Implementing strategies in synthetic biology to maximize membrane protein production

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Abstract

Membrane proteins represent a class of proteins that are difficult targets to characterize. Their structural and functional characterization requires that they first be produced at quantities that enable their biophysical and biochemical analysis. Because they are natively produced at levels much lower than their soluble counterparts, extraction from their natural sources is not sufficient to produce enough material for these studies. Recombinant protein expression and production has become a popular method to produce large amounts of proteins for research and industrial purposes. Significant effort has been spent finding new ways to optimize and increase protein expression. As cutting edge techniques in synthetic biology continue to advance they offer a potential well of opportunities to tune expression through better control of the transcription and translation processes. Many techniques being developed are geared toward the production of soluble proteins, but in the following review, a focus on effective strategies to maximize membrane protein production in yeast is presented and includes many of the most innovative approaches to maximize expression using synthetic biology. Synthetic biology utilizes modern techniques in molecular biology and genetic engineering to optimize the production of compounds produced in microbes by altering gene elements required for transcription and translation of critical genes responsible for their synthesis. Compounds include natural products, hydrocarbon-based compounds for biofuels, and therapeutic proteins. Producing membrane proteins recombinantly using similar methods to increase expression yields is described in this review along with cutting edge techniques like cell-free expression, which circumvents many of the common problems that plague overexpression of membrane proteins microbial-based platforms.

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Take Aways

- Membrane proteins are challenging targets for recombinant expression
- Overexpression results in misfolding, aggregation, degradation and apoptosis
- Synthetic biology helps tune protein expression and increase yields**Abstract**Membrane proteins represent a class of proteins that are difficult targets to characterize. Their structural and functional characterization requires that they first be produced at quantities that enable their biophysical and

biochemical analysis. Because they are natively produced at levels much lower than their soluble counterparts, extraction from their natural sources is not sufficient to produce enough material for these studies. Recombinant protein expression and production has become a popular method to produce large amounts of proteins for research and industrial purposes. Significant effort has been spent finding new ways to optimize and increase protein expression. As cutting edge techniques in synthetic biology continue to advance they offer a potential well of opportunities to tune expression through better control of the transcription and translation processes. Many techniques being developed are geared toward the production of soluble proteins, but in the following review, a focus on effective strategies to maximize membrane protein production in yeast is presented and includes many of the most innovative approaches to maximize expression using synthetic biology. Synthetic biology utilizes modern techniques in molecular biology and genetic engineering to optimize the production of compounds produced in microbes by altering gene elements required for transcription and translation of critical genes responsible for their synthesis. Compounds include natural products, hydrocarbon-based compounds for biofuels, and therapeutic proteins. Producing membrane proteins recombinantly using similar methods to increase expression yields is described in this review along with cutting edge techniques like cell-free expression, which circumvents many of the common problems that plague overexpression of membrane proteins microbial-based platforms.

Introduction

Membrane proteins (MPs) represent an important class of biomolecules that either closely associate with or almost completely reside within the membranes of cells. They are crucially important in cellular processes ranging from signaling, trafficking, and more recently, scaffolding and shaping of the plasma membrane. Regions of exposed hydrophobic amino acid residues form intimate contacts with membranes that help to stabilize their structure and function (Levental & Lyman, 2022). They also render these proteins remarkably challenging to study. Many biochemical and biophysical techniques used in the preparation of their soluble counterparts must often be adapted through the addition of detergents and lipid mimetic complexes that provide a membrane-like environment. One major obstacle to their study is producing quantities of biologically active proteins for structural characterization and other downstream analyses.

MPs can be divided into three broad classes: peripheral, integral, and lipid-anchored. Peripheral MPs interact with the plasma membrane superficially wherein only a small portion or region of exposed hydrophobic amino acid residues are in contact with the lipid bilayer. These proteins can typically be extracted using biochemical techniques suitable for soluble proteins and do not necessitate the addition of detergents or lipids to increase their solubility in aqueous buffers. Similarly, lipid-anchored proteins are mostly soluble in aqueous buffers and rely on the covalent attachment of a lipid (e.g. palmitoylation) or a glycolipid (e.g. glycophosphatidylinositol) to one or more residues to interact with membranes. Integral membrane proteins (IMPs), which will be primarily referred to in this article, are almost entirely, $> 50\%$ amino acid composition, embedded in the lipid bilayer of the plasma membrane of cells rendering them extremely insoluble. *In vitro* , a suitable detergent or lipid complex must be used to keep them soluble and functionally active (Czerski & Sanders, 2000; Levental & Lyman, 2022; Lin & Guidotti, 2009; Whiles et al., 2002). Methods used to obtain proteinaceous material for *in vitro* analyses can rarely be universally applied across the entire spectrum of IMPs. Efforts to optimize experimental conditions is resource and labor-intensive and hampers progress toward characterization. As a result, relatively few IMPs have known solved structures compared to soluble proteins (Carpenter et al., 2008; Pan & Vachet, 2022). G-protein coupled receptors (GPCRs), for example, are one of the largest classes of IMPs (Errey & Fiez-Vandal, 2020). They are a key player in signal transduction and are responsible for processing extracellular signals across cell membranes leading to a downstream response. They are nearly ubiquitous across all kingdoms of life, but their importance in critical cellular processes, specifically in humans, makes them popular targets for drug therapy. Elucidating the structure of GPCRs has direct implications for rational drug design. Until 2007, the three-dimensional structure of nearly all GPCRs remained uncharacterized (Cherezov et al., 2010; Qu et al., 2020). Fortunately, critical advances in experimental methods such as the advent of cryo-electron microscopy (cryo-EM) have enabled significant achievements to be made. Although, high-yield production and purification still remains

a formidable challenge to their understanding and behavior*in vivo* .

Synthetic biology is a field uniquely poised to address the expression problem in membrane protein research. In this review, techniques used in the field of synthetic biology are explored presenting potentially the most effective ways to fine-tune expression and production to maximize yields. Recombinant expression methods serve as the basis for this discussion to provide a background for understanding the underlying challenges associated with current methods in MP expression. Along these lines, the molecular biology steps that govern critical intracellular processes in yeast is described to highlight important areas that might be targeted to address some of the most difficult challenges. Transcription and translation are two processes that lie at the center of protein expression and production. They affect intracellular conditions in yeast that ultimately effect cell viability and final yields. Many of the synthetic biology strategies discussed take into account regulatory features involved in transcription such as choice of gene promoters, terminators and other genetic elements that provide a greater level of control, which affects the viability of yeast during expression as well as the quantity and potential quality of the protein produced.

Current challenges in recombinant membrane protein production

Determining the optimal expression cassette and culture conditions for maximum MP yield is largely an empirical process. Finding a singular, universal approach is far from realistic when considering the nuances of expression in any microbial platform including yeast. Yeast is the most commonly used eukaryotic system for recombinant protein expression. Prior knowledge of structural features of the target protein is primarily what drives the decision to use yeast over its common prokaryotic counterpart, *E. coli* . Bacterial and yeast strains, are selected for their well-characterized and robust transcription-translation machinery, ease of culturing and genetic alteration, and their ability to be scaled up to industrial levels. High culture densities can be attained while having direct implications for high-yield protein production. Protein expression targets that have posttranslational modifications (PTMs) critical to their structure and function may benefit from heterologous expression in eukaryotic yeast species. Yeasts are phenotypically better equipped to handle proteins that require PTMs as they are mostly unable to be carried out in prokaryotes. Eukaryotic yeast species such as *Saccharomyces cerevisiae* (*S. cerevisiae*) are equipped with a developed secretory pathway that carries out posttranslational processing such as the addition of sugar residues at asparagine within the consensus motif, Asn-X-Ser/Thr (*N* -linked glycosylation) and serine and threonine residues (*O* -linked glycosylation). Glycosylation occurs both during (co-translation) and after (post-translation) synthesis of the polypeptide chain. It can have important ramifications for proper structure and function. Glycosylation is often required for proper protein folding and transport of MPs through the secretory pathway. In some circumstances, glycosylation reportedly increases protein secretion leading to enhanced expression, and it facilitates proper folding by destabilizing the unfolded polypeptide state (Han & Yu, 2015; Shental-Bechor & Levy, 2008). Further, glycosylation reportedly enhances the stability, function and resistance to proteolytic degradation in cellulases (Greene et al., 2015). When MPs are not properly glycosylated and folding is negatively affected, proteins may become prematurely degraded through the ER-associated degradative pathway (Han & Yu, 2015). In *S. cerevisiae* , improper glycosylation can lead to irreversible misfolding, aggregation and degradation, impacting protein yields. This commonly occurs in *S. cerevisiae* where proteins often suffer from hyperglycosylation through the addition of excess mannose residues (Conde et al., 2004; Kastberg et al., 2022; Nakamura et al., 1993). Differences in glycosylation between mammalian and yeast cells give rise to changes in glycosylation patterning through differences in the identity of sugar residues attached. This can also impact protein yield through misfolding and premature degradation. Hyperglycosylation can be avoided altogether through site-directed mutagenesis of glycosylated residues, however, amino acid substitutions also introduce some risk of improper folding and degradation (Han & Yu, 2015). Other yeast species have been explored as an alternative to *S. cerevisiae* since they do not suffer from the same hyperglycosylation effects. Species such as methylotrophic, *Pichia pastoris* (*P. pastoris, syn. Komagataella phaffii*), have been used successfully for high-yield expression. *Yarrowia lipolytica* (*Y. lipolytica*) is an emerging species with potential for industrial applications due to its ability to produce commodity compounds and hydrocarbon-based compounds for biofuels, among others. Though, it has not been extensively studied for its MP production potential. The secretory pathway is considered the rate-limiting step in protein synthesis in

yeast. During expression, if proteins become misfolded, they accumulate in the ER and impede the synthesis of new proteins triggering a stress-related response called the unfolded protein response (UPR). When this happens it can induce premature degradation and cell death. These are just some of the factors that must be considered when deciding on the best expression platform when maximum yields are required. *E. coli* , *S. cerevisiae* , and *P. pastoris* (syn. *K. phaffii*) are among the best characterized organism genomes reported enabling different recombinant expression conditions to be tested and optimized.

Disulfide bonding is another type of PTM important for the stabilization of tertiary structural contacts in some IMPs. Bacterial expression systems lack an oxidative intracellular environment where disulfide bonds form. *S. cerevisiae* provides a better platform to support this type of PTM. Where certain PTMs are critical for structure formation and stabilization, *S. cereivisiae* is principally the better choice. Mammalian proteins are often post-translationally modified compared to prokaryotic proteins, reinforcing the importance of selecting the proper host (Macek et al., 2019).

Another challenge to successful MP expression is establishing optimal cell growth and culture conditions. High-yield recombinant protein production is achieved by applying rigorous growth conditions to coax the host into producing as much protein as possible. Under such conditions, deleterious effects can lead to truncated, misfolded, or degraded protein. For IMPs, overexpression also leads to an overabundance of the target protein in the plasma membrane after trafficking from the secretory pathway. This results in molecular crowding and alters both protein conformation and the morphology of the membrane itself (Chen et al., 2016; Guigas & Weiss, 2016; Löwe et al., 2020; H.-X. Zhou, 2009). The latter can have serious destabilizing effects and potentially lead to premature cell death (Figure 1).

The goals of synthetic biology are directly aimed at addressing challenges in expression and other compounds of industrial importance. It is for this reason that the next section is devoted to discussing techniques that have gained traction in the field. It is meant to suggest a practical set of parameters that can be used to guide the design of gene expression constructs to increase MP yields in yeast.

3. Strategies to modulate expression using synthetic biology

Synthetic biology encompasses a broad curation of methods aimed at reprogramming different cell types including microbial and mammalian cells for the purposes of producing compounds used in biofuels, pharmaceuticals, and materials-based applications. The synthetic processes necessary to produce these compounds requires the introduction of metabolic pathways not native to the original host species in many cases. One challenge synthetic biology has the potential to address is producing high-quality MPs for structural characterization and other biochemical analyses. Progress in MP research is often stymied by a lack of high-throughput, robust methods to produce high-quality material. The following summarizes and highlights some of the most effective tools available that can be used to tune MP expression and production in*S. cerevisiae* , Much of the progress made in synthetic biology has been possible using *E. coli* and *S. cerevisiae* – two of the best studied microbes. Application of these tools is slowly being introduced into other microbes. Processes in yeast will be the focus for the remainder of the review. The intracellular processes involved in protein production include transcriptional regulation through adaptation of genetic elements such as gene promoters, terminator regions, and other mechanisms of transcriptional control including transcription factors. A brief discussion on the use of fusion tags to boost protein yields follows along with whole genome editing strategies to generate selective knockout (KO) strains and down-regulate undesired proteolytic pathways. Conversely, metabolic engineering methods are discussed as a way of introducing novel pathways and genes identified to support MP production along with future insights into how this technology can be used to tune processes involved in expression. Innovative culturing methods that include comparative insights into differential protein induction methods, constitutive versus inductive, are discussed. Lastly, the latest developments in cell-free (CF) expression, a field that has seen significant advances in protein production applications, is presented with an emphasis on advantages over whole cell expression and future directions.

Tuning protein expression and yield in yeast using genetic elements and fusion tags

High-yield protein expression often employs rigorous expression conditions to achieve the highest yields

possible for a target construct. Under these conditions, the rate of protein synthesis exceeds the capacity of the intracellular protein quality control machinery leading to misfolding and premature protein degradation. In the case of the former, protein misfolding leads to the accumulation of aggregated material that collects inside the expression host called inclusion bodies. Inclusion bodies (IBs) are insoluble and resistant to proteolytic degradation. This can be advantageous if a target IMP is susceptible to premature degradation during expression or if overexpression leads to premature cell death, which is common in the case of MPs (Kesidis et al., 2020). Proteins can be coaxed into IBs by incorporating certain fusion tags into the gene expression construct (Esposito & Chatterjee, 2006). The use of fusion tags has seen much use in high-yield protein expression primarily as a handle for post-production purification. These Include the common hexahistidine tag (His6), Myc, thioredoxin (Trx), and maltose binding protein (MBP). Including either solubility or IB-directing tags within the gene expression cassette also has substantial effects on protein yields (Costa et al., 2014; Ki & Pack, 2020). Most reports of IB formation occur in *E. coli* and it is generally considered an undesired outcome of protein aggregation that results from poor expression conditions and the lack of PTMs. Where *E. coli* falls short in expression efforts, yeast has proved to be more successful (Cai et al., 2019; Duman-Özdamar & Binay, 2021). Taking advantage of IB formation can be used to effectively increase expression yields and is useful when a feasible refolding procedure has been established (Bhatwa et al., 2021). The use of IB tags for *S. cerevisiae* expression is not as well described, but there is evidence of IB formation under specific conditions depending on the expression target (Binder et al., 1991; Rueda et al., 2016). Interestingly, two identified prionogenic proteins which originated in yeast, were shown to form inclusion bodies when expressed in *E coli.* (Espargaró et al., 2012). Re-purposing these proteins, Sup35 and Ure2, may provide one possible strategy to initiate IB formation when used as a fusion tag for proteins expressed in*E. coli* and *S. cerevisiae* .

Directing protein expression, primarily MPs, into IBs increases yield, but does little to preserve protein structure and function. Solubilizing and refolding aggregated protein in the absence of chaperones can be complicated as protein size increases (molecular weight) due to the increasing number of associated protein folding intermediates that can be assumed (Kiefhaber et al., 1991; Mitraki & King, 1989; Silow & Oliveberg, 1997). Again, this is further complicated by any native PTMs required for proper structure and function (Roth et al., 2010; Shental-Bechor & Levy, 2008). In *S. cerevisiae* , the presence of the secretory pathway is accompanied by the unfolded protein response (UPR) that is triggered under stress-induced conditions (Gardner et al., 2013; Ng et al., 2000; Walter & Ron, 2011). High-level expression conditions can create an environment where protein misfolding is more likely to occur. A cascade of intracellular events results that can either increase the expression of molecular folding chaperones or it can lead to premature death through activation of apoptotic pathways (Hetz et al., 2020; Walter & Ron, 2011). Finding alternate ways to relieve cell-induced stress is necessary to avoid these unwanted outcomes and achieve maximum yields. Toward this end, attenuating induced expression conditions are required, which can be accomplished by choosing a compatible expression plasmid and tailoring incorporated genetic elements to optimize this process through better control.

Protein expression plasmids comprised of small circular pieces of DNA that are introduced to either bacterial or yeast organisms and are incorporated into the phenotype of the host expression system. The native transcription and translation machinery of the host is hijacked to produce the product of the target genes included within the plasmid DNA, usually at levels higher than the homeostatic levels of the native genes in the host genome. When successful conditions have been established, this results in high protein yields. Predicting optimal expression conditions *ab initio* is nearly impossible considering the intricate interplay of multiple processes involved. Rigorous expression conditions are usually employed, but this can lead to undesired outcomes including premature cell death. The use of genetic elements such as promoters to induce protein expression and impose more control on the process is one way to potentially mitigate this effect. Choice of plasmid also influences protein expression levels by their copy number within a cell and mitotic stability, which affects their ability to replicate through cell division during propagation. Plasmid copy number also affects protein expression levels and it has been suggested that when coupled with a strong constitutive promoter, plasmid copy number is reduced along with plasmid stability, which is enough to offset

expression "gains" achieved by a strong promoter (Stueber & Bujard, 1982). Although, the use of auxotrophic selection markers has a much greater effect on plasmid copy number (Karim et al., 2013). A variety of commonly used plasmids are readily available for protein expression in yeast. These include pRS plasmids and their variants, pYES, pESC and many others which are summarized by Da Silva and Srikrishnan (Da Silva & Srikrishnan, 2012; Fang et al., 2011). Many of the plasmids in these series spawned from the popular pRS plasmids and differ by slight variations in their promoter regions as well as other features including various selection markers and incorporated tags for assaying expression and intracellular trafficking or for purification. Promoters can be classified as either constitutive or inducible. While constitutive promoters; TEF1, ADH1, and GDP, offer a greater dynamic range of expression, inducible promoters, FIG1 and GAL, enable greater control over timing and transcriptional regulation of expression, which is especially useful when expressing targets that are inherently toxic to the host. MPs fall into this category. When they are overexpressed and trafficked to the plasma membrane, this can lead to membrane overcrowding causing destabilizing effects (Guigas & Weiss, 2016; Löwe et al., 2020; H.-X. Zhou, 2009). Waiting to induce expression until higher cell densities are achieved is a way to ensure expression will not trigger premature cell death before higher yields can be obtained.

Efforts continue to be directed toward engineering new synthetic promoters to optimize expression by incorporating elements that allow for greater transcriptional control (Alper et al., 2005). Synthetic promoters usually include hybrid features of the native promoter along with a tunable site upstream of the core promoter that can act to suppress its activity. This is effective particularly for a strong promoter such as GAL1 (Mazumder & McMillen, 2014). Including repressor sites can also change a constitutive promoter, PFY1, to an inducible one using a similar approach, by incorporating regulatory elements upstream of the main promoter. Alternatively, hybrid promoters take elements from at least two different promoters, combining them into a single new promoter with altered activity and regulation (Blazeck et al., 2012). Recombining promoter regions requires a detailed understanding of different native yeast promoters and regulatory elements. Some promoters, the tightly-regulated *HO* promoter for example, are only active in certain cell types and respond to transcription factor initiation under specific conditions. The *HO* promoter is activated only in mother-type yeast cells in response to the SWI5 transcription factor during G1 phase of cell cycle division (Nasmyth et al., 1990). This adds an additional layer of protein expression control through the use of transcription factors, which interact with promoter and repressor regions to up- or down-regulate expression. This further expands the capacity for fine tuning. Transcription factors are native to all organisms and their identification has traditionally encompassed experimental characterization (Ian A. Taylor et al., 2000; Vachon et al., 2013). These methods have largely been supplanted using genome sequencing and mapping methods along with bioinformatics to help identify conserved regulatory regions across species (Hahn & Young, 2011; Yu, 2006). To understand transcription activation or repression of a specific promoter region requires *a priori* knowledge of their native function, which genome mapping has helped elucidate. These efforts have resulted in the curation of libraries of promoters enabling high-throughput screening and the construction of synthetic promoters (Gordân et al., 2011). Beyond that, functional assessment is needed to assay transcription factor and regulatory motif compatibility with the host organism expression machinery. This can be accomplished using a fluorescence reporter gene to measure expression levels or by measuring mRNA (Blazeck et al., 2012). Using a repressor in conjunction with a promoter provides a means of tighter control over inducing expression and could help to avoid the negative effects that result from overwhelming the intracellular expression machinery under rigorous expression conditions, this includes when using a strong promoter. Coupled with an in-depth analysis of expression for a target construct, a linked repressor can be activated at different time points throughout the period of expression to avoid aggravating the UPR or inducing apoptosis (Kaneko & Nomura, 2003).

Another strategy to allow for better control over protein expression is by engineering non-native terminator sequences. Though, perhaps not as commonly addressed as a means of tailoring protein expression, adjusting terminator sequences can impact the completion of transcription, dissociation and recycling of transcription machinery and other important parameters such as mRNA half-life. They can be versatility introduced across different yeast expression platforms like industrial, *Y. lipolytica* , and they can be completely synthe-

tically constructed resembling few to no features of any native terminator sequences (Curran et al., 2015). Short terminator sequences have been engineered for yeast and showed increased protein expression levels by 3.7-fold when compared with the commonly used *CYC1* terminator in yeast. A weak terminator can lead to transcriptional read-through and delayed dissociation of RNA polymerase. In *E. coli* , it has been suggested lower transcription efficiency can lead to slowed expression, reducing the chance of overwhelming the translation machinery (Swartz, 2001). Using similar rationale, this may be necessary to attenuate the effects of a strong promoter and avoid triggering a stress-related cellular response such as misfolding, degradation (mRNA and protein) and cell death. One other approach that has been used in *P. pastoris*(syn. *K. phaffii*) using hybrid promoters that contain both bacterial and yeast derived elements (Liu et al., n.d.). The purpose is to employ a yeast species that could produce proteins with desired PTMs rather than the hyperglycosylation *S. cerevisiae* is known for, while adapting it to high-level expression by introducing promoter features from a related high-producing species. Using a synthetic hybrid promoter along with a transactivator enabled methanol-free activation of protein production and increased the yield of a recombinant α-amylase expression target produced in *P. pastoris (* syn. *K. phaffii*). This avoided the need to use toxic methanol to induce expression.

Metabolic and genome engineering efforts to increase membrane protein yield

In recent years, whole genome methods to increase recombinant protein production have emerged. This has been mostly reported for *E. coli* . However, *S. cerevisiae* still remains one of the more popular eukaryotic expression platforms. Both model microbe systems are well-characterized and contain reasonably-sized genomes that enable genetic manipulation to be feasible. As our understanding of the interplay between multiple intracellular processes continues to evolve, this has shed light on additional indirect factors that influence the outcome of protein expression. This requires a top-down systems level analysis of simultaneous cell processes that take place under specific environmental conditions. Metabolic engineering aims to address these challenges. To look at how this approach can be applied to MP expression requires a recognition and understanding of the processes not only directly involved in but also those affected downstream by their synthesis, folding, PTM, and translocation. Transcriptomics and metabolomics analyses are two ways that the effects of growth conditions can be assessed by looking at changes in transcript levels, mRNA, of genes involved in certain metabolic pathways (R. Carlson et al., 2002; Chae et al., 2017; Dromms & Styczynski, 2012; Guan et al., 2018; Park et al., 2005; Trethewey, 2004; Yuan et al., 2018). Similarly, metabolomics is used to assess signature metabolites and their relative quantities, which are by-products from these pathways and interconnected processes. Information can be gleaned from these studies about the pathways that are most affected by expression conditions. Metabolic engineering aims to divert resources that support identified systems away from non-essential pathways and redirecting them toward recombinant expression. The rationale is that this results in prolonged expression leading toward increased production levels. Employing a rational approach to achieve this requires a systems level understanding of the host metabolism including those involved in transcription, translation and flux of metabolites, which also includes proteins. An analysis of the host expression platform helps in deciding which pathways are critically effected during protein expression. As a result of these analyses one strategy is to engineer KO yeast strains that are lacking pathways least critical for survival of the organism. Also, targeting genes responsible for the translation bottleneck created when strong promoters generate higher rates of mRNA synthesis helps alleviate this stress and associated degradation of mRNA. This was successfully demonstrated in *E. coli* when 36 genes responsible for non-essential functions were selectively knocked out (Sharma et al., 2020; L. Zhou et al., 2022). The results showed that protein expression could be increased by 1.5-fold when carried out at lower temperatures, 25 °C.

Alternatively, knockin (KI) strains also show promise as a means of increasing production. In this way, strains are engineered to carry out important processes that lead to proper protein function. This is a common strategy employed in the latest gene editing technologies such as CRISPR/Cas9. This allows for targeted gene insertion to coax the host into producing gene products that are either non-native to the host organism or to restore gene function that has been lost (Giuliano et al., 2019; Ye et al., 2021). Recently, CRISPR/Cas9 technology was used successfully to introduce the T7 RNA polymerase gene, an established

robust expression system, into a strain of *E. coli* naturally lacking this system (Ye et al., 2021). While some genome-wide strategies have been used successfully in *E. coli* , an extensive exploration of these methods in *S. cerevisiae* has not yet been reported. The addition of folding chaperones such as Hsp150 and PDI1 has seen some success, mostly for the recombinant expression of soluble proteins (Kim et al., 2014). Yeast strains deficient in lysosomal compartment proteases along with other organellar proteases is another strategy that has been implemented to circumvent protein degradation, truncation and increase yields by at least 10-fold (Tomimoto et al., 2013). Processes such as glycosylation also have significant effects on protein yield and quality control. Mutagenesis efforts have been directed toward humanizing yeast strains to produce glycosylation patterns on recombinantly expressed proteins similar to those produced in humans (Hamilton & Gerngross, 2007) (Figure 2). As proof of concept, humans, for example, lack the ability to produce sialic acid, *N* -glycolylneuraminic acid, while other mammals can produce it. This leads to aberrant sialylation. Knocking out its production in mammalian and other cell lines can potentially alleviate this problem. This is an important consideration in the design and production of therapeutic proteins where posttranslational differences in the resulting protein can have severe immunological effects. For MPs along with other non-therapeutic proteins, glycosylation affects folding, stability, half-life and function (Helenius & Aebi, 2001; Mitra et al., 2006). All of which are important considerations for high-yield, high-quality recombinant expression.

Changing culture conditions to tune expression

Before some of the cloning and engineering strategies previously discussed, "simpler" approaches may also prove helpful. High-yields and high-quality are not necessarily linked and higher-yields may, in fact, produce large quantities of unfolded, non-functional protein. When this outcome is not intended, for example, if avoiding inclusion body formation is desired, lowering the culture temperature during protein induction can lead to higher yields of functional, high-quality material (Francis & Page, 2010; Li et al., 2001; San-Miguel et al., 2013; Weickert et al., 1997). This has mostly been explored in E. coli, however, and little is reported on low temperature cultivation effects in yeast. The use of synthetic media also facilitates slowed expression and production compared to nutrient-rich media. Lastly, like strong promoters, growth conditions can also affect plasmid copy number (Stueber & Bujard, 1982).

A promising field that has gained much traction over the last decade is the use of CF systems for high-yield expression. Once used primarily as a research tool to understand transcription and translation has now been adapted to produce protein products at scale for synthetic biology (Garenne et al., 2021; Silverman et al., 2020). CF systems do not suffer from the production bottlenecks presented by their whole-cell counterparts, but there are other important considerations. The intracellular systems and processes that accompany traditional *in vivo* expression have effectively been removed, leaving only the essential protein expression and production machinery. Prokaryotic CF systems emerged using components isolated from *E. coli* where recombinantly expressed, purified, components have been used successfully – "Protein synthesis Using Recombinant Elements" (PURE system) (E. D. Carlson et al., 2012; Klammt et al., 2006; Ohashi et al., 2010). Success has also been met using crude cell lysis extract (Kigawa et al., 2004). Eukaryotic CF expression has been developed from a variety of sources ranging from wheat germ to Chinese hamster ovary (CHO) cells to HeLa cells (Anderson et al., 1989; Brödel et al., 2014; Harbers, 2014; Weber et al., 1975). There are only a few reports of CF systems using expression machinery isolated from yeast and most utilize soluble proteins to demonstrate feasibility of the system (Hodgman & Jewett, 2013). Extracts from *E. coli* have been utilized successfully in CF expression on a multi-liter scale (Figure 3). The reactions are stable for several hours and can be scaled to produce gram-level quantities of material (E. D. Carlson et al., 2012). CF yeast systems have also been utilized successfully to produce active firefly luciferase at µg-scale (Hodgman & Jewett, 2013). While most of the large-scale production efforts described utilize soluble proteins, strategies to adapt this technique to MPs are summarized nicely in several reviews (Klammt et al., 2006; Sachse et al., 2014). CF In Vitro Transcription/Translation reaction (IVTT) systems provide a solution to the potentially limited space available at the plasma membrane that is necessary to support properly folded, functional MPs. Arguably, it also leaves proteins less susceptible to the structural and conformational defects that arise from overcrowding in the membrane during overexpression. Rather, in the absence of the native cell-

membrane, CF systems rely on artificially supplemented media to support MP solubility. Mild detergents and other lipids that assume a bilayered morphological structure are added to the synthetic milieu to keep newly synthesized hydrophobic MPs soluble and conformationally active (Klammt et al., 2006). CF systems streamline the production process by omitting the need for any additional protein refolding protocols to be established prior to characterization. One important consideration is the compatibility of detergent and lipid additives not only to the target protein, but also their effects, if any, on the viability of the IVTT reaction components. Also, the concentration of detergent and lipids required to maintain protein solubility and active reaction components must be determined as well. Detergents must be maintained at a concentration above their critical micelle concentration, CMC, to maintain effective solubility properties (Kalipatnapu & Chattopadhyay, 2005; Sachse et al., 2014). Though this is perhaps a slight simplification of the complex biophysical interactions between MPs and solubilizing detergents and lipids. At any given time during the course of the reaction, the concentration of freely available detergent and associated micelles fluctuates during expression as proteins are synthesized and occupy micelle aggregates. Further complicating this process are the changes in by-product accumulation accompanying the reaction. These are important considerations when optimizing the synthesis reaction, but advantageously, carrying this out in a CF system also provides flexibility in the amount of detergent that can be added, changing the solubilizing capacity of the reaction mixture. Generally, nonionic detergents such as Brij and Triton X-100 detergents can be scaled at minimal cost. Other proprietary detergent mixtures such as Empigen BB®, which is comprised of a heterogeneous distribution of varying chain length molecules, is also mild and nondenaturing (Lowthert et al., 1995). These types of detergents are least likely to cripple the functionality of the components in the reaction mixture leading to longer reaction times and increased protein yields. Further, orchestrating a system to remove reaction by-products as they accumulate and adding substrates can facilitate longer reaction times and improve yields (Schoborg et al., 2014). Detergent solubilized proteins can be later reconstituted into bilayered systems for downstream analysis (Rieth et al., 2020).

Conclusions and Future Directions

A plethora of strategies along with synthetic biology tools for high-yield recombinant MP production is described. Integral MPs remain one of the most challenging expression targets due to their extreme hydrophobicity, which presents challenges to most host organisms when expressed at high levels. Overexpression often involves recruiting rigorous gene expression conditions that lead to activation of stress-related intracellular pathways in yeast that can thwart efforts to achieve high yields. Engineering native and nonnative hybrid promoters can help alleviate these problems as well as coupling activator and repressor sites upstream of the target protein. Consideration of terminator sequences provides another way to fine-tune expression and slow the coupled transcription-translation process to help avoid overwhelming the intracellular translation machinery. Many of the strategies summarized in this regard relate to the use of expression plasmids to achieve maximum yields. Indirect approaches have also been described using metabolic engineering and other genome engineering techniques to generate KO and KI strains with the aim of redirecting yeast metabolism and nutrient resources toward producing the target protein. The use of metabolic/genome engineering to halt non-essential processes has been demonstrably successful in some cases for soluble proteins and has yet to be applied to MP expression. Generating KOs to remove unwanted protease activity can also help increase yields, although increasing yields is not always coupled with high-quality, functional proteins. This review is intended to summarize the tools available to MP researchers looking to produce proteins for characterization. It describes best approaches that can be applied to maximize yields, which is often a bottleneck to progress in areas of structural biology and other types of analyses. It is also intended to help define areas in need of further development and where future efforts could be beneficially directed so that more can be learned about the processes critical to expression. While still somewhat a new methodology, CF expression for MPs holds tremendous promise as an alternative to heterologous expression in microbes by removing some of the critical barriers to overexpression and maximizing yields. Further, similar strategies used for synthetic biology in microbial systems have been attempted in CF systems with some success. Genetic components; transcription/translation, promoters, regulatory elements are beginning to be explored in CF systems and already make use of well-characterized, robust systems such as the *E. coli* -based T7 transcription system

(Garenne et al., 2021). Much of the progress seen with CF systems has been derived from *E. coli* . However, the same limitations present in *E. coli* expression still plagues its CF counterpart. Namely, the ability to carry out PTMs. Although, less is known about CF systems using yeast-derived components much of its progress has been pioneered by Jewett and co-workers and could potentially complement *E. coli* -derived systems for this reason, but without the limitations of the finite space in the plasma membrane. Along these lines, it may be advantageous to use CF in tandem with microbial systems by testing an expression system *in vitro* prior to transferring the genetic components to a cell-based system. CF systems are amenable to laboratory automation and potential scale-up making high-throughput testing possible, which can help supplement arduous efforts to screen single expression conditions at one time. CF also makes incorporation of unnatural amino acids possible for biophysical and other downstream analysis.

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Figures

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