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Review

Engineering *Saccharomyces cerevisiae* for efficient production of recombinant proteins

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ABSTRACT

Saccharomyces cerevisiae is an excellent microbial cell factory for producing valuable recombinant proteins because of its fast growth rate, robustness, biosafety, ease of operability via mature genomic modification technologies, and the presence of a conserved post-translational modification pathway among eukaryotic organisms. However, meeting industrial and market requirements with the current low microbial production of recombinant proteins can be challenging. To address this issue, numerous efforts have been made to enhance the ability of yeast cell factories to efficiently produce proteins. In this review, we provide an overview of recent advances in *S. cerevisiae* engineering to improve recombinant protein production. This review focuses on the strategies that enhance protein production by regulating transcription through promoter engineering, codon optimization, and expression system optimization. Additionally, we describe modifications to the secretory pathway, including engineered protein translocation, protein folding, glycosylation modification, and vesicle trafficking. Furthermore, we discuss global metabolic pathway optimization and other relevant strategies, such as the disruption of protein degradation, cell wall engineering, and random mutagenesis. Finally, we provide an outlook on the developmental trends in this field, offering insights into future directions for improving recombinant protein production in *S. cerevisiae*.

1. Introduction

Proteins play a crucial role as biomacromolecules in living organisms and are significant components of many consumer products such as pharmaceuticals, food, and feed. They are frequently used in industrial production of biofuels and chemicals [1]. However, the extraction and purification of valuable proteins from natural sources pose a challenge because of their low yields, making them unsuitable for industrial processes [2]. The use of recombinant proteins for disease treatment has become a well-established option, with therapeutic biopharmaceuticals being one of the fastest-growing multibillion-dollar industries. The vaccination industry generated approximately US $28 billion in 2015 and is projected to reach US $39 billion by 2022 [3]. Enzyme usage in various industrial sectors has grown continuously, particularly over the past two decades. These sectors include dairy product manufacturing [4], starch transformation [5], baking [6], and beverage production [7]. For industrial production, microbial organisms are widely used to express recombinant proteins of medical or industrial value [8]. Bacteria, particularly *Escherichia coli*, are the most efficient producers because of their rapid growth and simple culture conditions. However, they cannot complete the post-transcriptional processes required for eukaryotic proteins, such as protein folding, glycosylation, phosphorylation, and removal of signal peptides [9]. In contrast, mammalian cells such as *CHO* cells have an effective post-translational modification process for eukaryotic proteins [10]. Nevertheless, the cost of recombinant protein production using mammalian cells is high due to their slow growth rate and complex culture conditions, limiting their application in this field [11]. In comparison, yeasts such as *Saccharomyces cerevisiae* and *Pichia pastoris* offer sev-

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eral advantages. Yeasts have a rapid growth rate and strong robustness, making it compatible with large-scale cultivation in bioreactors [12,13]. Additionally, they have a clear genetic background and use diverse genetic modification methods, such as CRISPR/Cas9 technology, making it easy to optimize recombinant protein production [8,14]. Importantly, as a eukaryotic model organism, yeast can efficiently complete the post-translational modification process [15]. *S. cerevisiae* and *P. pastoris* have been used to produce many FDA-approved recombinant protein drugs [16,17], including insulin and insulin analogs, human serum albumin, hepatitis vaccines, and virus-like particles for vaccination against human tumor viruses [18,19]. Furthermore, *S. cerevisiae* is often used to produce industrial enzymes, including α-amylase and cellulase [20–24].

Secretion of recombinant proteins does not require cell lysis, resulting in fewer interfering proteins. This simplifies the separation and purification processes and reduces production costs, making it a widely used method for recombinant protein production. The surface-display of recombinant proteins has extensive applications in vaccine and antibody development, library screening, biosensor detection systems, biodegradation, and biofuels [25]. Therefore, it has become a powerful tool for biotechnological and biomedical applications [26,27]. However, production of secreted or surface-displayed proteins by *S. cerevisiae* is often limited to low amounts. Consequently, various approaches, such as optimizing gene transcription levels and engineering secretory pathways, have been explored to enhance recombinant protein production. In this review, we summarize the function of these strategies for improving the production of recombinant proteins in *S. cerevisiae*.

2. Increasing transcription levels to improve recombinant protein production

Transcription levels directly affect recombinant protein yield. To increase production in *S. cerevisiae*, various strategies have been used to enhance the transcription levels of recombinant proteins. These strategies include promoter engineering, codon optimization, and expression system optimization.

2.1. Promoter engineering for enhancing recombinant protein production

Controlling gene expression through promoters is essential for heterologous genes [28]. Continuous exploration and characterization of endogenous *S. cerevisiae* promoters has been conducted for better expression of heterologous genes under different culture conditions [29–33]. Two main types of endogenous promoters have been studied in *S. cerevisiae*: constitutive promoters that provide relatively stable expression under various culture conditions [34] and inducible promoters that specifically express genes under particular conditions [35]. Commonly used high-strength constitutive promoters include P_{TEF1}, P_{TDH3}, P_{PGK1}, P_{TPI1}, P_{CCW12}, and P_{ENO2} [36], whereas inducible promoters include P_{GAL1}, P_{GAL2}, P_{GAL7} and P_{GAL10} (Fig.1A) [37].

In addition to endogenous promoters, heterologous promoters were screened for their higher strength in *S. cerevisiae* (Fig. 1B). Eleven galactose-inducible promoters from *Saccharomyces* species were compared in *S. cerevisiae*, and most have stronger than native P_{ScGAL1} activity, especially P_{SeGAL2} and P_{SkGAL2} from *Saccharomyces eubayanus* and *Saccharomyces kudriavzevii*, respectively [38,39]. Promoter engineering has recently been used to construct synthetic promoters for various applications. Synthetic promoters can be created by combining core promoter elements and upstream activating sequences [40]. Compared to natural promoters, some synthetic hybrid promoters have shown higher promoter strengths [28]. Synthetic hybrid promoters have been successfully used to improve the expression of surface-displayed and secreted β-glucosidase in *S. cerevisiae* [40]. In addition, synthetic P_{ENO2} and synthetic P_{PDC2} obtained from random mutagenesis of their native sequences improve the expression of the cellobiose transporter and β-glucosidase by 24.4-fold and 3.0-fold, respectively [41]. In summary, promoter engineering is a common and effective strategy for increasing recombinant protein production in *S. cerevisiae*.

2.2. Codon optimization improves the production of recombinant proteins

Codon optimization is an approach used in gene engineering to enhance heterologous gene expression by changing synonymous codons based on the codon bias of an organism. The degeneracy of the genetic code gives rise to the occurrence of synonymous codons [42,43]. Different hosts exhibit distinct frequencies of synonymous codons used in the translation process, resulting in a codon bias during the expression of recombinant proteins [44,45] and leading to a host-specific codon usage bias [46]. Initially, codon optimization was used to promote recombinant protein production in prokaryotic production systems [47] and has been extensively used in *S. cerevisiae* [48–50].

The replacement of rare codons with high-frequency ones using synonymous substitutions is the conventional codon optimization method for improving recombinant protein expression. For example, Kim et al. achieved a 2.5-fold increase in the yield of recombinant proteins in *S. cerevisiae* using conventional codon optimization [51]. However, in some cases, replacing synonymous codons with high-frequency ones may result in no increase or decrease in recombinant protein expression [52,53]. This phenomenon arises because this strategy overlooks the kinetic effects of protein translation and assumes a direct linear relationship between tRNA abundance and protein translation [54]. It relies on a high abundance of tRNA corresponding to high-frequency codons to directly enhance protein translation efficiency. However, numerous studies have demonstrated that tRNA abundance is not directly related to translation levels [55–57] and can be affected by growth conditions and cell cycle [58–60]. Moreover, tRNA abundance is not the sole determinant of the translation rate of recombinant proteins [61], as specific codon combinations also play a crucial role in regulating the speed of ribosomal transcription and translation and promoting proper protein folding [62]. When modifying the coding sequence, factors such as the average GC content of the host gene, mRNA secondary structure, codon adaptation index, and tRNA concentration should be considered to avoid potential adverse effects on recombinant protein expression [63]. In contrast to traditional codon optimization approaches, specific codon optimization based on system-level information, as well as the upstream and downstream regions of codons, has been shown to effectively enhance catechol 1,2-dioxygenase activity by 2.9-fold [64]. Similarly, Cripwell et al. increased extracellular α-amylase activity by 3.3-fold using specific codon optimization [65]. Therefore, codon optimization can effectively improve the production of recombinant proteins.

2.3. Optimization of protein expression systems to improve recombinant protein production

Recombinant proteins are commonly introduced into *S. cerevisiae* using gene expression cassettes, which can be delivered via plasmids or integrated into chromosomes [66]. Plasmids carrying the 2μ origin of replication can maintain multiple copies (5–30 copies) in *S. cerevisiae*, resulting in an increase in the transcription level of the recombinant protein [67]. However, plasmids often fail to remain stable under non-selective conditions, which significantly affects the recombinant protein yields, especially during large-scale and long-term industrial fermentation [68].

The use of novel selection markers has shown a positive effect on maintaining plasmid stability. Liu et al. identified that the *POT1* gene can be used as a selective marker in *S. cerevisiae* [69,70]. This is because the *TPI1* gene encodes a triose-phosphate isomerase in the EMP pathway and deleting *TPI1* significantly hampers cell growth when glucose is the sole carbon source. In contrast, the expression of the exogenous *POT1* gene from *Schizosaccharomyces pombe* can recover the deletion of the *S. cerevisiae TPI1* gene and is used as a selective marker

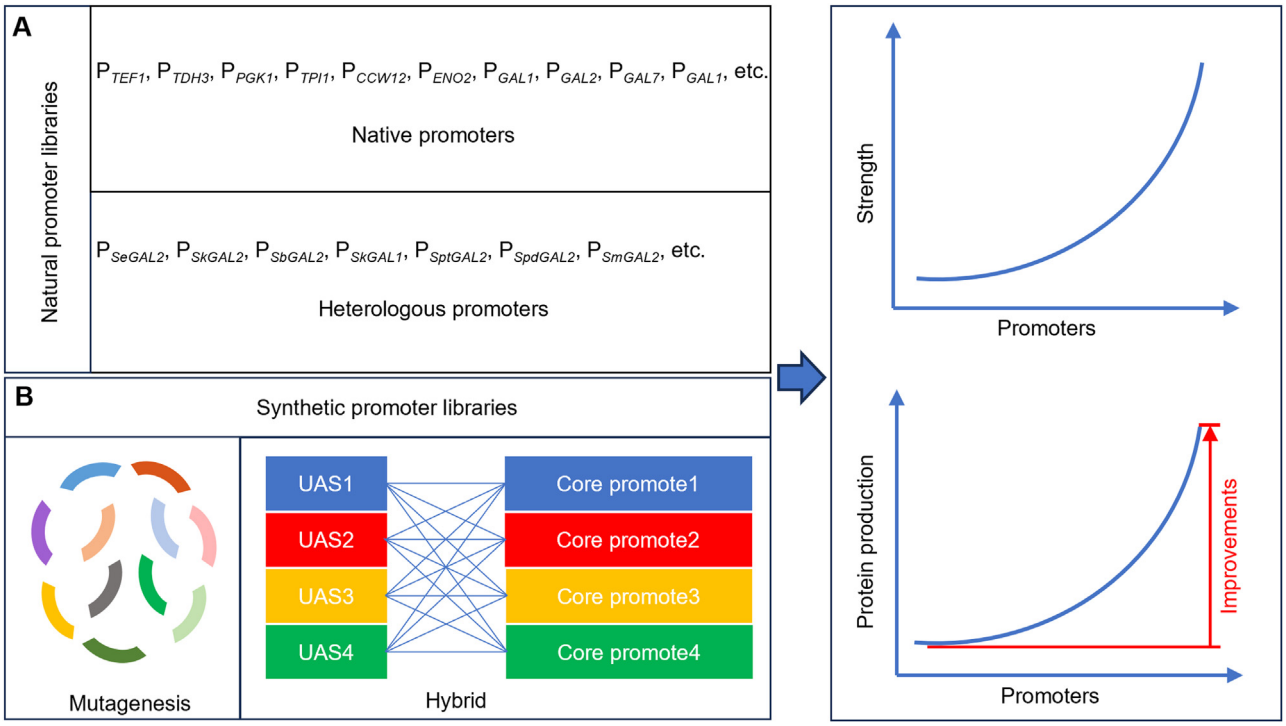

Fig. 1. Promoter engineering to enhance recombinant protein production. A. Identification of robust promoters from natural libraries. B. Development of synthetic promoter libraries via mutagenesis and hybrid approaches.

to maintain plasmid stability under glucose-only conditions. Exogenous Pot1p may not function optimally in *S. cerevisiae* and may require multiple copies to complement the host cell defect, resulting in high copy numbers of the plasmid [71]. Several studies have used the *POT1* maker to achieve high production of recombinant proteins, including α-amylase, β-glucosidase, cellobiohydrolases, glucan 1,4-α-glucosidase, and insulin, via secretion and surface display in *S. cerevisiae* [8,13,69,71,72].

With advancements in *S. cerevisiae* genome modification technology, particularly the CRISPR-Cas9 system, integrating a recombinant protein expression cassette into the chromosome has become a more advantageous option [73]. However, only a limited number of genes can be integrated into this system. To obtain multiple copies of the target gene in one round of construction, delta integration is an essential approach to achieve multi-copy numbers in one step since there are approximately 425 copies of δ sequences distributed throughout the yeast genome [74]. The introduction of several types of cellulases using delta integration has made it easier to obtain strains with high cellulolytic activity [75]. To reduce false positives during antibiotic selection and the low integration efficiency of auxotrophic selection, the *POT1* selection marker is used for genomic delta integration. Song et al. used this selection marker to achieve 32-copy integration of a green fluorescent protein expression cassette and 35-copy integration of a cellobiohydrolase I expression cassette, resulting in 67% Avicel conversion and the highest CBHI activity and protein yield [71]. Coupling delta integration with the CRISPR-Cas9 system can provide a simple platform for high-efficiency, single-step, marker-less, multicopy chromosomal integration of target genes, which has potential applications in optimizing recombinant protein production [76]. In addition to delta integration, rDNA integration is a good choice for obtaining multiple copy numbers, because 100–1000 copies of *rDNA* sequences are present in all eukaryotic genomes [77]. Eight copies of the xylose isomerase gene were integrated into the genome in a single step via rDNA integration [78]. In the future, we speculate that these methods could be applied to efficiently enhance recombinant protein expression in *S. cerevisiae*.

3. Improving the production of recombinant proteins by engineering the secretory pathway

Protein secretion in *S. cerevisiae* begins with the translocation of polypeptides into the endoplasmic reticulum (ER). Molecular chaperones assist in the initial protein folding and modification, such as disulfide bond formation and the addition of central sugar chains, ensuring structural correctness. Protein precursors are then transported to the Golgi apparatus through membrane vesicles, where further processing and modifications, such as sugar chain extension, occur. Finally, the mature and active proteins are transported out of the cell or to the cell surface through membrane vesicles. However, several limiting factors in the secretory pathway hinder the efficient production of recombinant proteins in *S. cerevisiae*. These factors include protein translocation, protein folding, insufficient transport capacity of membrane vesicles, excessive glycosylation, modification, and protease degradation. Numerous studies have focused on optimizing secretory pathways in *S. cerevisiae* (Fig. 2).

3.1. Engineering protein translocation to facilitate recombinant protein production

Secretory proteins begin their journey through the secretory pathway via co- or posttranslational translocation into the ER lumen. In cotranslational translocation, the signal peptide of newly synthesized proteins is recognized by a signal recognition particle (SRP) when it emerges from the ribosome during translation. Signal peptides and SRP can affect translocation efficiency, and in turn, protein production [79]. Signal peptide optimization has been widely used to increase the secretion of various recombinant proteins. Different signal peptides from *S. cerevisiae* proteins have been used to secret recombinant proteins, and the activity of β-galactosidase with signal peptides from Aga2p, Crh1p, Plb1p, and MFα1p is higher than that with the wild-type sequence [80]. The signal peptide Ncw2p, coupled with *SEC72* deletion, lead to a 9-fold improvement in protein secretion [81]. Heterologous and synthetic signaling peptides have been used to improve protein production. For

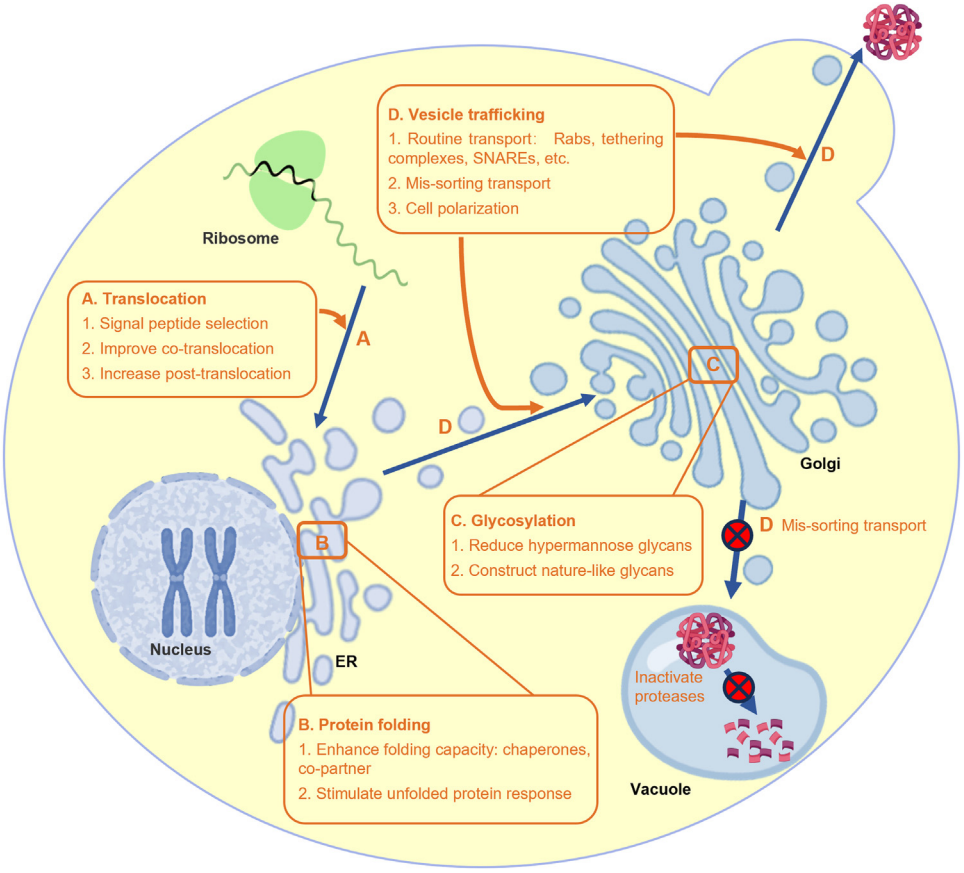

Fig. 2. Regulation of the secretory pathway to improve recombinant protein production. A. Reinforcing the protein translocation pathway. B. Streaming the protein folding process. C. Optimizing protein glycosylation. D. Enhancing the vesicle trafficking pathway.

instance, β-glucosidase with its native signal peptide and sequence from *Kluyveromyces* Inu1p yields the highest activity compared to that of signal peptides from *S. cerevisiae* such as Suc2p, Pho5p, and MFα1 [82]. Additionally, a synthetic propeptide increases the production of human granulocyte colony-stimulating factor by 109% compared to that of the native propeptide region [83]. However, no universal signal peptide sequences have been identified for the efficient secretion of all recombinant proteins. Therefore, building signal peptide libraries to screen for optimal libraries for high production of target proteins has become increasingly important [84,85]. Additionally, the overexpression of SRP components can be considered an alternative strategy to enhance protein secretion. The expression of SRP subunits Srp54p and Srp14p enhances the secretion of several recombinant proteins [86].

In *S. cerevisiae*, post-translational translocation occurs independently from the SRP, with the newly synthesized protein supported by cytosolic chaperones. Subsequently, the protein is pulled into the ER through a translocon, which is a complex of Sec61p, Sec63p, Sec71p, and Sec72p [87]. The cytosolic SSA subfamily, consisting of 70 kDa heat shock proteins, plays an essential role as molecular chaperones during posttranslational translocation [88]. Engineering posttranslational translocation has been shown to increase the production of recombinant proteins. For example, the regulation of Sec61p expression [89] and overexpression of Ssa1p [86] improves protein secretion.

Therefore, protein translocation may be a restrictive step in the secretory pathway for recombinant protein production, and the optimization of this process is important for improving protein secretion (Fig. 2A).

3.2. Engineering protein folding to enhance recombinant protein production

After translocation to the ER, the recombinant protein is folded into its native spatial structure with the assistance of the ER-resident protein-folding machinery. This process is regulated by strict quality control [90]. Only correctly folded and assembled proteins are exported from the ER to the Golgi apparatus [91], where they undergo further modifications. However, accumulation of misfolded proteins in the ER leads to stress and triggers an unfolded protein response [92]. *S. cerevisiae* has a limited availability of natural folding partners, which results in a lower ER protein folding capacity compared to animal cells, and this becomes a major constraint in the production of recombinant proteins [66].

Increasing the number of folding partners is a viable alternative for improving folding efficiency and reducing misfolded proteins (Fig. 2B). Numerous studies have shown that enhancing the expression of the chaperone BiP (Kar2p) significantly increases the yield of different recombinant proteins by 5-to 26-fold [86,93,94]. However, in some cases, the overexpression of Bip does not always yield positive results for all target recombinant proteins and may even have negative effects [95]. For instance, Kauffman et al. discovered that Bip overexpression leads to increased long-term binding between Bip and target proteins, resulting in ER-associated degradation rather than enhanced protein expression [96]. This finding highlights the importance of considering the effects of Bip overexpression on different target proteins. The involvement of Bip in these processes relies on its ATPase activity, which is regulated by its co-partner DanJp and its homologs Jem1p [97], Sls1p/Sil1p [98], and Lsh1p [99]. Enhancing the expression of one or more of these co-partners can boost the production of various recombinant proteins in *S. cerevisiae* by stimulating Bip ATPase activity [100].

Protein disulfide isomerase (Pdi1p) is another crucial protein involved in disulfide bond formation and plays a role in assisting the correct folding of proteins. Therefore, it is often overexpressed to enhance recombinant protein production [101–103]. Additionally, the co-overexpression of Bip and Pdi1p has been used to enhance recombinant protein production. Shusta et al. achieved an 8-fold increase in SCFV(Single-Chain Fragment Variable) production by co-overexpressing Bip and PDI1p [104]. However, the effect of this opti-

mization strategy varies for different recombinant proteins. For instance, co-overexpression of PDI1p and Bip does not increase the expression of β-glucosidase expression in *Pyrococcus furiosus* [105]. These results demonstrate that the overexpression of chaperones in the ER is an effective strategy for enhancing the production of recombinant proteins. However, it is important to note that this approach may not work optimally for all target proteins.

UPR maintains ER proteostasis by regulating the expression of genes involved in various secretory pathways. When cells are under stress due to unfolded proteins, the transcription factor Hac1p is translated into active proteins by cleaving inhibitory introns, which then systematically activate the expression of chaperones and other components involved in the secretory pathway [106]. Valkonen et al. observed that the deletion of the *HAC1* gene results in decreased production of two heterologous proteins, α-amylase and endoglucanase [107]. In contrast, overexpression of *HAC1* enhances the production of heterologous a-amylase by 70% and endogenous invertase by 2-fold [107]. Additionally, the introduction of heterologous HAC1p enhances the expression of α-amylase by 2.4-fold [107]. Therefore, the regulation of the URP pathway can effectively improve protein production in *S. cerevisiae* (Fig. 2B).

3.3. Engineering glycosylation modification improves the production of recombinant proteins

Currently, more than 70% of biopharmaceuticals are glycoprotein products [108]. Glycosylation is an important process in biopharmaceuticals that involves the addition of various sugar molecules to a protein through covalent bonds at specific amino acids such as asparagine, serine, and threonine [109]. *N*-linked and *O*-linked glycosylation are the two forms of glycosylation. Glycosylation modifications affect the pharmacokinetics and pharmacodynamics of therapeutic proteins [110]. Additionally, protein glycosylation is usually responsible for maintaining protein stability, thereby improving the protein half-life and protecting against thermal and chemical denaturation or proteolytic degradation [111].

S. cerevisiae, a model eukaryotic organism, can glycosylate proteins. However, when expressing heterologous proteins in *S. cerevisiae*, hypermannose glycan structures are often formed, resulting in reduced activity and altered immunogenicity [108,112,113]. Strategies have been used to engineer glycosylation modification processes in *S. cerevisiae* to enhance the production and bioactivity of recombinant proteins.

One important strategy is to reduce hypermannose glycans by deleting the essential genes involved in glycosylation. Disruption of Golgi mannosyltransferase Och1p has been found to increase the production of the active form of human tissue-type plasminogen activator [114] and cellulase, including β-glucosidase, endoglucanase, and cellobiohydrolase [115]. Deletion of other Golgi mannosyltransferases, such as Mnn2p, Mnn9p, Mnn10p, and Mnn11p, enhances the secretion of multiple recombinant proteins and endogenous invertase [116–119]. However, knockout of these mannosyltransferases can reduce normal cell growth and cell wall integrity of the host. The growth defect of the *OCH1*-deleted strain is alleviated by combining genetic engineering and mutagenesis, resulting in the increased secretion of recombinant proteins [120]. This study facilitates the practical application of this strategy.

Another approach is to introduce heterologous glycosylation modification pathways in *S. cerevisiae* instead of relying on endogenous pathways, to produce recombinant proteins with nature-like glycan structures that facilitate protein bioactivity and stability. For instance, to produce recombinant proteins carrying human-like *N*-glycans in *S. cerevisiae*, Alg3p and Alg11p are deleted to disrupt maturation of the endogenous high-mannose structure. Complex human-like N-glycan structures on secreted monoclonal antibodies are successfully synthesized by expressing human N-acetylglucosaminyltransferases I and II [121]. Recent advances have led to the formation of galactosylated complex-type glycans in *S. cerevisiae*. This is achieved by deleting Alg3p and Alg11p and subsequently overexpressing a human galactosyltransferase fusion protein with or without the UDP-glucose 4-epimerase domain [122].

These strategies offer promising avenues for tailoring glycosylation modifications in *S. cerevisiae*, resulting in recombinant proteins with improved bioactivity and stability, similar to the natural glycan structures found in human proteins (Fig. 2C).

3.4. Engineering vesicle trafficking for the improvement of recombinant protein production

Vesicle trafficking plays a crucial role in the secretory pathway responsible for the transport of proteins through various cellular compartments, such as the ER, Golgi apparatus, and endosomes, and to their destination at the cell membrane or vacuole. This process involves four key steps: vesicle budding, delivery, tethering, and fusion [123], which are tightly regulated by various components, including Rabs, coats, tethering factors, soluble N-ethylmaleimide-sensitive factor attachment receptor proteins (SNAREs), and a range of regulatory factors.

In *S. cerevisiae*, even correctly folded recombinant proteins can accumulate intracellularly [124], indicating that vesicle trafficking is a limiting factor in protein secretion. Rab proteins function as molecular switches and play crucial roles in the regulation of vesicle formation, transport, docking, and fusion [125]. Overexpression of Rabs Ypt31/32p has been shown to increase vesicle trafficking [126], and overexpression of Sec4p results in a 3-fold increase in the secretion of α-amylase [127]. Tethering complexes are important for proper localization of vesicles [128]. Increasing the expression of tethering complexes components, such as Sec10p and Exo70p, has been demonstrated to increase β-glucosidase activity, though not endoglucanase activity [129]. SNAREs are responsible for the fusion of vesicles with the target membranes. Overexpression of SNAREs containing Sso1/2p and Snc1/2p has been widely used to improve the production of α-amylase and cellulase [130,131]. Regulatory factors such as the Sm-like proteins Sec1p and Sly1p play a role in vesicle docking and fusion. Overexpression of Sec1p improves the secretion of α-amylase and insulin by 62% and 30%, respectively, whereas overexpression of Sly1p enhances their secretion by 43% and 37%, respectively [132]. These results highlight that strengthening vesicle trafficking through the overexpression of key components is an effective strategy for improving recombinant protein expression.

In situations where heterologous proteins are overexpressed, the limited protein transport capacity in *S. cerevisiae* can lead to missorting of properly folded proteins into vacuoles through two separate pathways, the CPY and ALP pathways [133]. The secretion of β-lactamase, an enzyme from *E. coli*, is very low in *S. cerevisiae*, and pulse-chase experiments have revealed that β-lactamase is missorted into vacuoles for degradation [134]. Deletion of Vps10p, the sorting receptor for multiple vacuolar hydrolases involved in the CPY pathway, can prevent vacuolar targeting [135] and increase the production of recombinant proteins [131,136]. However, engineering the vacuolar transport pathway is challenging because of the physiological importance of vacuoles in *S. cerevisiae* [137,138]. Zhang et al. found that knockout of *VPS10* has no positive effect on the secretion of insulin-containing fusion proteins, whereas deletion of certain proteins involved in vesicle trafficking from endosomes to the Golgi, such as *VPS4, VPS8, VPS13, VPS35* and *VPS36*, enhances the secretion of these fusion proteins [136]. In addition, Huang et al. found that knockout of Vps26p, Vps29p, Vps35p, Vps5p, and Vps17p, which are also involved in vesicle trafficking from the endosomes to the Golgi, increases α-amylase secretion [8]. Kanjou et al. found that the knockout of *MON2*, which encodes a protein for vesicle formation located in the late Golgi, enhances the secretion of heterologous luciferase [139].

Cell polarization, which involves the asymmetric organization of the plasma membrane cytoskeleton and organelles, is critical for regulating vesicle trafficking and protein transport [140]. In *S. cerevisiae*, engineering cell polarization was reported to increase α-amylase production. Coexpression of Bud1p and Cdc42p, involved in bud site selection and

cell polarity, respectively, increases the production of surface-displayed α-amylase by 100%, and introducing two genomic copies of Bud1p improves α-amylase secretion by 92% [13].

Vesicle trafficking is an important process in secretory pathways and involves complex regulatory networks. Engineering vesicle trafficking can significantly enhance protein production, and further systematic investigation of this complex process is necessary to identify new target components that not only improve the production of recombinant proteins but also contribute to a better understanding of the underlying mechanisms (Fig. 2D).

4. Global metabolic pathway optimization for the improvement of recombinant protein production

Protein production is governed by a series of biological processes, such as transcription, translation, posttranslation, and cell wall biosynthesis [141,142]. Although individual modifications of these processes can bolster protein production, the resulting improvements often have restricted magnitudes [143]. Consequently, the global optimization of metabolic pathways is indispensable for constructing an efficient microbial cell factory tailored for protein secretion. Huang et al., isolated a yeast mutant with a 5.2-fold increase in α-amylase production using UV mutagenesis and microfluidic screening. Subsequent whole-genome sequencing revealed that chromosome III in the mutant is duplicated. Moreover, changes occur in the components involved in various processes related to protein production, including organelle fusion, vesicle organization, membrane fusion, endocytosis, transcription from the RNA polymerase II promoter, lipid metabolic processes, and cytoskeleton organization [144]. In their follow-up research, Huang et al. used RNA sequencing to study transcriptional dynamics and found that the mutant exhibits a comprehensive modification in cell metabolism, which contributes to augmented extracellular protein production. Leveraging inverse engineering and physiological assays, they demonstrated that reduced respiration and increased fermentation caused by altered energy metabolism, as well as an equilibrium in amino acid synthesis and a decline in thiamine biosynthesis, are pivotal for protein production enhancement [145]. Additionally, they used RNAi-mediated perturbations in cellular metabolism, protein modifications, degradation processes, and regulation of cell cycle genes to augment protein synthesis after global metabolic regulation [146]. Systematic engineering of cell wall biosynthesis significantly enhances the production of cellulase and α-amylase. Proteomic investigations coupled with reverse engineering revealed an overarching upregulation of secretory and protein translocation pathways [147]. These studies demonstrated that optimizing global metabolic regulation is an attractive strategy for enhancing protein production. However, owing to the complex regulatory mechanisms within cells, current global metabolic regulation still relies on non-rational and semi-rational platforms. More efficient and precise strategies for global metabolic regulation require further research.

5. Other strategies to enhance recombinant protein production in *S. cerevisiae*

Other strategies include reducing intracellular proteolysis, fusion with enhanced domains, and nondirected mutagenesis screening. Proteases can degrade recombinant proteins, thereby reducing their production. Deletion of the vacuolar protease Pep4p has been shown to be effective in increasing the production of several proteins [148,149], and similar deletions of other proteases have resulted in the improved production of affibody molecules [150]. Fusion of recombinant proteins with effectively expressed domains can also be used to increase protein production. For example, L1 lipase expressed with an N-terminal fusion of the cellulose-binding domain from *Trichoderma harzianum* endoglucanase II increased production up to 7-fold [151]. High-throughput microfluidics and UV mutagenesis screening have been used to obtain yeast strains with increased α-amylase production 3-fold [144]. Additionally, RNA-seq has been used to investigate the genome-wide transcriptional response in yeast strains with efficient protein production capacity, revealing that altered energy metabolism, balanced amino acid biosynthesis, and reduced thiamine biosynthesis are closely related to protein secretion, demonstrating the need for global tuning of metabolism for efficient protein production [145].

6. Conclusions

Numerous efforts have been made to improve the production of recombinant proteins in *S. cerevisiae* based on multiple aspects including transcriptional and translational levels, posttranslational modifications in the secretory pathway, and recombinant protein transport levels. Although individual or combinatorial modifications of these strategies have shown positive effects on protein secretion, it is important to consider that protein secretion involves numerous intracellular processes, which present a significant challenge for the design of global engineering strategies. In the future, robust and comprehensive engineering strategies such as pcSecYeast, a genome-scale secretory model that can predict targets for global engineering to improve protein production [152], should be explored.

Declaration of Competing Interest

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

CRediT authorship contribution statement

Shuo Yang: Investigation, Writing – original draft. **Liyun Song:** Investigation, Writing – original draft. **Jing Wang:** Investigation, Writing – original draft. **Jianzhi Zhao:** Writing – review & editing. **Hongting Tang:** Conceptualization, Writing – original draft, Writing – review & editing. **Xiaoming Bao:** Conceptualization, Writing – review & editing, Funding acquisition.

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