells can undergo enzyme-associated autolysis after sporulation, thus cell lysis is not required for dsRNA extraction (Park et al., 2020).

With the increase of insect resistance to Bt, the use of Bt as a platform for dsRNA expression would help with pest control *via* a Bt + RNAi strategy (Caccia et al., 2020; Kang et al., 2021). Therefore, the Bt dsRNA expression system could be a useful dsRNA production platform for the introduction of RNAi in organisms.

Insect-Symbiotic Bacteria dsRNA Expression Systems

There are abundant symbiotic bacteria in the oral tract and gut of insects, and they interact directly with the insects and plants. Some symbiotic bacteria can easily be genetically manipulated, so they might be potentially efficient dsRNA production platforms for insect control. The use of insect-symbiotic bacteria to express dsRNA for insect control is known as symbiont-mediated RNAi (SMR) (Taracena et al., 2015; Whitten et al., 2016; Whitten and Dyson, 2017; Whitten, 2019; Asgari et al., 2020).

Rhodococcus rhodnii (*R. rhodnii*), a symbiotic bacterium of the triatomine *Rhodnius prolixus*, was used to express RHBP-specific hairpin RNA; the gene expression products of RHBP can suppress *R. prolixus* by affecting its adult oviposition (Taracena et al., 2015). Subsequently, two symbiotic bacterial strains, *R. rhodnii* and BFo2 (a member of the *Enterobacteriales*), were isolated from the insects *R. prolixus* and *Frankliniella occidentalis* (western flower thrips), respectively. The RNase III gene was knocked out and dsRNA expression cassettes was expressed in these two insect symbiotic bacteria; when the engineered bacteria were taken up by insects, the dsRNA functioned in the hosts, inducing RNAi effects (Whitten et al., 2016).

Snodgrassella alvi, a core gut symbiotic bacterium of the honeybee *A. mellifera*, was modified as a dsRNA-producing host. The dsRNA produced by the engineered *S. alvi* can suppress gene expression in *A. mellifera*. Moreover, this dsRNA can suppress genes of parasitic *Varroa* mites and kill them, which protects the honey-bees from the *Varroa* mites, the most threatening pest to the world's beekeeping industry (Leonard et al., 2020). Based on this technology, a new bioproduct, "BioDirect" was registered as dsRNA for the prevention and control of *Varroa mites*. This is the first dsRNA biopesticide active ingredient submitted to the U.S.

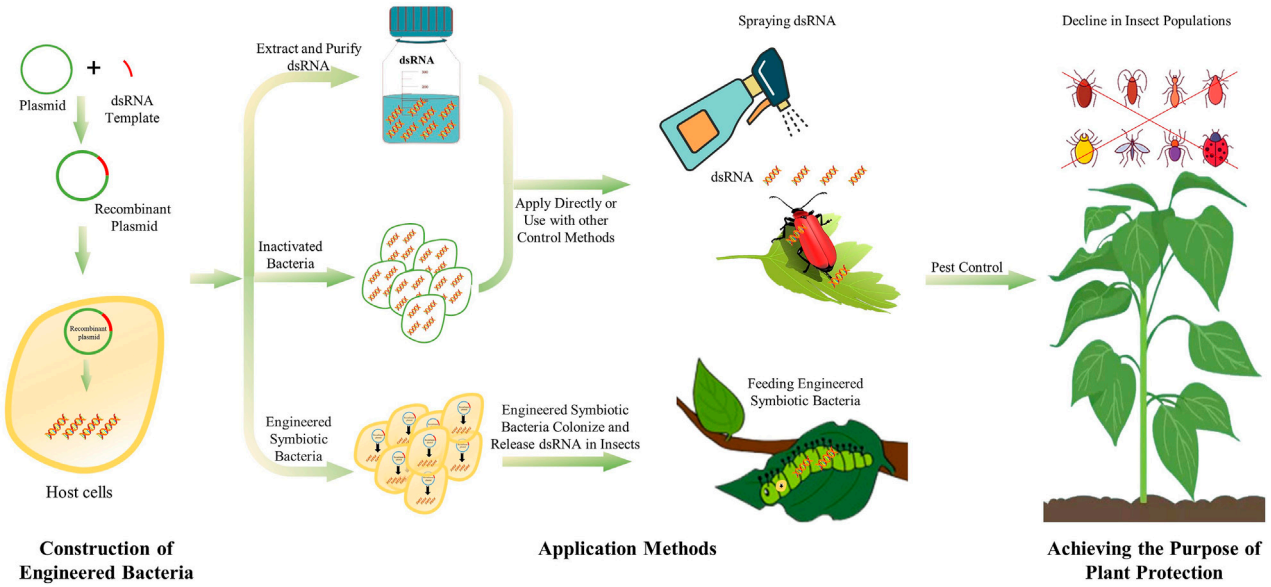

FIGURE 1 | The process and use method of microbial dsRNA production system.

Environmental Protection Agency (EPA) for exogenous application in agriculture.

Thus, SMR is not only potential pest control agents, but can also be beneficial for insect protection. SMR depends on both the specificity of RNAi toward the targeted insect gene, and the specificity of the symbiotic bacterium for its host. This dual specificity makes SMR a precision control tool, and this tool is obviously different from chemical insecticides. However, there are issues that need to be addressed before symbiotic bacteria can reliably serve as dsRNA expression hosts. The first is to find suitable symbiotic bacteria that stably colonize the host insects; the bacterial content should also be relatively high in the host insect. The second is that the symbiotic bacteria must be able to express dsRNA efficiently and stably. Thus, the acquisition and modification of symbiotic bacteria and colonization of the engineered symbiotic bacteria in the host need to be addressed before applying SMR for dsRNA production.

Nevertheless, this SMR strategy is specific for pest control without increasing environmental stress, and it might be widely used in the future.

CONCLUSION AND PERSPECTIVES

Genetic engineering of microorganisms for large-scale production of dsRNA is feasible. Currently, *E. coli*, *Bacillus, S. cerevisiae* and several other symbiotic bacteria have mature expression systems for dsRNA production. As most of these bacteria are probiotics and/or model species, they might be the most suitable microbial hosts for diverse dsRNA production. *Corynebacterium glutamicum* has also been shown to efficiently synthesize dsRNA longer than 1 kbp in a yield >1 g/L of culture (Hashero et al., 2021). Besides, microalgae can also be engineered as dsRNA expression vectors, and shrimps and crabs can be protected from bacterial or viral infection by feeding on microalgae expressing dsRNA (Saksmerprome et al., 2009; Somchai et al., 2016; Charoonnart et al., 2019). Fungi (Chen et al., 2015) and viruses (Dubreuil et al., 2009; Kumar et al., 2012) have also been engineered to produce dsRNA, and better results have been obtained.

The dsRNA synthesized by microbes can be used directly in live or inactivated microbes. However, engineered microbes entering a host induce immune responses, which might compromise the desired RNAi effects. Moreover, the engineered microbes may spread into the environment, and lead to sustainable expression of dsRNA, which might affect non-target species in the environment. Besides, plasmid-based expression elements may be transferred inter-species, resulting in biological contamination problems (Mendelsohn et al., 2020). dsRNA produced in engineered bacteria cannot be secreted directly outside the cell. Therefore, lysis, extraction and purification are required to obtained dsRNA production. The lysis of cells can be performed by ultrasonication, enzymatic lysis, boiling lysis, while sodium dodecyl sulfate (SDS) can be used to enhance the lysis (Posiri et al., 2013). After the cell wall is broken, the nucleic acid can be released to obtain a crude extract of dsRNA. Then, use appropriate RNA extraction methods, such as TRIzol reagent or other RNA extraction reagents, to obtain pure dsRNA production. Extracting and purifying dsRNA from engineered bacteria will avoid or reduce the problems mentioned above. However, these processes are relatively cumbersome and need to be further optimized. The dsRNA obtained through microbial-production can be directly applied to pest control by spraying, and the nanocarrier-mediated transdermal dsRNA delivery system can facilitate the development of sprayable RNA pesticides (Zheng et al., 2019; Yan et al., 2020). Which method to use also needs to be selected according to different environments (**Figure 1**).

There are also some further technical issues in this field that need to be solved. For example, substrates for industrial fermentation can be contaminated with various bacteria, and such contaminants can inhibit growth of the desired (dsRNA-expressing) bacteria and reduce the efficiency of the fermentation process, thus significantly reducing productivity (Seo et al., 2020). Antimicrobial decontamination strategies have been developed, but the metabolites produced and antibiotics used to avoid contamination by other microorganisms are released, inevitably putting pressure on the environment and increasing risks to human health (Kraemer et al., 2019). Once these problems are solved and dsRNAs can be produced by large-scale fermentation, they will have broad application prospects and bring huge economic benefits.

dsRNA production methods have been continuously optimized in recent years to adapt to production needs and promote the application of this technology. The cost of dsRNA was approximately US$12,000/g in 2008, dropping to US$60/g in 2018, and in 2020, RNAGri had the ability to produce tons of dsRNA at a cost of US$1/g, Greenlight's GreenWorX™ system can further reduce the cost of dsRNA synthesis to < US$0.5/g (Cagliari et al., 2019; Suhag et al., 2020; Taning et al., 2020), which will provide material for the economical large-scale application of dsRNA-based pesticides.

The large-scale application of RNAi technology relies on the construction of efficient and appropriate microbial cell factories for dsRNA production. With the development of synthetic biology, global rewiring of the expression systems of model species to increase dsRNA expression levels will be possible. In the future, active engineered microorganisms for dsRNA production and low-cost purified dsRNA will become available leading to greener agriculture without chemical pesticides to protect plants from insects and microbial infections.

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Advances and Opportunities of CRISPR/Cas Technology in Bioengineering Non-conventional Yeasts

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Non-conventional yeasts have attracted a growing interest on account of their excellent characteristics. In recent years, the emerging of CRISPR/Cas technology has improved the efficiency and accuracy of genome editing. Utilizing the advantages of CRISPR/Cas in bioengineering of non-conventional yeasts, quite a few advancements have been made. Due to the diversity in their genetic background, the ways for building a functional CRISPR/Cas system of various species non-conventional yeasts were also species-specific. Herein, we have summarized the different strategies for optimizing CRISPR/Cas systems in different non-conventional yeasts and their biotechnological applications in the construction of cell factories. In addition, we have proposed some potential directions for broadening and improving the application of CRISPR/Cas technology in non-conventional yeasts.

Keywords: CRISPR/cas, cell factories, microbial production, non-conventional yeast, genome engineering

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INTRODUCTION

Non-conventional yeasts have been considered as potential eukaryotic chassis for scientific research and industrial application. Owing to their outstanding natural characteristics. These advantageous attributes include thermotolerance, utilization of extensive carbon sources, and the capacity to produce high-titer proteins, lipids, or other commercial metabolites. For instance, *Scheffersomyces stipitis* is continually used in ethanol fermentation via lignocellulosic feedstock due to its inherent xylose metabolism (Agbogbo and Coward-Kelly, 2008). Methylotrophic yeasts like *Ogataea polymorpha*, *Pichia pastoris* and *Ogataea thermomethanolica*, possess an efficient ability to secrete heterologous protein and glycosylate, and were utilized commercially for producing a variety of proteins (Gellissen, 2000; Hartner and Glieder, 2006; Bredell et al., 2018). Oleaginous yeasts *Rhodotorula toruloides* and *Yarrowia lipolytica*, are capable of storing large amounts of cellular lipids from low-cost carbon sources (Castañeda et al., 2018; Chattopadhyay et al., 2021). The heat resistance of *Kluyveromyces marxianus* allows it to ferment at higher temperature, thus decreasing the probability of contamination (Marcišauskas et al., 2019). Furthermore, *Kluyveromyces lactis* finds extensive usage in lactose metabolism to secrete proteins (Spohner et al., 2016).

Genetic engineering is fundamental to study gene functions and control the expression of genes for producing specific compounds or otherwise regulating the gene expression when these yeasts are employed for scientific research or industrial applications. The efficient genome editing approaches and the corresponding tools are critical for rapid genome and metabolic engineering. The traditional gene manipulation tools such as *Cre-loxP* system can improve the genome editing efficiency to some

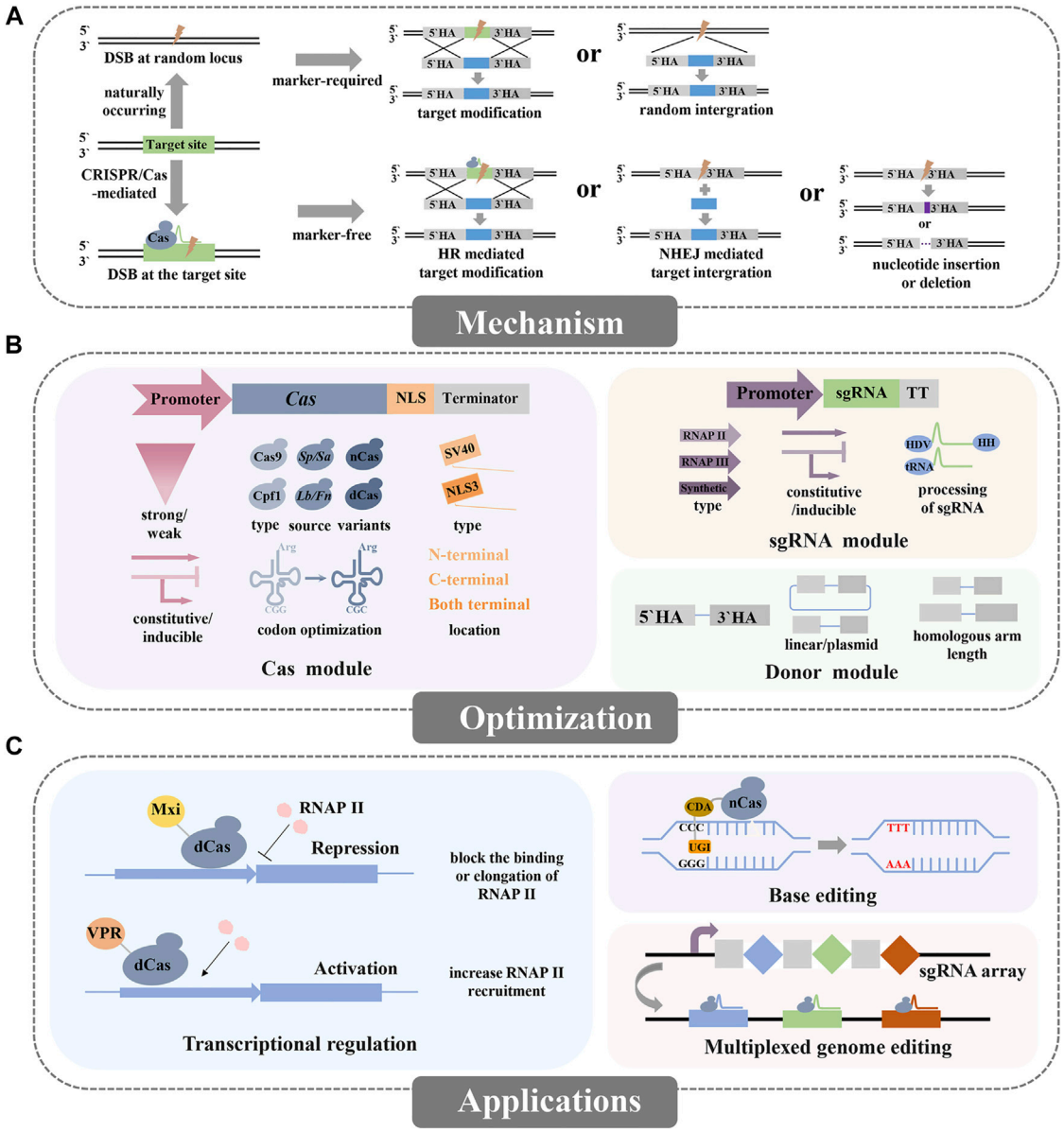

FIGURE 1 | Overview of the mechanism, optimizations, and applications for CRISPR/Cas mediated genome editing in non-conventional yeasts. **(A)** Genome editing via naturally occurring and CRISPR/Cas-mediated DNA repair mechanisms. **(B)** The optimizations of the CRISPR/Cas system include the Cas module, sgRNA module, and donor module. **(C)** The applications of the CRISPR/Cas system mainly include the basic applications (knockin or knockout) and advanced applications (repression, activation, base editing or multiplexed genome editing).

extent, but these systems can only modify single locus in one step, and the marker recycling is time-consuming and left many scars in genome, which is not conducive to genomic stability (Xie et al., 2014). Moreover, the natural homologous recombination (HR) depends on a DNA break which occurs accidentally at the target locus (Raschmanová et al., 2018). In the last few decades, several new, better, and more accurate genetic tools have been developed to improve the efficiency of genome editing, such as zinc-finger nucleases (ZFNs) (Doyon et al., 2011) and transcription activator-like effector nucleases (TALENS) (Li et al., 2011). The targeting of specific DNA sequences by ZNFs and TALENs depends on the protein-DNA interaction, and then the DNA break is introduced by FokI. However, the construction of specific DNA binding proteins is still a laborious and time-consuming task.

Recently, the CRISPR/Cas system has revolutionized genome editing technology due to its efficiency, accuracy, and convenience (Hsu et al., 2014). This system essentially comprises a DNA endonuclease (e.g. Cas9 or Cpf1) which can bind to the target DNA sequence by the guidance of sgRNA, and then generating DNA double-strand breaks (DSB), and later the repair mechanism including HR, non-homologous end-joining

(NHEJ), or microhomology-mediated end joining (MMEJ) is activated (Zetsche et al., 2015; Burstein et al., 2017) (**Figure 1A**). The CRISPR/Cas system relies on the DNA-RNA recognition for inducing precise DNA cleavage, which is marker-free and capable of simultaneous multi-loci editing, thus greatly accelerates the genome editing.

The genome-editing system mediated by CRISPR/Cas has already been widely employed in the genetic engineering of non-conventional yeasts and promoted the biotechnological development of these yeasts. Considering the increasing attention of non-conventional yeasts as the chassis for synthetic biology, we systematically summarized the optimization strategies for highly efficient adopting CRISPR/Cas systems in non-conventional yeasts and highlighted the advanced applications of this technology on functional genomics and constructing non-conventional yeast cell factories. Based on current achievements and challenges, we presented our perspectives on building more efficient and adaptable CRISPR/Cas derived system, which would provide new insights in further study CRISPR/Cas technology in non-conventional yeasts.

OPTIMIZATION OF CRISPR/CAS SYSTEM IN NON-CONVENTIONAL YEAST

CRISPR/Cas system comprises two main components, namely Cas protein and sgRNA. To ensure efficient genome editing in non-conventional yeasts, species-specific optimizations of these two components are essentially required (**Figure 1B**).

Optimization of sgRNA Expression Through Different Promoters

Sometimes the efficient expression of sgRNA comes out to be a challenge in non-conventional yeasts due to the lack of suitable promoters. The promoters used for sgRNA expression should have suitable strength and the sgRNAs require nuclear localization and both ends trimming. In non-conventional yeasts, the transcription of functional sgRNA is usually achieved by four types of promoters: 1) The RNA polymerase II (RNAP II) dependent promoters; 2) The RNA polymerase III (RNAP III) dependent promoters; 3) Synthetic hybrid promoters; 4) T7 polymerase dependent artificial promoter.

The RNAP II dependent promoters are usually responsible for producing mRNAs, so the ribozymes executing cleavage sequences are generally flanked on both ends of sgRNAs for modification and maturation. Hammer head ribozyme (HHR) and hepatitis delta virus (HDV) ribozyme are the commonly used elements, which have been employed in building CRISPR/Cas9 system in *S. cerevisiae* (Gao and Zhao, 2013), *P. pastoris* (Weninger et al., 2016), *O. thermomethanolica* (Phithakrotchanakoon et al., 2018), and *Y. lipolytica* (Gao et al., 2016). However, the genome editing efficiency was lower in comparison to RNAP III dependent promoters based CRISPR/Cas system. This may be presumably due to the extension of the sgRNA variable region from 20 bp to 69 bp because of the homologous arm of the 5′ hammerhead ribozyme (Gao and Zhao, 2013), another reason may be the sgRNAs transcribed by these promoters were exported from the nucleus into the cytosol for translation. Hence RNAP II dependent promoters have rarely been used in sgRNA expression. Interestingly, in the CRISPR/Cpf1 system of *Y. lipolytica*, the editing efficiency reached 93.3% ± 11.5% where sgRNA expression was controlled by RNAP II dependent promoter *TEFin* without ribozymes (Yang et al., 2020), which may be caused by the inherent capacity of Cpf1, thereby enabling to produce mature sgRNA from pre-sgRNA array (Fonfara et al., 2016).

RNAP III dependent promoters such as *SNR52*, *RPR1* have been considered as the most suitable candidates for the expression of sgRNA, because the RNAs transcribed by them remain in the nucleus. By far, the *SNR52* promoter is the most commonly employed promoter for sgRNAs expression, which allowed for gene editing in various non-conventional yeasts, including *S. stipites*, *K. marxianus*, *K. lactis*, *C. albicans and O. polymorpha*. The *RPR1* promoter was also employed in *P. kudriavzevii* and *I. orientalis*. In **Table 1**, we summarized the great and broad effect of these type of promoters in genetic engineering of non-conventional yeasts.

The RNAP III binding sites of some RNAP III dependent promoters are located within their mature transcript, which may add additional nucleotides to the sgRNA, thereby preventing the maturity and release of sgRNA. The sgRNAs expressed by these promoters are thus fused with tRNA, then the tRNA is isolated through its internal maturation mechanism. In *P. pastoris*, the orthogonal tRNA-sgRNA cassettes were expressed by the *tRNA* promoter that enabled multiplexed genome integration of three genes (*gnt1*, *mns1*, and *mnn2*) involved in glycosylation (Dalvie et al., 2020). In *O. polymorpha*, an improved system with $tRNA^{Leu}$-sgRNA fusion was constructed to enhance the sgRNA expression. The efficiencies of indel mutations were significantly improved to 17–71% in comparison to native RNAP III dependent promoter *OpSNR6* which resulted in less than 1% gene disruption (Gao et al., 2021). In *I. orientalis*, a series of native and synthetic promoters used for sgRNA expression were evaluated, and the synthetic *RPR1'-tRNA*^{Leu} promoter was identified as the most effective promoter; the efficiency of single, double, and triple gene disruption was recorded as 97, 90, and 46.7% (Tran et al., 2019), respectively. A similar study was up taken in *Y. lipolytica* where synthetic promoters based on the RNAP III dependent promoters and $tRNA^{Gly}$ were employed for the expression of sgRNA. The editing efficiency of *PEX10* reached up to 92% by the synthetic promoter *SCR1'-tRNA*^{Gly}, which enabled more than a 2-fold increase over the native *SNR52* promoter (Schwartz et al., 2016). In *K. marxianus*, three synthetic RNAP III dependent promoters, including *RPR1-tRNA*^{Gly}, *SCR1-tRNA*^{Gly}, and *SNR52-tRNA*^{Gly} promoter, were applied to optimize the expression of sgRNA. The highest editing efficiency observed in this case was 66% which was achieved by the *RPR1-tRNA*^{Gly} promoter (Löbs et al., 2017). Similarly, in the CRISPR/Cpf1 system, synthetic *SCR1'-tRNA*^{Gly} promoter and native the RNAP III dependent promoter *5sRNA* were tested for their potential to enhance sgRNA expression in *Y. lipolytica*. The highest efficiency was

TABLE 1 | Genetic editing applications of CRISPR/Cas in non-conventional yeasts.

Organism	Host strains	Expression cassette for cas	Promoter of sgRNA	Application	References
Y. lipolytica	PO1f	$P_{UAS1B8\text{-}TFF\ (136)}$-*Sp_Cas9*-$T_{CYC1}$ (*Y. lipolytica* codon-optimized)	[a]*SCR1'-tRNA*^{Gly}	Lycopene biosynthesis pathway (*crtB*, *crtE*, *crtI* and *Ggs1*) were integrated into different loci, resulting in an 8.6 folds increase in lycopene production over the wildtype strain	Schwartz et al. (2017b)
—	PO1f	$P_{UAS1B8\text{-}TFF\ (136)}$-*Sp_Cas9*-$T_{CYC1}$ (*Y. lipolytica* codon-optimized)	[a]*SCR1'-tRNA*^{Gly}	A dual-cleavage strategy for gene excision and targeted integration	Gao et al. (2018)
—	PO1f *ku70Δ*	P_{TFFIn}-*Sp_dCas9*-T_{XPR2} (*Y. lipolytica* codon optimized)	[a,b] $(A1R1)_{x2}A3$	Combing of CRISPRi and a sensor of fatty acid to achieve negative autoregulation of the lipogenic pathway, the naringenin production was increased by 74.8% resultantly	Lv et al. (2020)
—	PO1f	$P_{UAS1B8\text{-}TFF\ (136)}$-*Sp_dCas9-VPR*-$T_{CYC1}$ (*Y. lipolytica* codon-optimized)	[a]*SCR1'-tRNA*^{Gly}	Two genes for cellobiose metabolism were activated through CRISPRa, enabling growth with cellobiose as the single carbon source	Schwartz et al. (2018)
—	PO1g *ku70Δ*	$P_{UAS1B8\text{-}TEF\ (136)}$-*Sp_nCas9-pmCDA1-UGI*-$T_{CYC1}$ P_{TFF}-*Sp_nCas9-pmCDA1-UGI*-T_{CYC1} P_{EXP}-*Sp_nCas9-pmCDA1-UGI*-T_{CYC1} P_{TFFIn}-*Sp_nCas9-pmCDA1-UGI*-T_{CYC1} (*Y. lipolytica* codon-optimized)	[a]*SCR1'-tRNA*^{Gly}	The C to T mutation enabled to introduce a stop codon to achieve the disruption of target gene. The efficiency of single and simultaneous double gene disruption was 94% and 31% *via* optimizing expression level (P_{TFFIn}-*nCas9-pmCDA1-UGI*-T_{CYC1})	Bae et al. (2020)
O. polymorpha	CGMCC7.89	P_{TEF1}-*Sp_Cas9*-T_{ADH2} (*H. sapiens* codon optimized)	[c]*SNR52*	A CRISPR–Cas9-assisted multiplex genome editing (CMGE) system was applied for multigenic editing include multiloci and multicopies. The efficiency of triple different genes knockout and knockin was 23.6% and 30.5% respectively. Multicopies GFP and resveratrol biosynthesis pathways were integrated into the rDNA site	Wang et al. (2018)
—	BY4330	P_{TEF1}-*Sp_Cas9*-T_{TEF1} (*H. sapiens* codon-optimized)	[a]*SNR6-tRNA*^{CUG}	A tRNA-sgRNA fusion was developed for efficient genome editing, the efficiency of disrupting target genes (four genes) via indel mutations ranged from 17% to 71%	Gao et al. (2021)
I. orientalis	SD108	P_{TEF1a}-*Sp_iCas9*-T_{PGK1} (*iCas9*: *SpCas9* with two mutations D147Y and P411T; *H. sapiens* codon optimized) (Bao et al., 2015)	[c]*tRNA*^{Leu} [c]*tRNA*^{ser} [c]*RPR1* [c]*5S rRNA* [a]*RPR1'-tRNA*^{Leu} [a]*5S rRNA-tRNA*^{Leu}	A series of native and synthetic promoters for sgRNA expression were characterized. The highest efficiency was achieved by *RPR1'-tRNA*^{Leu} promoter with the efficiency of single, double, and triplexed gene disruption was 97%, 90%, and 46.7%	Tran et al. (2019)
P. kudriavzevii	YB4010	P_{TDH3}-*Sp_iCas9*-T_{TEF1a} (*H. sapiens* codon optimized)	[c]*RPR1*	Deleting the downstream competing pathway and integrating the itaconic acid (IA) biosynthesis pathway into the genome. The production of IA was achieved at 1,232 mg/L in fed-batch fermentation	Sun et al. (2020)
O. thermomethanolica	TBRC656	P_{AOX}-*Sp_Cas9*-T_{AOX} (*H. sapiens* codon-optimized)	[b]*AOX*	Integrative and episomal CRISPR systems were developed for gene disruption via indel mutations. Via the integrative system, three genes (*OtHAC1*, *OtMAL1* and *OtMAL2*) were disrupted with the efficiency of 63%, 97%, and 93%, individually. Utilizing the episomal system, the efficiency of one gene (*OtMAL1*) disruption was 92%	Phithakrotchanakoon et al. (2018)
C. albicans	SC5314	P_{ENO1}-*Ca_Cas9*-T_{ENO1} (*C. albicans* and *S. cerevisiae* codon-optimized)	[c]*SNR52*	Four loci (*CDR1* and *CDR2*, two alleles each) were targeted by a single sgRNA via homozygous knockout, with an efficiency of 20%	Vyas et al. (2015)
K. lactis	ATCC 8585 *ku80Δ*	P_{FBA1}-*Sp_Cas9*-T_{CYC1} (*S. cerevisiae* codon-optimized)	[c]*SNR52*	The muconic acid biosynthesis pathway (*AroF*, *AroB*, *AroD*, *AroZ*, *AroY* and *CATA*) was divided into three fragments targeting	Horwitz et al. (2015)

(Continued on following page)

TABLE 1 | (*Continued*) Genetic editing applications of CRISPR/Cas in non-conventional yeasts.

Organism	Host strains	Expression cassette for cas	Promoter of sgRNA	Application	References
				different loci. The efficiency of triple integrations was 2.1%	
K. marxianus	NBRC1777	P_{PDC1}-*Sp_nCas9-CDA*-T_{TDH3} P_{PDC1}-*Sp_Cas9*-T_{TDH3} (*H. sapiens* codon optimized)	[c]*SNR52*	The Target-AID base editor was applied to introduce stop codon into *Nej1* and *Dnl4* genes through C-T mutagenesis. The efficiency of the mutant allele (*Nej1* and *Dnl4*) was 12.5%. Three DNA fragments were assembled and integrated with a 50 bp homology arm in NHEJ null mutants. The efficiency of integration was 100%	Nambu-Nishida et al. (2017)
—	CBS 6556	P_{TEF1}-*Sp_Cas9*-T_{CYC1} (*H. sapiens* codon-optimized)	[c]*SNR52* [c]$tRNA^{Gly}$ [a]*SNR52-*$tRNA^{Gly}$ [a]*SCR1-*$tRNA^{Gly}$ [a]*RPR1-*$tRNA^{Gly}$	A variety of natural and synthetic RNAP III dependent promoters were designed for the expression of sgRNA. The CRISPR/Cas9 system was employed to disrupt the genes of ethyl acetate biosynthesis for gene functional characterization. The highest editing efficiency (66%) was provided by the *RPR1-*$tRNA^{Gly}$ promoter. The efficiency of genes disruption ranged from 10% to 67%	Löbs et al. (2017)
—	CBS 6556	P_{TEF1}-*Sp_dCas9*-T_{CYC1} (*H. sapiens* codon-optimized)	[c]$tRNA^{Gly}$	A multiplexed CRISPRi approach was used for regulating the central carbon flux to increase the production of ethyl acetate. The production of ethyl acetate is 3.8-fold higher than the natural capacity	Löbs et al. (2018)
R. toruloides	NP11	P_{GPD}-*Sa_Cas9*-T_{HSP} (*R. toruloides* codon optimized)	[c]*U6b*	The CRISPR/Cas9 system was developed for gene disruption. The genes (*CRT1*, *CAR2*, *and CLYBL*) disruption efficiency via indel mutations was 66.7%, 75%, and 75%, respectively. The disruption efficiency of gene *CRT1* via HR was 8%	Jiao et al. (2019)
—	NP11	P_{GPD1}-*Sp_Cas9*-T_{NOS} P_{FBA1}-*Sp_Cas9*-T_{NOS} P_{PGI1}-*Sp_Cas9*-T_{NOS} P_{PGK1}-*Sp_Cas9*-T_{NOS} P_{TEF1}-*Sp_Cas9*-T_{NOS} (*R. toruloides* codon-optimized)	[b]*GPK* [c]$tRNA^{Gly}$ [c]*5S* [a]*5S-*$tRNA^{Gly}$ [a,b]*GPK-5S-*$tRNA^{Gly}$	An optimal CRISPR/Cas9 system was developed for multiplexed gene disruption. *PGK1* promoter for Cas9 and *5S-*$tRNA^{Gly}$ promoter for sgRNA are the best combination, the efficiency of duplexed gene disruption was 78%	Schultz et al. (2019)
S. stipitis	Y-21448	P_{ENO1}-*Sp_Cas9*-T_{TEF1} (*S. stipitis* codon-optimized)	[c]*SNR52*	The efficiency of gene disruption *via* indel mutations was 80%	Cao et al. (2017)
—	Y-21448	P_{ENO1}-*Sp_Cas9*-T_{TEF1} P_{ENO1}-*Sp_dCas9-Mxi1*-T_{TEF1} (*S. stipitis* codon-optimized)	[c]*SNR52*	Gene knockin via HR in NHEJ-deficient strain. The dCas9-Mxi1 was used to repress the expression of eGFP. The efficiency of gene knocking was 73–83%. The eGFP expression was repressed by 32–40% via targeting to different loci	Cao et al. (2018)
P. pastoris	GS115	P_{HXT1}-*Sp_Cas9*-T_{DAS1} (*H. sapiens* codon optimized)	[b]*HXT1*	High-efficiency sites were screened for multiloci gene integration. Three high-efficiency sites were screened, the efficiency of double-locus and triple-locus integration was 57.7–70% and 12.5–32.1%	Liu et al. (2019)
—	GS115	P_{GAP}-*Sp_Cas9*-T_{AOX1} P_{GAP}-*Sp_dCas9*-T_{AOX1} (*H. sapiens* codon optimized)	[b]*HTX1*	Episomal expression of sgRNA was used for CRISPR/Cas9, and the dCas9-based CRISPRi was introduced for gene interference. The editing efficiency of each targeted gene reached or exceeded 75%, and a precise sequence of P_{AOX1} which can control the transcription and translation of *AOX1* was obtained *via* CRISPRi	Hou et al. (2020)

[a]*Synthetic promoter.*
[b]*RNAP II dependent promoter.*
[c]*RNAP III dependent promoter.*
Sp_Cas9: Streptococcus *pyogenes Cas9, Sa_Cas9:* Staphylococcus aureus *Cas9, Ca_Cas9: non-canonical codon Cas9 for C. albicans.*

achieved in the case of *SCR1'-tRNA*^{Gly} promoter; the editing efficiency of *CAN1 via* indels mutation was found to be as high as 86.6% ± 5.7% (Yang et al., 2020).

However, finding a suitable RNAP III dependent promoter in some hosts is still a challenge. In a recent study, an artificially constructed promoter based on the T7 expression system was successfully used for sgRNA expression in some yeasts. An optimized T7 polymerase mutant (P266L) fused with an SV40 nuclear localization sequence (NLS) was developed to ensure a functional T7 promoter for the expression of sgRNA. This system was widely applied in *Y. lipolytica*, *K. lactis*, and *S. cerevisiae* and its editing efficiency was more than 60% (Morse et al., 2018). The optimized T7 expression system from bacteria provides an alternative tool for hosts with no suitable promoters for sgRNA expression. Overall, these innovative approaches of sgRNA expression have great potential for enhancing genome editing in non-conventional yeasts.

Strategies for Optimal Cas Protein Expression

Nuclear localization, codon optimization, and promoter screening (strong/weak or constitutive/inducible) were often adapted for optimizing the expression of Cas protein. In eukaryotic chassis, the Cas protein should be localized in the nucleus to generate DSB, the most common strategy being the fusion of an NLS with the Cas to achieve nuclear targeting. The most typical strategy involves the fusion of an SV40 NLS to the N- or C-terminal of Cas (Schwartz et al., 2016; Yang et al., 2020), or even both C- and N-terminal (Cao et al., 2017; Tran et al., 2019; Hou et al., 2020). It has been reported that in *R. toruloides*, an SV40 NLS fused to the C-terminal of Cas9 is insufficient to achieve its nuclear targeting. Therefore, NLS3 (an endogenous NLS) was appended to the C-terminus of the Cas9 to ensure its import to the nucleus (Schultz et al., 2019).

Codon optimization of the *Cas* gene can affect the functionality of the CRISPR/Cas system, however, in terms of the efficiency of genome editing, it does not seem necessary in some non-conventional yeasts. The *Homo sapiens* codon-optimized *Cas9* has been used for genetic editing in *P. pastoris* (Weninger et al., 2016) and *Y. lipolytica* (Gao et al., 2016). *ScCas9* (*S. cerevisiae* codon-optimized) has been used in *K. lactis* (Horwitz et al., 2015) and *C. glabrata* (Enkler et al., 2016). Both *C. glabrata* and *S. cerevisiae* codon-optimized sequences of *Cas9* enabled the genetic editing in *C. glabrata* (Enkler et al., 2016) and both *H. sapiens* and *Y. lipolytica* codon-optimized sequences are functional in *Y. lipolytica* (Gao et al., 2016; Schwartz et al., 2016). In contrast, the codon optimization of *Cas9* seriously influenced the efficiency of editing in *P. pastoris* (Weninger et al., 2016) and must be required for the non-canonical codon assignment yeast *C. albicans* (Vyas et al., 2015).

In non-conventional yeasts, the promoters used for *Cas* expression commonly focus on strong and constitutive promoters, such as P_{TEFin} *or* P_{TEF} in *Y. lipolytica* (Holkenbrink et al., 2018; Bae et al., 2020), P_{ENO1} in *C. albicans* (Vyas et al., 2015), P_{FBA1} in *K. lactis* (Horwitz et al., 2015), and P_{ScTEF1} in *C. glabrata* (Enkler et al., 2016). In *O. thermomethanolica*, the expression of Cas9 was controlled by an inducible promoter P_{AOX}. The high expression of Cas9, when triggered by methanol, has a detrimental influence on its fitness. This negative effect was ameliorated when the expression of Cas9 was induced by glycerol instead (Schwartz et al., 2017a). However, the inducible promoter tested in *P. pastoris* was not successful (Schwartz et al., 2018). In *C. glabrata*, the choice of the promoter may influence the type of mutation, a single base pair or larger insertions were observed when different promoters were used to express *Cas9* (Enkler et al., 2016). In *Y. lipolytica*, different promoters were screened for *nCas9-pmCDA1-UGI* expression, and the highest efficiency was achieved by *TFFin* promoter (Ramesh et al., 2020). These findings show that an appropriate level of *Cas9* expression is beneficial to the strain's resilience and genome editing efficiency. As a result, species-specific improvements of the CRISPR system are required for it to function properly.

Except for the expression of sgRNA and Cas, the targeted gene loci and the sequence of sgRNA also have a significant impact on the genome-editing efficiency. In addition, if the DSB must be repaired by HR, a donor DNA must be given. The type (linear or plasmid) and homologous arm length of donor DNA also impact HR effectiveness.

ADVANCED CRISPR/CAS TECHNOLOGY IN NON-CONVENTIONAL YEASTS

Besides the exploitation of the basic function of the CRISPR/Cas system like gene deletion or integration, some advanced CRISPR/Cas technologies have been applied in non-conventional yeasts such as the regulation of transcription, base editing and homology-independent gene integration (**Figure 1C**).

CRISPR Interference and CRISPR Activation

Metabolic engineering and functional genomics both benefit from targeted gene transcriptional regulation. CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi) have been constructed to enhance or weaken the expression of the target gene in non-conventional yeasts. These technologies are based on inactive Cas (dCas), which still preserving the capacity to target and bind to particular DNA sequences.

CRISPRi is a simple but useful tool that can down-regulate the expression level of the target gene. By binding to region of the promoter, dCas could sterically block the binding or elongation of the RNA polymerase, resulting in transcription repression. The effect of repression can be further strengthened by fusing a repressor domain like Mxi1 to dCas. For example, a CRISPRi system based on dCas9 was developed in *P. Pastoris*, and through this system, a more precise sequence of P_{AOX1} was obtained which can control the transcription and translation of *AOX1* (Hou et al., 2020). In *Y. lipolytica*, the CRISPRi system was also developed to repress NHEJ. By multiplexed targeting to *ku70* and *ku80*, the HR efficiency, in this case, is as high as 90% compared to the *ku70* deficient strain (Schwartz et al., 2017a). In *K. marxianus*, a

multiple CRISPRi system was developed for redirecting carbon flux of the central metabolic pathway towards ethyl acetate production, causing an improved ethyl acetate titer by 3.8-fold (Löbs et al., 2018). Alternatively, the selection of sgRNA has a great influence on the repression efficiency of CRISPRi. Zhang *et al.* designed a multiplexed sgRNA targeting strategy in *Y. lipolytica*. Through simultaneous targeting to *gfp* gene with three different sgRNAs, the repression efficiency reached 92% and 85% with dCas9 and dCpf1 respectively. Furthermore, the efficient repression of three target genes (*vioA*, *vioB*, and *vioE*) in protodeoxy-violaceinic acid (PVA) synthetic pathway was also realized in one step by this strategy, the content of PVA was reduced by 61% and 75% with dCpf1 and dCas9 separately compared with their corresponding control strains (Zhang et al., 2018).

In general, the fusion of transcriptional activators like VP64 and VPR with dCas9 causes gene upregulation by increasing RNA polymerases recruitment. In *Y. lipolytica*, a dCas9-VPR fusion was used to activate β-glucosidases that allow its growth with cellobiose as the single carbon source (Schwartz et al., 2018). Truncating sgRNA could inhibit the nuclease activity of Cas protein, but not influence the targeting effect. On this basis, a Cpf1-VPR fusion with truncated sgRNA (16 bp) increased hrGFP expression by 10-fold in *Y. lipolytica* (Ramesh et al., 2020).

In addition to the on/off states of gene expression, controlling the gene expression at a suitable level allows for the creation of the desired phenotype. Graded gene expression strengths were obtained by altering the sgRNA target site in the promoter region using CRISPRi or CRISPRa. It resulted in a dynamic gene expression range from zero to several 10-fold improvement, allowing for fine-tuning of metabolic pathway expression and optimization of optimal phenotypes. The balance between cell growth and products biosynthesis is the major issue to be addressed in construction cell factories. CRISPR/Cas-mediated multiple genes synchronized regulation maybe a powerful tool to build a highly efficient non-conventional yeast cell factories.

Base Editing

Base editing is a valuable tool with a lot of potential in genetic editing. By fusing deaminase with the nCas or dCas proteins, these fusion proteins may directly create precise point mutations in genomic DNA. A Target-AID (target activation-induced cytidine deaminase) base editor based on the nCas9-pmCDA1 fusion protein was created to execute the conversion of C to T in human cells and *S. cerevisiae*, which was tested by inserting a nonsense mutation into the coding sequence.

In non-conventional yeasts, the Target-AID base editor was employed to realize gene disruption in *Y. lipolytica* (Bae et al., 2020). Similarly in *K. marxianus*, this base editor was also constructed to disrupt *Nej1* or *Dnl4* which was involved in NHEJ to enhance the proportions of HR-mediated integration (Nambu-Nishida et al., 2017). Apart from gene disruption, this base editor was further employed for situ mutagenesis, thus enabling it to obtain the desired phenotype. Recently, the general transcription factor gene *SPT15* in *S. cerevisiae* was mutated by Target-AID base editor to enhance the stress tolerances (Liu et al., 2021). Furthermore, this strategy has also been applied in mammals (Ma et al., 2016) and plant (Li et al., 2020) but has not been widely reported in non-conventional yeasts. Though the overall usage of base editing is less in comparison to conventional CRISPR/Cas genetic editing, it has the potential to complement genetic editing tools because of its procedures being donor-free and DSB-free.

Homology-independent Targeted Genome Editing

Constructing homologous arms for homology-dependent genome editing is laborious and time-consuming. In most non-conventional yeasts such as *Y. lipolytica*, *S. stipites*, *R. toruloides*, *P. pastoris*, and *K. marxianus*, NHEJ is the dominant repair pathway of DSB. A recently study took advantage of this inherent property and constructed a CRISPR/Cas9-mediated homology-independent gene integration tool in *Y. lipolytica*. The targeted gene integration rate was up to 55% by optimizing the cleavage efficiency of Cas9, manipulating repair fidelity of NHEJ, cell cycle and integration sites. By using this tool, iterative integration of canthaxanthin biosynthesis pathway including four genes (*GGS1*, *carB*, *carRP* and *CrtW*) was achieved (Cui et al., 2021). It is worth noting that integration of an 8,417 bp fragment composed of *GGS1*, *carB*, and *carRP* into genome by one step may still be a challenge for the HR dependent targeted genome integration, indicating this tool paves a new avenue to realize the accurate and efficient targeted genome integration in some non-conventional yeasts.

CRISPR/CAS TECHNOLOGY IN FUNCTIONAL GENOMICS AND CELL FACTORY CONSTRUCTION OF NON-CONVENTIONAL YEASTS

Relative to the conventional yeast *S. cerevisiae*, the genome annotations for non-conventional yeast are not particularly thorough. In *Y. lipolytica*, CRISPR/Cas9 has been applied to research functional genomics through the construction of a sgRNA library that covers the whole genome and targeting 7,845 coding sequences (CDS). A total of 1,377 CDSs were identified as necessary CDSs by employing this approach. This sgRNA library facilitates the screening of growth and non-growth related phenotypes, such as canavanine resistance (Schwartz et al., 2019). In *P. pastoris*, one to three nucleotides have been precisely inserted or deleted at the S215 of the methanol expression regulator Mxr1, and the S215 also has been mutated to A215 through a single base replacement. The frameshift mutation of *Mxr1* resulted in almost no transcription of its target genes *DAS1*, *DAS2* and *AOX1*, with *AOX2* transcription, decreased by 40%. For the $Mxr1^{S215A}$, the transcription of these four targeted genes was decreased by nearly 60% (Hou et al., 2020). In *K. marxianus*, the CRISPR/Cas9 system was developed to characterize functional genes within the ethanol and ethyl acetate biosynthesis pathway by disrupting the genes involved, thereby demonstrating that *ADH7* (alcohol dehydrogenase) played a major role as an alternative pathway for the biosynthesis of ethyl acetate (Löbs et al., 2017). These researches

demonstrated that CRISPR/Cas was a valuable method for determining gene function and identifying candidate genes.

The creation and application of strains often require repetitive design-build-test cycles, the CRISPR/Cas method can speed up this process because it enables marker-free gene editing. Wang *et al.* used CRISPR/Cas9 to engineer *O. polymorpha* to produce resveratrol. By targeting the rDNA site, 10 copies of resveratrol biosynthetic pathway (three genes, composed of *TAL*, *4CL* and *STS*) were integrated into the genome, resulting in higher resveratrol production, which reached as high as 97.23 ± 4.84 mg/L, representing a 20-fold increase compared with single-copy integration (Wang et al., 2018). In *Y. lipolytica*, CRISPR/Cas9 was used by *Schwartz et al.* to screen integration loci that not only allow for high expression of the integrated gene but also have no detrimental impact on cell resilience after the gene has been integrated. Five of the 17 loci were found, and four genes (*crtB*, *crtE*, *crtI* and *Ggs1*) involved in the lycopene production pathway were each integrated into a separate locus enabling an 8.6 folds increase in lycopene production in comparison to the wildtype strain (Schwartz et al., 2017b). In *P. kudriavzevii*, the CRISPR/Cas9 was adapted to deleting the downstream competing pathway and then integrating the itaconic acid (IA) biosynthesis pathway into the genome. The production of IA achieved afterward was 1,232 mg/L in fed-batch fermentation (Sun et al., 2020). Overall, CRISPR/Cas technology is a powerful tool in bioengineering these traditional hardly genetically engineered yeasts for basic research or industrial applications (**Table 1**).

DISCUSSION

Despite the numerous successful applications of CRISPR/Cas technology in different non-conventional yeasts, there are still many obstacles to overcome and CRISPR/Cas based techniques to develop in non-conventional yeasts.

In most non-conventional yeasts, NHEJ weakens their precise genome engineering. To increase the HR efficiency, the most common strategy is to knock out or inhibit NHEJ-related genes such as *ku70/80*, *Nej1*, and *Dnl4*, which have proven effective in *Y. lipolytica* (Kretzschmar et al., 2013), *K. marxianus* (Nambu-Nishida et al., 2017). However, many studies reported that the robustness of NHEJ-deficient cells is poor in comparison to NHEJ-proficient cells (Kretzschmar et al., 2013). Alternatively, some studies show that the HR efficiency can be enhanced by overexpressing HR-associated genes like *Rad51/52* and *Sae2*. Expression of the codon-optimized *ScRad52* demonstrated an obvious improvement in the HR efficiency of *Y. lipolytica* (Ji et al., 2020). The combined expression of *ScRad51*, *ScRad52*, and *ScSae2* significantly improved the HR rate of *O. polymorpha* (Gao et al., 2021). On the contrary, *ScRad51/Rad52* expressed in *P. pastoris* (Cai et al., 2021) and *S. stipitis* (Cao et al., 2018) had no distinct improvement in HR efficiency, but overexpression of endogenous *Rad51* and *Rad52* resulted in higher HR activity of *P. pastoris* (Cai et al., 2021). Presumably, the mechanism of DSB repair is mysterious and complex, the choice of proper genes and the expression strength of these genes are important to increase HR efficiency, which is essential for efficient operation of the multiple genes simultaneously in non-conventional yeasts. Alternatively, CRISPR/Cas9-mediated homology-independent targeted gene integration maybe a potential tool for precise genome engineering.

In addition to the current commonly used Cas9 and Cpf1, other Cas proteins have gradually started receiving attention. Recently, Walton *et al.* developed a near-PAMless SpCas9 variant (SpRY), which can target almost all PAMs (Walton et al., 2020). For Cpf1, two AsCpf1 variants were developed, which recognize TYCV and TATV PAMs, respectively (Gao et al., 2017). Cas13a previously known as C2c2 can induce precise cleavage of RNA, therefore, it can perform RNA interference without DNA damage (Cox et al., 2017). Furthermore, gene editing at the RNA level is reversible and changeable, it is a viable method for developing dynamic regulatory elements to control the level and timing of Cas13a expression. Cas14, which has only 400 to 700 amino acids, may target ssDNA and induce cleavage without requiring a specific sequence (Harrington et al., 2018). Up to now, the applications of Cas9, Cpf1 variants, Cas13a, and Cas14 are mainly focused on nucleic acid testing (Gootenberg et al., 2017; Ge et al., 2021) or mammalian (Gao et al., 2017; Walton et al., 2020) and plant cells (Abudayyeh et al., 2017). Based on their advantages, they will have promising potentials to be developed in non-conventional yeasts.

The use of the CRISPR/Cas system in conjunction with other methods or effectors has considerably increased the total capability of cellular engineering. In the realm of synthetic biology, the combination of dynamic regulation with CRISPR is highly efficient in improving biological processes. Recently, the muconic acid production in *E. coli* was increased by 1.3-fold through using an optogenetic CRISPRi system (Wu et al., 2020). Similar to *Y. lipolytica*, by the combination of a fatty acid biosensor and CRISPRi, the production of naringenin was increased by 74.8% (Lv et al., 2020). On the other hand, the target nucleotide diversification was performed by the fusion of nCas9 and error-prone DNA polymerase in *E. coli* (Halperin et al., 2018) and *S. cerevisiae* (Tou et al., 2020) respectively. Although the base editing accomplished by Cas-deaminases fusion has been widely investigated, it has only been reported in few non-conventional yeasts. The introduction of these systems into non-conventional yeasts would be a huge help in fine-tuning gene expression or global genome engineering, both of which are important techniques for building highly efficient cell factories or scientific research.

AUTHOR CONTRIBUTIONS

LS: writing-original draft. ZD and QW: writing-review and editing and project administration. All authors contributed to the article and approved the submitted version.

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