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ARTICLE

Recent Advances in Engineering Yeast for Pharmaceutical Protein Production

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Recombinant pharmaceutical proteins account for a significant portion of the multi-billion-dollar pharmaceutical industry. Among various potential cell factories, yeast has attracted great attention in pharmaceutical protein synthesis due to its unicellular and eukaryotic properties, easy genetic manipulation, fast growth, as well as capability of post-translational modifications. In this review, recent advances in glycoengineering of yeast and secretory mechanisms in yeast for the production of biopharmaceutical proteins with appropriate pharmacokinetic properties were overviewed. To further improve those two aspects of yeast engineering, strain and pathway engineering studies is necessary to unveil engineered yeast cell factories providing humanized glycosylation with appropriate homegeneity and high secretory therapeutic production with high yield. In addition, current systems and synthetic bioloy tools and omics technologies to enhance the production of pharmaceutical proteins were briefly discussed. Integration of comprehensive systems biology models with omics technologies will open new doors to better understanding of yeast glycosylation and secretory mechanism, which will help obtain valuable information for strain and pathway engineering approaches. On the other hand, the applications of currently available synthetic biology tools such as CRISPR/Cas9 and TALENs in yeast engineering will further help researchers manipulate yeast strains for high secretory recombinant therapeutic protein production with desired features. All in all, currently available systems and synthetic biology tools can be applied to yeast engineering for improved biopharmaceutical protein production.

Introduction

Many valuable natural products have been used as flavors, pharmaceuticals, nutraceuticals, and fragrances. Many of these compounds are either plant-derived, possessing complex extraction process and low yield, [1–3] or produced by organisms that cannot be easily utilized in industrial scale production.[4] Despite the efforts to enhance the productivity of these natural producers, there are still limiting factors for industrial production such as low growth rate and variability in yield.[5] Microbial production, therefore, has been attracting great attention because of several advantages such as faster, more economical, easier genetic manipulations, less complex downstream process, and more adaptable to industrial application.[6] In particular, yeast has started taking the lead in biotechnology for natural product synthesis because it exhibits the properties of both unicellular and eukaryotic organisms, including easy genetic manipulation, fast growth, and post-translational modifications. Thus, unlike most bacteria, yeast can achieve high cell density and high titer in an economically-viable way, but does not include pathogens, viral inclusions or pyrogens.[7]

The most known and widely studied yeast is *Saccharomyces cerevisiae*. It has been used for the production of primary metabolites, secondary metabolites, and biopharmaceutical proteins from various carbon sources. Non-*Saccharomyces cerevisiae* yeasts are denoted as non-conventional yeasts such as *Pichia pastoris*, *Hansenula polymorpha*, *Yarrowa lipolytica*, *Kluyveromyces lactis*.[8] *S. cerevisiae* grows in high sugar content media, whereas some of non-conventional yeasts do not require such media. Therefore, non-conventional yeasts have great potential to offer alternative metabolic pathways to utilize various carbon sources and produce novel products.[9]

As a host organism, yeast has been used to produce a wide variety of products including sterols, terpenoids, lactic acid, citric acid, alcohols, sugar derivatives, organic and fatty acids, terpenes, aromatics, polyketides, peptides and recombinant therapeutic proteins.[10–15] Among all, recombinant therapeutic proteins, also known as biopharmaceutical proteins, are one of the fast-growing and attractive classes of medicine. These proteins have an estimated market value of 125 billion USD in 2012 with an expected annual increase rate of 7-15%.[16,17] Biopharmaceuticals such as anti-TNF (TNF: tumour necrosis factor) antibodies, cancer antibodies, insulin and its analogues, human growth hormone, and so on occupy 25% of commercial pharmaceutical products and 40% of total pharmaceuticals sales.[18,19]

Three commonly used hosts for biopharmaceutical production are *Escherichia coli*, yeast, and mammalian cells with the share of 32%, 15% and 43% of total biopharmaceuticals.[20] Mammalian cells like Chinese Hamster Ovarian cell line have the advantages of human-like *N*-glycosylation, thus showing better pharmocokinetics.[16] However, as compared to other commonly used hosts, they require complex media and are more sensitive to growth conditions than *E. coli* or yeast. They are also relatively slow and susceptible to viral contamination.[21] On the other hand, as a cell factory, *E. coli* uses simple medium, is incubated under easy cultivation conditions, and reaches high cell density with high growth rate and protein production yield. In spite of these positive aspects, *E. coli* has the drawbacks of poor protein folding capacity, limited secretion capacity, lack of post-translational modifications and toxicity.[22] However, yeast can potentially solve these challenges because it is able to synthesize many recombinant therapeutic proteins, and provides less susceptible to phage contaminations

and better secretory mechanism compared to bacteria.[23] As a generally recognized as safe (GRAS) organism, yeast has a good tolerance to low pH and fermentation inhibitors.[24] More importantly, yeast allows the post-translational modifications for pharmaceutical proteins.[25]

Fig. 1 overviews the popular fields of systems and synthetic biology and the classes of substrates and products in yeast biotechnology. As shown in the figure, this review is aimed at recent technologies of systems and synthetic biology tools to engineer yeast for the production of a wide range of products. It also covers some of the recent advances in post-translational modifications techniques and secretory mechanisms in yeast as well as the applications of systems and synthetic biology tools to yeast glycoengineering and secretory machinery to enhance biopharmaceutical recombinant protein production.

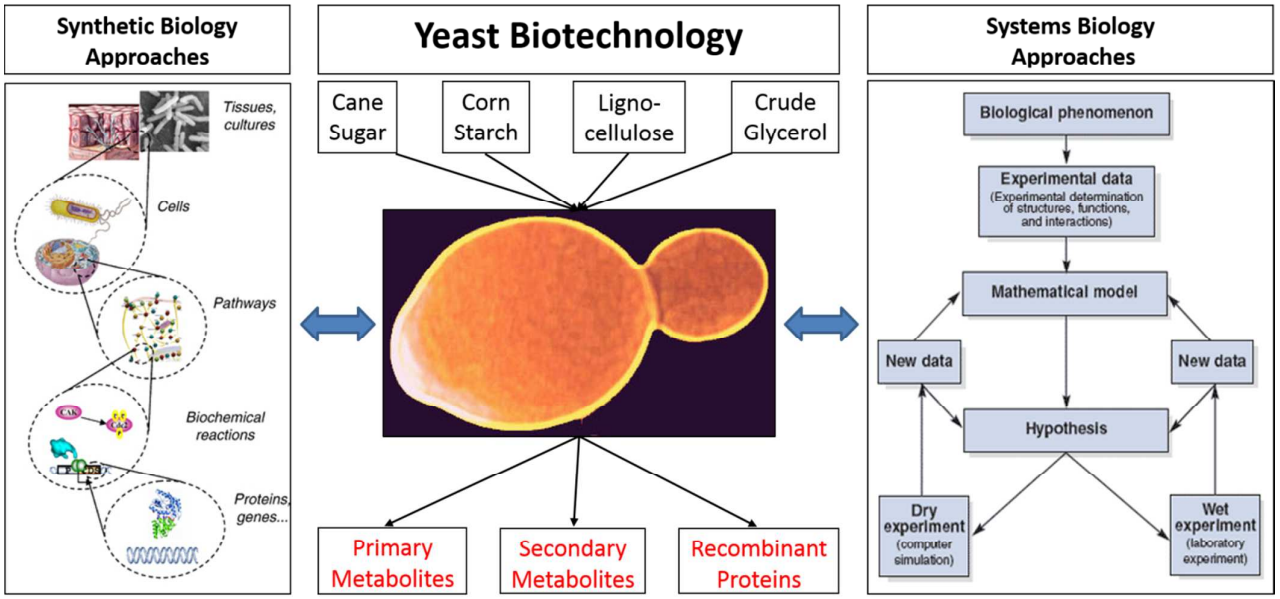

Fig. 1 Substrates used in yeast biotechnology, major classes of products produced by yeast, and the approaches of fast-growing fields of systems and synthetic biology to enhance the production of various products in yeast.[26,27] Various natural and engineered yeast strains are able to utilize the listed carbon sources for the production of wide variety of products classified in there major groups; primary and secondary metabolites, and recombinant proteins. Primary metabolites: Alcohols, lactic acid, citric acid, etc. Secondary metabolites: Polyketides, antibiotics, isoprenoids, terpenes, sterols, aromatics, etc. Recombinant Proteins: Insulin, vaccines, human serum albumin, human growth hormone, collagen, single-chain antibodies, epidermal growth factor, etc.

Glycoengineering of yeast

Glycoengineering modifications play significant roles in proper folding, half-life, and pharmacokinetic properties of recombinant therapeutic proteins. Even though yeast can potentially carry out *N*- and *O*-glycosylation, native high-mannose yeast *N*-glycans are not appropriate for medical use without modifications due to the interaction of mannose glycans with human *C*-type lectins of immune system.[28] Both the structure and the number of mannose glycans can potentially affect not only pharmacokinetic properties but also efficacy, immunogenicity, and half-life of therapeutic proteins.[29–31] Therefore, yeast strains with the ability to synthesize human-like

glycans instead of yeast-specific ones, as schematically illustrated in Fig. 2, have been developed, which are called glycoengineered yeast.[32] By glycoengineering of yeast, *N*-glycan humanization mainly takes place in three stages. The first stage is to eliminate or limit the yeast hypermannolysation through metabolic engineering perturbations.[33] One way is to disrupt or delete glycotransferase genes such as *OCH1* while expressing mannosidase genes, resulting in human-like $Man_5GlcNAc_2$ glycoform.[34,35] In another study, *ALG3* gene was deleted with various modifications to obtain human-like $Man_3GlcNAc_2$ glycoform.[35] In the second stage, further modification of terminal mannose substrate is performed using *N*-acetylglucosamine transferase, *GnT-I*, generating a glycoform with GlcNAc terminal. The addition of second GlcNAc sugar to mannose is carried out using *GnT-II* gene, resulting in $GlcNAc_2$-$Man_3GlcNAc_2$. The last stage is the sialylation of those human-like glycoforms by introducing heterologous synthetic genes to produce sialylated glycoproteins with more than 90% terminal sialylation.[36]

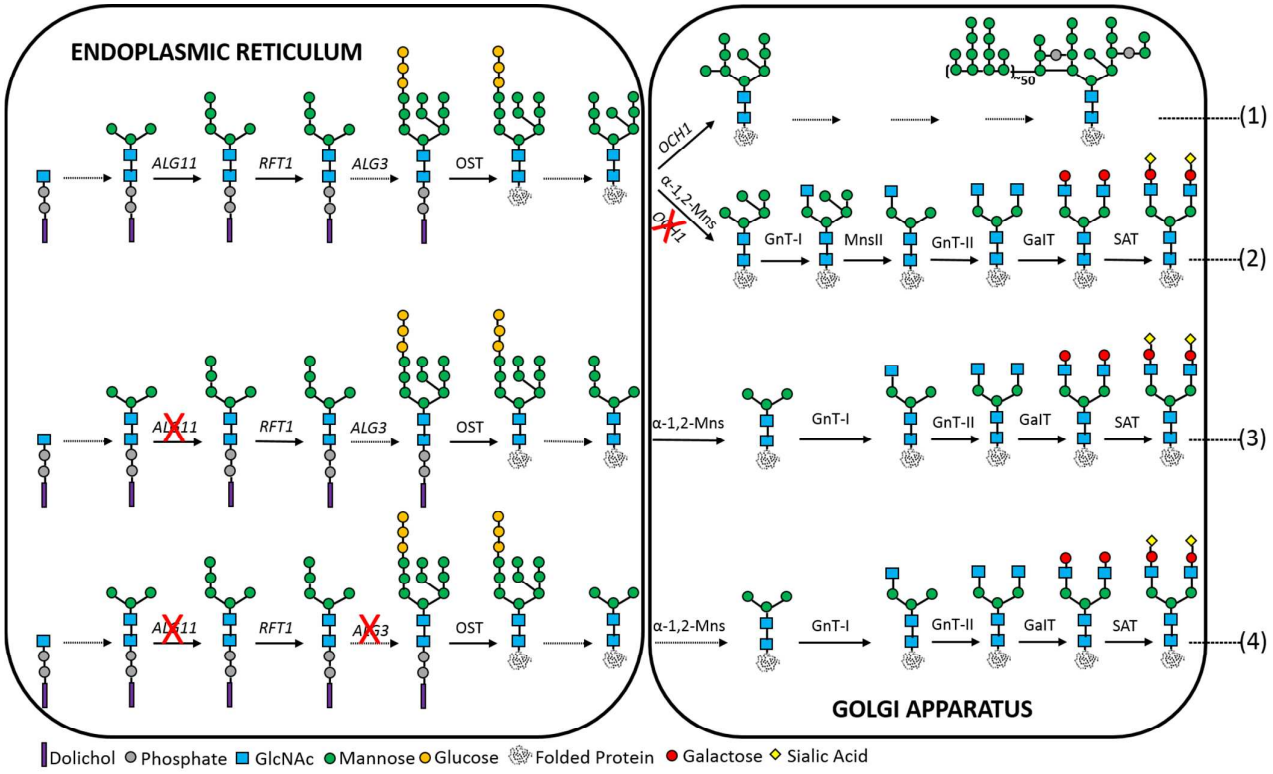

Fig. 2 Representative pathway of *N*-glycosylation pathways in various yeasts for glycoengineering to produce humanized *N*-glycosylation. (1) Highly mannosylated *N*-glycan synthesis in *S. cerevisiae* without *OCH1* gene deletion. *N*-glycan processing of *S. cerevisiae* in the Golgi apparatus is further attachment of mannose residues mannosyltransferases. In this case, the final compound is highly mannosylated *N*-glycan with various numbers of added mannose up to approximately 50. (2) Human-complex type *N*-glycan synthesis in *S. cerevisiae*, *P. pastoris*, *H. polymorpha*, *K. lactis* through *OCH1* gene deletion. (3) Human-complex type *N*-glycan synthesis in *S. cerevisiae*, *P. pastoris*, *H. polymorpha* through *OCH1* and *ALG3* gene deletions. (4) Human-complex type *N*-glycan synthesis in *S. cerevisiae* through *ALG11* and *ALG3* gene deletions.

Similarly, biopharmaceutical proteins with *O*-glycans interact with the human mannose lectins, causing inferior pharmacokinetic properties.[30] *PMT* gene was disrupted in order to reduce the presence or occupancy of *O*-glycan, but this approach was not sufficient to eliminate all *O*-glycans due to the presence of multiple *O*-mannosyltransferases in yeast. Also, the expression of mannosidases limited *O*-mannose chain length to some extent, giving rise to a decrease in lectin binding by glycoprotein.[33] In addtition to these metabolic engineering approaches, there are some *O*-glycosylation inhibitors added during protein expression phase, but these small molecules can only reduce *O*-glycosylation.[37] Instead of elimination of *O*-glycans, reengineering *O*-glycans in yeast has been performed through mimicking mammalian *O*-glycosylation. For instance, the human-like sialylated glycoforms have been produced by the introduction of α-1,2-mannosidase and β-1,2-*N*-acetylglucosaminyltransferase enzymes.[38] Another example is mucin-type *O*-glycosylation engineered in *S. cerevisiae* by introducing genes encoding *Bacillus subtilis* UDP-Gal/GalNAc 4-epimerase, human UDP-Gal/GalNAc transporter, human ppGalNAc-T1, and *Drosophila melanogaster* core1 β1–3 GalT.[39]

In brief, glycoengineering of yeast to remodel *N*- and *O*-glycosylation helps to optimize the pharmacokinetic properties of recombinant therapeutic proteins as reviewed in literature in detail.[28,40] However, there is still a need for studies to fully humanize *N*- and *O*-glycosylation pathways in yeast and maintain the uniformity of glycosylation.[41] In the meantime, the effect of glycoengineering on protein quality, folding, stability and solubility needs to be investigated. Also, a recent glycoengineering strategy for mammalian cells achieved a balance between retaining necessary *N*-glycan functions and decreasing the complexity of *N*-glycosylation.[42] A similar approach can be applied to yeast strains to reduce *N*-glycan heterogeneity for the sake of better pharmacokinetic properties. Lastly, glycoengineering of yeast to produce application-specific glycoproteins with customized properties should be the next focus in glycoengineering.

Recent progress in understanding of secretory mechanisms in yeast

Secretory mechanism in yeast is quite complex and similar to animal cell secretion system as it handles more than 550 proteins with signal peptides. Nevertheless only a few endogenous proteins are secreted out, thus facilitating the purification of secreted recombinant protein.[21,23] The improvement of protein expression efficiency and quality of protein can be achieved through engineering the protein secretory machinery. Fig. 3 explains schematically the details of secretory machinery in yeast including possible modifications and transport steps. For the further detailed information, one can read the review articles in the literature.[43–46]

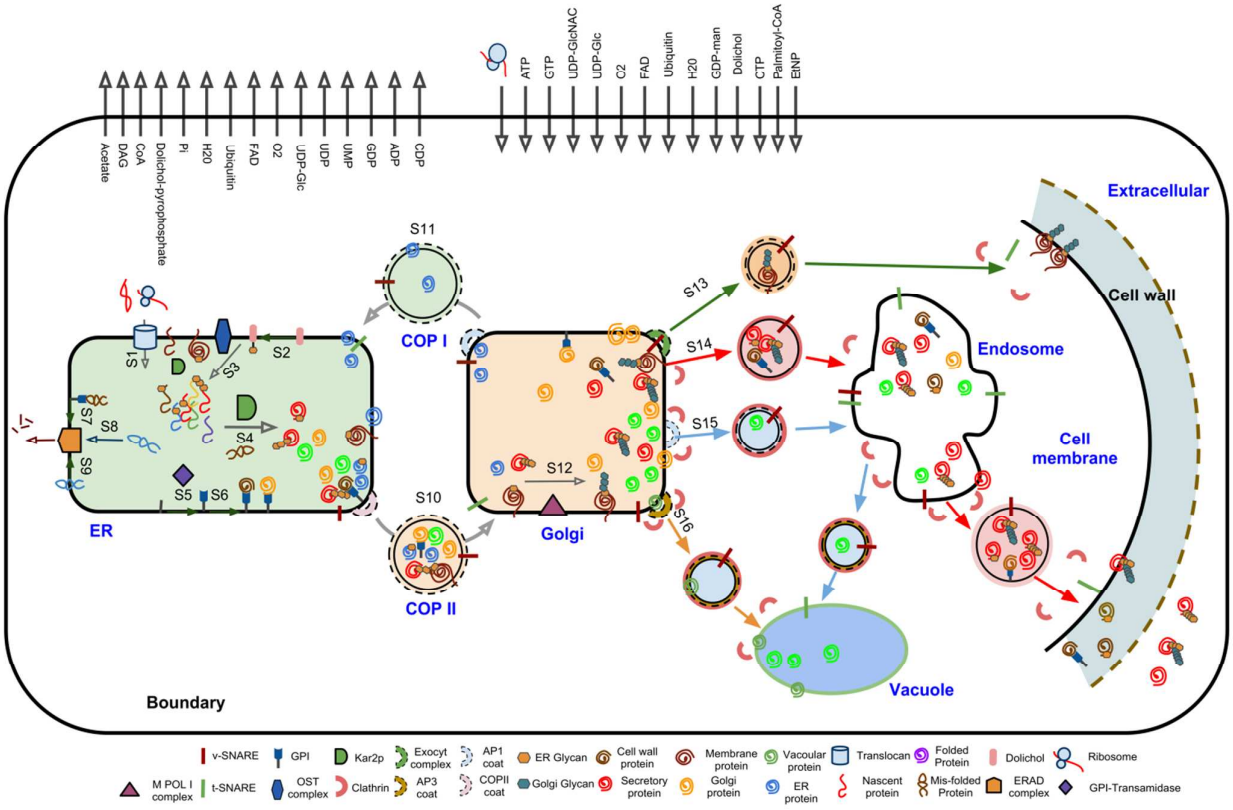

Fig. 3 Schematic representation of yeast secretory machinery including the possible modification and transport steps.[47] This model covers all the possible post-translational modifications and transport routes of secretory machinery. This secretion machinery is divided into 16 subunits (S1-S16). These subunits are: S1: Translocation, S2: Dolichol pathway, S3: ER glycosylation, S4: Folding, S5: GPI biosynthesis, S6: GPI transfer, S7: ERADC, S8: ERADL, S9: ERADM, S10: COPII, S11: COPI, S12: Golgi processing; S13: LDSV (low density secretory vesicle); S14: HDSV (high density secretory vesicle); S15: CPY pathway, S16: ALP pathway. Each subunit is indicated with an arrow. This model has 8 compartments including endoplasmic reticulum (ER), Golgi, COPI, COPII, vacuole, endosome, membrane and extracellular. The proteins located in the cell wall are considered to be extracellular proteins. The black rectangle around the machinery show a virtual system boundary that separates secretory machinery from the rest of cell and the exchange reactions are shown by arrows crossing this boundary.

The first step of secretion mechanism is the transfer of target protein through endoplasmic reticulum (ER) into the secretion pathway which requires secretion signal peptides for the translocation of proteins into ER.[21] Thus, the efficiency of leader sequence plays a significant role in the yield of secreted proteins. Compared to the prepro-leader sequences of native and alpha-mating factor, synthetic prepro-leader sequence exhibited more efficient protein secretion.[23] The correct folding of proteins in the ER is also very critical since it designates whether the protein is transfered into a secretion pathway or it is targeted towards ER-associated degradation (ERAD).[46] Based on the location of misfolded protein, ERAD has three distinct subtypes; luminal region (ERAD-L), membrane region (ERAD-M), and cytosolic region (ERAD-C). This protein misfolding management mechanism is mostly adequate to process misfolded proteins in yeast during unvegatative growth.[48] Once the protein misfolding causes a luminal burden due to higher level of unfolded proteins, unfolded protein

response (UPR) mechanism is activated to reimburse the elevated ER stress.[49] In addition to these two mechanisms, Miller et al. proposed UPR/yapsin-mediated pathway by which yeast can eliminate the excess or misfolded proteins when other mechanisms are saturated or out of function.[50] By ER luminal environment manipulation, secretion efficiency was enhanced through overexpression of chaperones and redox enzymes in ER.[51] For example, activated heat shock response by the overexpression of the activated mutant of heat shock factor 1 leads to improved protein secretion mechanism.[46] Also, the overexpression of Hac1p, which expresses a set of ER chaperones, improved the secretion capacity of heterologous proteins in *P. pastoris*.[52]

Although the recombinant proteins can be folded correctly through engineering protein expression and folding mechanisms in yeast, the secretion of the desired protein is often poor because the protein secretion machinery takes place in several cell compartments. The secretory mechanism in yeast contains the protein trafficking through ER, Golgi, trans-Golgi network, endosome, and either cell membrane or vacuole.[53] At each vesicle trafficking step, there are responsible proteins such as Sec1p, Sly1p, Vps45p, Vps33p. Hou et al. overexpressed these proteins, resulting in 70% increase in overall α-amylase secretion.[54]

Additionally, yeast produces plenty of proteases that may not only degrade the expressed protein and reduce the yield, but also damage the quality of protein of interest. Pathways from endocytosis to vacuole and from post-Golgi sorting to vacuole are the two potential degradation routes.[55] Multiple gene deletions, therefore, have been implemented in order for the efficient secretory production of recombinant therepeutic proteins.[21] As an example, the multiple deletions of *YPS1*, *YPS2*, *YPD3*, *YPS6*, and *YPS7* genes in *S. cerevisiae* were employed. This approach diminished the cleavage of recombinant parathyroid hormone protein.[56] Thus, the elimination of proteases can potentially increase the efficacy of protein secretion.

All of these aforementioned examples of genetic manipulations has improved protein secretory machinery in yeast. However,the most significant expression parameters regarding secretion machinery are protein type, expression system, promoter, and leader sequence that have to be essentially engineered for the higher secretory pathway efficiency in order to optimize the overall recombinant protein production in yeast.[57] Thus, simultaneous or multi-targeted genetic strategies will lead to further improvement of secretory production of biopharmaceutical proteins.[58] Channeling the flux towards to necessary precursors may also enhance the protein secretion.[59] More research on engineering secretion pathway, perfecting protein trafficking between different organelles, and preventing degradation of desired recombinant protein in addition to protein expression studies should be performed. The combination of studies on different aspects of improvement of secretion mechanism and protein production will reveal an engineered yeast strain as an improved cell factory for secretory biopharmaceutical production.

Recent technologies to enhance biopharmaceutical protein production

Systems biology tools

Systems biology makes a significant contribution to metabolic engineering studies through advanced analysis of cellular phenotypes and metabolic modeling to design enhanced cell factories as it bridges the gap between wet and dry labs.[60,61] Systems biology offers a

comprehensive analysis of physiology of microorganism by the integration of various omics technologies such as genomics, transcriptomics, proteomics, metabolomics, and fluxomics as well as computationally derived model and networks as overviewed in Fig. 4 with details.[27,62,63]

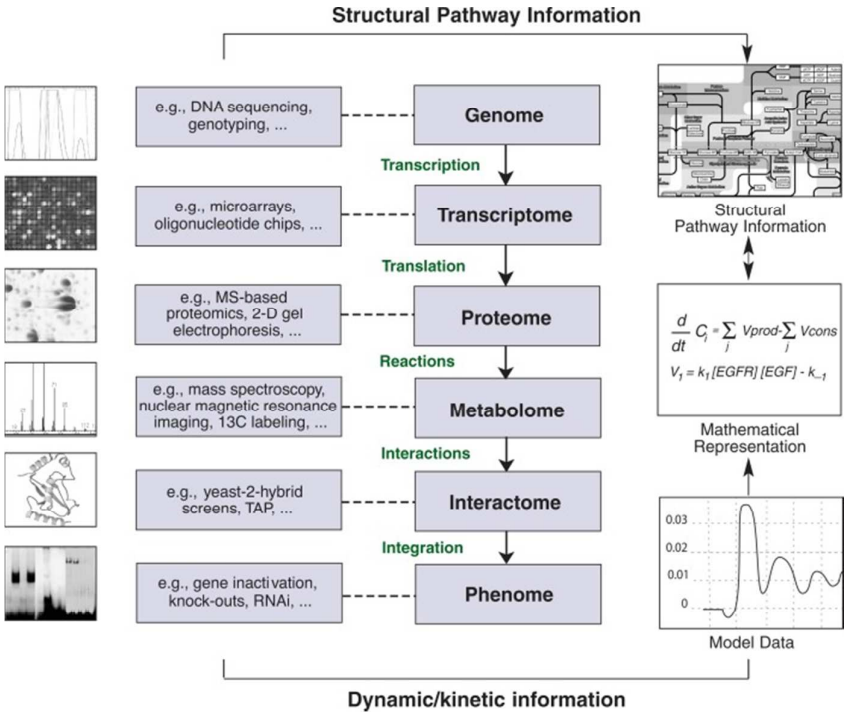

Fig. 4 The driving force behind systems biology known as omics technologies provide information on various levels such as genome, transcriptome, proteome, metabolome, interactome and phenome levels. To collect these data, a wide variety of complementary technologies and techniques such as DNA microarrays, RNA-Seq, mass spectrometry (MS), etc. are used. The experimental data reveal structural and dynamic information that is used to generate mathematical formulas, resulting in the development of comprehensive models and pathway maps. These models allow scientists to evaluate the potential effects of modifications or perturbations on/in the system and to design further experiments for the analysis of additional biological situations.[27]

Genomics reveals the genetic information stored in the genome by which the basic level of understanding of microorganisms has been carried out. With the advances in DNA sequencing techniques and bioinformatics, now it is much easier to identify and access the function and structure of genome.[64] *Saccharomyces* Genome Database (SGD) has potential to address the roles of uncharecterized genes and to map new functional connections to unrelated processes.[65,66] Additionally, evolutionary engineering studies with the help of genomics and other omics technologies can find out the casual relationships between identified mutations and phenotypes. Furthermore, the integrated analysis of genomics with reverse metabolic engineering may potentially help to improve the strains with higher performance.[67]

Transcriptomics is a highly dynamic and comprehensive way to evaluate the gene expression level through the quantification of all RNA molecules.[64] For instance, RNA-seq, a revolutionary quantitative technique for transcriptome analysis, is a cost-effective sequencing

technology with the accurate measurement of levels of transcripts and their isoforms, revealing the data on the gene activity quantification at different environmental conditions.[68] Due to lack of correlation between mRNA levels from transcriptome analysis and protein levels, proteome analysis with the help of advanced mass spectrometry has been popular and aids in identifying the cellular regulation mechanism following transcription.[60,69]

Proteomics validates transcriptomes and reveals all properties of proteins such as protein quantity, PTM, and protein interactions.[69] Another very beneficial omics technology is metabolomics that generates a global analysis of all intracellular and extracellular metabolites and their variations over the time under specific genetic and/or environmental perturbations.[64] Metabolomics combined with other omics data has potential to provide the comprehensive map of matabolic pathways under different conditions, thus facilitating a deeper understanding of cell factories.[70]

Lastly, fluxomics reveals the data of metabolic fluxes by involving the quantification of the rate of metabolites through metabolic pathways or reactions in biological systems. It is a dynamic indication of how cells utilize carbon and energy sources. With the help of other omics technologies, fluxomics is a robust technique to shed light on the regulation of metabolic networks.[71] For fluxomics analysis, two mathematical models have been available (i) steady-state models such as flux balance analysis (FBA) and ^{13}C-based metabolic flux analysis (^{13}C-MFA) that concentrate on the stoichiometric properties of the metabolic networks, and (ii) kinetic models such as dynamic FBA (dFBA) focusing on cell-wide dynamic regulation. These model studies have shown to enhance the production of various chemicals and therapeutics in yeast.[72]

Systems biology with the integration of aforementioned omics technologies and kinetic models has a great potential to comprehend the cell factories in deep, thus helping to improve biotherapeutic production with proper pharmacokineticsin yeast. In particular, modelling of both macro- and microheterogeneity of glycans have been studied for glycoengineering in order to understand underlying mechanism of glycosylation. For instance, Jedrzejewski et al. developed a modelling framework to establish a link from the extracellular environment and its effect on intracellular metabolites to the distribution of glycans. This systems glycobiology approach provided *in silico* prediction of glycoform of a biopharmaceutical protein based on extracellular conditions as well as optimization of bioprocess conditions.[73] Some of such computational models with potential aplications in glycoengineering, parameter calibration, and parameter-free analyses were recently overviewed.[74] Nevertheless, these approaches mostly focuses on the detailed understanding of a single biochemical process. Thus, systems-level understanding through the combination of computational and experimental tools has been attracted attentions because it will provide deeper insights into how multiple biochemical reactions and transport mechanisms interact with each other in order for the control of glycan biosynthesis.[74] Currently, the systems glycobiology has the obstacles of lack of an accepted standard for modelling, insufficient quantitative data from proteomics experiments, and limited glycoinformatics databases. For standardization, it is required to integrate glycan structure information and glycosylation-related enzyme definitions into computational models. Additionaly, integration of mass spectrometry derived site-specific glycosylation data into those models along with glycoproteomics analysis softwares will help improve glycoengineering of yeast. Lastly, more glycoinformatics data should be utilized to link glycan structure with function.[75]

Secretion mechanism of biotherapeutic proteins in yeast, which is another reviewed aspect in this article, can also be improved through aforementioned systems biology approaches. For example, Feizi et al. performed genome-scale modelling of protein secretion machinery in yeast. Based on protein specific information matrix model, they developed an algorithm to mimic secretory machinery by assigning each secretory protein to a particular secretory class that determines the specific transport steps for each corresponding protein. In addition, they gained system-level prediction of energy and metabolic demands and the activity of each component in secretory machinery through the integration of protein abundances into the model.[47] Such systems biology approaches should be integrated with omics data to elucidate deeper insights in yeast secretion machinery.

Synthetic biology tools

Cell factories exhibiting high flux from carbon substrate towards the product of interest and low or no by-product formation are required for industrial processes. The optimization of flux through metabolic pathways, therefore, is essential to maintain the balance for optimal enzyme expressions.[24] The fast-growing field of synthetic biology can potentially provide well-characterized biological parts to fine-tune the gene expressions in yeast as well as to construct novel genetic devices and cell-based systems.[21]

For instance, the semi-synthetic artemisinin project was a successful model for the use of synthetic biology to develop pharmaceutical drugs. The main purpose of this project was to engineer a microorganism to produce artemisinin precursor at high rates, yields, and titres. The production of artemisinin was successfully achieved using *E. coli* as chassis organism.[76] However, the shortcomings of *E. coli* such as inhibition of growth by *mevT* operon led to the switch to yeast-based production of artemisinic acid (oxidized derivative of amorphadiene).[76,77] A cytochrome P450 enzyme, which is responsible for the oxidation of amorphadiene, was identified in natural producer of artemisinin (*Artemisia Annua*) and functionally expressed in *S. cerevisiae*.[78] To increase titres of artemisinic acid, additional synthetic biological approaches such deletion of *GAL80* gene and expressions of various genes suh as *ALDH1*, *CYP71AV1*, *CPR1*,and *ADH1* were applied, resulting in the high titre of 25 g per liter artemisinic acid.[79]

As an encouraging story of artemisinin production, synthetic biology has enormous potential to improve the host organisms for enhanced production of various chemicals including pharmaceuticals as well as drug discovery through enabling a wide variety of tools.[80–82] Table 1 summarizes various synthetic biology tools at different modification levels that has recently been reviewed and applied for pharmaceutical production by yeast.[83–85]

Tabke 1 Synthetic biology tools at different modification levels to enhance the synthesis of products of interest.

Synthetic Biology Tools	Modification Levels	Function and Application	References
Gibson Assembly, Gateway Cloning, DNA Assembler	Pre-transcriptional	Quick pathway construction	86–88
Yeast Oligo-mediated Genome Engineering	Pre-transcriptional	Rapid genome modification	89
CRISPR-Cas Systems, ZFNs, TALENs	Pre-transcriptional, Transcriptional	Genome editing, transcriptional regulation	90–92
Synthetic Promoters	Transcriptional	Tuning synthetic genetic systems	83,84
RNA Control Modules	Post-transcriptional	Tuning gene expression	93
Synthetic Ribosome Binding Sites	Post-transcriptional, Translational	Controlling protein expression	94

Gene Codon Optimization	Transcriptional, Translational	Improving translational rates	95
Synthetic Protein Scaffolds	Translational	Optimization of metabolic pathways	96,97
Genetically-encoded Biosensors	Translational	Modular control over metabolic fluxes	98,99

In pre-transcriptional modification level, some synthetic biology tools for the modular assembly of multi-gene pathways are listed as follows: gateway recombination, Gibson assembly, DNA assembler and standard assembly techniques such as BioBrick™ and ePathBrick.[100,101] Yeast oligo-mediated genome engineering, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) systems, Zinc Finger Nucleases (ZFNs), and Transcription Activator-Like Effector Nucleases (TALENs) are the synthetic genome editing and transcriptional regulation tools.[16,24]

Recently discovered genome editing technique of CRISPR-Cas9 systems have attracted huge attention due to the ability to easily, quickly, and efficiently modify endogenous genes in a wide variety of biomedically significant cell types and in organisms having been challenging to manipulate genetically with traditional methods.[102] This cutting-edge technology has successfully been implemented in model yeasts (*S. cerevisiae* and *Schizosaccharomyces pombe*) for genome engineering, resulting in increased homologous recombination efficiency and feasibility for genetic manipulations. Thus, CRISPR-Cas9 systems hold a great potential to edit genome in yeast with the simultaneous modification of multiple targets using guided RNA.[90,103,104] In addition to CRISPR-Cas9, CRISPR-associated systems such as CRISPR interference, CRISPR-mediated activation through dCas9 have been discovered and utilized in order for modulating complex gene expression, silencing gene expression, upregulating gene expression, and gene activation.[105,106]

In transcriptional level, synthetic biology provides synthetic promoters that are well-characterized constitutive or inducible promoters with strong transcriptional activities.[83,84] To fine-tune and control gene expression precisely, synthetic ribosome binding sites and RNA-based control modules in post-transcriptional level can be employed with synthetic promoters.[94,107] Another synthetic biology tool is codon optimization to reduce mRNA secondary structure and improve translational rate.[95] Synthetic biology also offers synthetic protein and RNA scaffolds that can provide precise modular control over metabolic flux to improve cell factories[96] and genetically-encoded biosensors and riboswitches by which metabolic pathways can be optimized and regulated.[85,108–110]

Synthetic biology includes the design, construction, and analysis of biological parts that do not exist in nature. It provides tools operating at different levels as summarized above in order to bring yeast metabolic engineering closer to industrial biotechnology and facilitate metabolic engineering methods for engineering biosynthetic pathways.[111,112] We covered various metabolic engineering approaches for glycoengineering, indicating the potential power of synthetic biology tools for the production of therapeutic proteins with humanized glycosylation. For exapmle, synthetic glycosylation pathways producing *N*-humanized glycans in *S. cerevisiae* have been achieved through deletion of *ALG3* and *ALG11* genes.[113]

Similar synthetic biological approaches can be applied to engineer secretory machinery. For instance, synthetic prepro-leader sequence have improved the secretion of recombinant proteins with higher titer compared to α-factor leader sequences.[114] In another study,

Tomimoto et al. disrupted *PEP4* and *PRB1* genes to obtain a protease-deficient *S. cerevisiae* strain using metabolic engineering approach, resulted in enhancing the secretory production of human interferon-β by 10-fold.[115] Application of the recent synthetic biology techniques such as CRISPR/Cas9 or TALENs that have already been integrated into engineering yeast for accelerated genome editing [89,116] will further improve the secretory mechanicery with high yield and productivity and glycoengineering of yeast. Such attempts involving synthetic biology tools to improve glycosylation process and secretory mechanisms in yeast will lead to not only help to produce biopharmaceuticals with desired pharmacokinetic properties and yields but also facilitate strain engineering, enhance metabolic and pathway engineering, and develop various screening techniques.

Conclusions and Future Perspectives

Microbial production of pharmaceutical products has been grabbing great attention due to various advantages such as faster, more economical, easier genetic manipulations, less complex downstream process, and more adaptable to industrial application. Particularly, yeast has been playing a significant role in biotechnology for natural product synthesis because it possesses the properties of both unicellular and eukaryotic organisms, resulting in easy genetic manipulation and fast growth as well as post-translational modifications. Recent advances in the glycoengineering of yeast lead to the production of biopharmaceutical proteins with appropriate pharmacokinetic properties. To further improve glycoengineered yeast, pathway engineering studies should be the future focus for the production of fully human-like glycosylated pharmaceutical proteins with appropriate homegeneity. Pathway engineering should also be performed to advance the secretory machinery of yeast through engineering to enhance secretion efficiency, perfect protein trafficking, and prevent degradation of desired pharmaceutical protein. Another useful approach is strain engineering of yeast, revealing an improved cell factory for biopharmaceutical production with desired pharmacokinetics, high yield, and advanced secretory machinery. Unlike most of past and ongoing studies, multiple genetic and metabolic engineering strategies should be simeltaneously applied to yeast for both pathway and strain engineering approaches. The fast-developing fields of systems and synthetic biology integrated with omics technologies will facilitate the use of multi-target strategies in the engineering of yeast. In particular, in the dry lab side, system-level models should be the future outlook in yeast systems biology to deeply understand metabolic systems and pathways related to glycosylation, protein production, and secretory machinery. Integration of the system-level models with omics data will reveal plenty of valuable validated information for pathway and strain engineering of yeast. In the wet lab side, synthetic biology provides powerful tools to open new doors for improved cell factories. The application of current synthetic biology tools in yeast pathway and strain engineering studies will yield new engineered yeast strains allowing high secretory recombinant therapeutic protein production. In a nutshell, available systems and synthetic biology tools can be applied more to yeast engineering for biopharmaceutical production with desired features.

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