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Engineering the supply chain for protein production/secretion in yeasts and mammalian cells

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Abstract Metabolic bottlenecks play an increasing role in yeasts and mammalian cells applied for high-performance production of proteins, particularly of pharmaceutical ones that require complex posttranslational modifications. We review the present status and developments focusing on the rational metabolic engineering of such cells to optimize the supply chain for building blocks and energy. Methods comprise selection of beneficial genetic modifications, rational design of media and feeding strategies. Design of better producer cells based on whole genome-wide metabolic network analysis becomes increasingly possible. High-resolution methods of metabolic flux analysis for the complex networks in these compartmented cells are increasingly available. We discuss phenomena that are common to both types of organisms but also those that are different with respect to the supply chain for the production and secretion of pharmaceutical proteins.

Introduction

The efficiency of recombinant protein production both in yeasts and in mammalian cells can be limited by a variety of factors (Fig. 1). In yeasts, the secretory pathway presents a major bottleneck in protein production and thus, many studies dealt with overcoming limitations in the secretory pathway by genetic engineering of the host cell [29, 31]. In mammalian cells, secretory capacity is usually not the limiting factor and engineering strategies rather focus on the construction of stable cell lines and on process, media and feeding conditions which result in high culture longevity, high integral viable cell density, process robustness and eventually high titer of a high-quality product [6, 101, 144]. In general, recombinant protein production results in a metabolic burden for the cell, which can cause limitations in the supply of precursors, energy and redox equivalents [89, 90, 102]. Recent studies focus on this issue in yeasts and mammalian cells, describing the impact of recombinant protein production on the metabolism of the host cell and the interaction between recombinant protein production and host cell physiology. Systems biology approaches aim at understanding global changes in proteome, transcriptome and metabolome of the cells in response to the burden of recombinant protein production [47, 57, 70, 106]. In this review, we will focus on the metabolic burden of recombinant protein production in yeast and mammalian cells: we will describe the impact of recombinant protein production from transcription and translation to protein processing and secretion, and summarize recent strategies by genetic, media and bioprocess engineering to overcome this metabolic burden.

Metabolic demand for protein synthesis

Biosynthesis of proteins is associated with a certain metabolic cost. Amino acids are the building blocks of proteins and thus contribute strongest to the metabolic demand for recombinant protein production. In yeast, often glucose or

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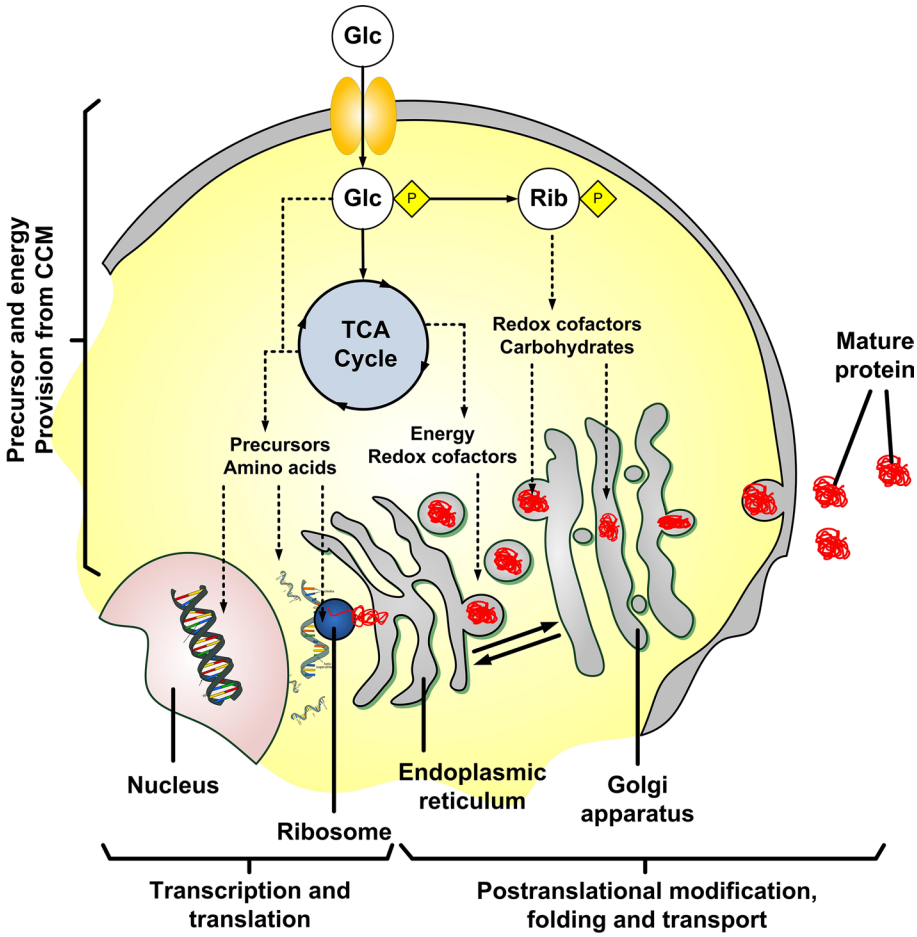

Fig. 1 Major connections between central metabolic pathways providing precursors, cofactors and energy for protein synthesis and secretion

glycerol serves as single carbon sources, which requires de novo biosynthesis of all amino acids. Though only part of the amino acids requires additional energy carriers for their biosynthesis when starting from these substrates, amino acids are characterized by a higher degree of reduction and therefore higher energy content per carbon atom [67, 119, 125]. Their synthesis redirects precursor molecules from the central carbon metabolism and consumes energy in the form of ATP or electron carriers as NADPH provided by catabolic reactions. The energetic cost for the biosynthesis of amino acids can be estimated by the number of high-energy phosphate bonds cleaved thereby [117]. Table 1 comprises the cost for de novo biosynthesis of the 20 proteinogenic amino acids exemplarily for *S. cerevisiae*. The impact of de novo synthesis of amino acids on recombinant protein production was demonstrated in *P. pastoris* by Heyland et al. [53], where selected feeding of only energy-intensive amino acids increased recombinant protein production significantly.

Translation and transcription pose the second major expense during protein production. Overexpression of the protein-of-interest will at least initially results in increased levels of mRNA [109, 111], increasing the anabolic demand for nucleotides as well as energy for transcription and transport into the cytosol. Here, translation takes place at the ribosome complex and increased production of a recombinant protein will also affect the biosynthesis of tRNAs and ribosomes and the associated energetic costs for the cell [17, 123]. Finally, biosynthesis of the protein itself is associated with a high energetic cost. Recruitment and release of tRNAs and peptide bond formation is usually associated with hydrolysis of 3–4 GTP per peptide bond [74, 137].

An important difference between mammalian cell culture processes and protein production in yeast is the media applied. Whereas, yeasts are generally cultured in media containing only a very limited number of carbon sources and no or only few amino acids, mammalian cell culture media are generally supplemented with all amino acids, with some exceptions depending on media and feed strategy [33, 37, 43, 145]. Therefore, amino acid synthesis is generally not a burden for mammalian cells, since the amino acids are provided in the media and are directly taken up by the cell. However, one important bottleneck might be transporters and transport kinetics. Depending on the protein product, uptake of amino acids might be too slow and might result in intracellular limitation of selected amino acid availability. Increased uptake of amino acids

and inefficient amino acid transport and its impact on productivity have been discussed for different producer cell lines [4, 34, 130], but there is a need for extended studies here, since there are, to our knowledge, no studies or data available that claim to understand and, most importantly, predict this issue.

Metabolic demand for biosynthetic and secretory machineries

The metabolic demand of secreted proteins is by far higher compared to cytosolic expression. After co- or posttranslational entry of the protein-of-interest into the endoplasmic reticulum (ER), active transport through the secretory machinery is associated with a high energetic cost of GTP [21, 150]. Especially in the case of recombinant proteins, repeated misfolding and refolding in the ER and the Golgi apparatus can occur. This is again associated with high energy costs regarding ATP [7, 85] as well as a steady drain on NADPH and $FADH_2$ for oxidative refolding of disulfide bonds [85, 134]. Using strong promoters can quickly result in the accumulation of misfolded proteins in the ER and the Golgi apparatus. Here, a high number of misfolded proteins can induce the unfolded protein response (UPR). This global response of the cell to folding stress in the secretory pathway will on the one hand induce expression of chaperones to support folding, thus increasing the metabolic demand for the upkeep of the secretory machinery [126]. On the other hand, the expression of secreted proteins will be suppressed to allow the cell to deal with the amount of misfolded proteins already present in the secretory pathway, which can be reflected in strong changes in the metabolism [120]. Thus, transport and folding are carbon draining, energy-intensive processes for the cell, but a proper estimation of the actual costs is complex and difficult.

Posttranslational modification of secreted proteins is a further drain on cellular resources and glycosylation plays a major role. In yeast, hyperglycosylation is always an important topic, and while mostly the negative consequences on activity of recombinant proteins or immunogenicity are discussed, also the anabolic demand of high glycosylation for the host cell must be considered. Nucleotide-activated sugars such as UDP-GlcNAc, GDP-mannose or UDP-glucose are the substrates for the many glycosyltransferases located in ER and Golgi apparatus [27, 50, 91]. The biosynthesis of these sugar metabolites is often associated with complex and energy-intensive pathways [91], and the supply of these sugars in fast-growing cells can even restrict the capacity of the secretory machinery [32]. Engineering of yeast strains toward human-like glycosylation patterns can also result in severe impairments of growth and productivity [28, 95]. Aberrant glycosylation of host proteins can directly affect their functionality. The altered demand for nucleotide sugars, on the other hand can also result in a limitation of these precursors in the different compartments of the secretory pathway, which in turn will further limit the secretory capacity of the cell [45, 132].

Finally, also the anabolic demand of lipid biosynthesis during recombinant protein production must be considered. Secretion of recombinant proteins can result in elevated lipid formation in yeast [74], and is associated with a high consumption of acetyl-CoA as main carbon precursor and cytosolic NADPH as main source of electrons for the reductive steps of the biosynthetic pathway [25, 52].

Table 1 Precursor and energy demand of amino acid biosynthesis in *Saccharomyces cerevisiae*

Precursor	Amino acid	Energy demand [117]
2-Oxoglutarate	Arginine	20.5
	Glutamine	10.5
	Glutamate	9.5
	Isoleucine	38
	Lysine	36
	Proline	14.5
3-Phosphoglycerate	Cysteine	26.5
	Glycine	14.5
	Serine	14.5
Oxaloacetate	Asparagine	18.5
	Aspartate	15.5
	Methionine	36.5
	Threonine	21.5
PEP + E4P	Phenylalanine	61
	Tryptophan	75.5
	Tyrosine	59
Pyruvate	Alanine	14.5
	Leucine	37
	Valine	29
Ribose-5-phosphate	Histidine	29

The protein production costs were taken from Raiford et al. [117] by calculating the average (per amino acid) number of high-energy phosphate bonds (*PO4) required for the synthesis of the average cellular protein's constituent amino acids

Metabolic flux analysis and modeling of protein biosynthesis and secretion

Metabolic flux analysis is one of the most important techniques to study the impact of recombinant protein secretion on the metabolism of the host cell. Metabolic flux analysis has been performed for various yeasts producing recombinant proteins, including *S. cerevisiae*, *P. pastoris* and *S. pombe* [46, 53, 63, 74]. The presence of organelles

in eukaryotic cells leads to separation of metabolic pathways between these compartments. This compartmentation can tremendously influence precursor and energy availability for certain pathways and thus the metabolic flux distributions in the cell [75]. Here, an intelligent choice of ^{13}C-labeled substrates can help to elucidate compartmentation in eukaryotic cells [24, 44, 127]. Careful experimental design and balancing of cellular and reactor compartments permitted a non-stationary ^{13}C metabolic flux analysis in CHO-K1 batch culture solely based on extracellular metabolite measurements [98]. These results showed that this basal cell line of many producer cells has very high activities of the pentose phosphate pathway as well as the TCA cycle. Jordà et al. [65] carried out a comprehensive metabolic analysis in *P. pastoris* growing on glucose–methanol mixtures. They analyzed the intracellular metabolome and the dynamics of intracellular metabolite labeling in continuous culture.

With respect to protein production, especially amino acid biosynthetic pathways are of interest, where compartmentation between mitochondria and cytosol must be considered. Blank et al. [14] described the compartmentation of amino acid biosyntheses for a variety of yeasts, which was identical for almost all amino acids and yeast species. However, compartmentation of some key amino acids can vary between cytosol and mitochondria. In *S. cerevisiae*, alanine biosynthesis took place in the mitochondria and was partly shifted to the cytosol when switching to respiro-fermentative growth conditions [44]. While valine and leucine biosynthesis takes place in the mitochondria of *S. pombe*, this pathway is located in the cytosol of *P. pastoris* [42, 73]. This difference will directly influence the demand for the precursor pyruvate between both compartments as well as the supply of NADPH, which cannot be directly transported over membrane boundaries. In this context, malic enzyme plays a major role in mitochondrial NADPH supply in *S. cerevisiae* [44, 93]. In contrast, malic enzyme is located in the cytosol in *S. pombe* and with cofactor specificity for NAD^+ instead [15], which leaves isocitrate dehydrogenase as sole source of mitochondrial NADPH [73, 74]. In summary, compartmentation may differ only for few but significant reactions of the central carbon metabolism, and thus must be investigated in detail for the construction of reliable metabolic networks as basis for rational network design, metabolic flux analysis and other metabolic modeling approaches [75].

As regards mammalian cells, metabolic flux analysis has been applied in recent years mostly to understand the influence of multiple conditions on energy metabolism [64, 100, 104, 139]. Fluxes to cellular proteins and the protein product are generally low compared to the ones in the central metabolism, and are usually directly determined through the composition of the cell and of the product (amino acids, sugars) and the specific growth rate. Importantly, reversibility and compartmentation of the metabolism are mostly neglected. Knowing the reversibility of many reactions and the contribution of extracellular amino acids versus intracellularly synthesized amino acids to the protein product would be interesting data, which are not generally generated. This could shed light on potential limitations. If, e.g., a high amount of a particular amino acid in the protein is not coming from an amino acid, which is provided in excess in the medium, this may indicate that, e.g., transport of the amino acid is a bottleneck. Such aspects could be nicely studied applying specific ^{13}C-labeled amino acids and measuring the resulting labeling patterns in the protein produced. Similar studies have already been performed to understand the channeling of substrates to different metabolic pathways and finally metabolite products, but not systematically to understand the contribution of synthesis versus uptake concerning the amino acids in a protein [103].

A deeper understanding of production and transport processes of recombinant proteins is of great interest. Pioneering studies for kinetic modeling of antibody formation and secretion in hybridoma cells were performed in the early 1990s by Bibila and Flickinger [11–13]. Other kinetic models followed, describing recombinant protein production in insect cells and filamentous fungi [108, 142]. In the latter study, ^{35}S-labeling of methionine was used to trace the biosynthesis of cellobiohydrolase in *Trichoderma reseii* throughout the different cellular compartments and estimate an average time for complete transport and secretion between 4 and 11 min. In a recent study, Pfeffer et al. [115] used an analogous approach, feeding ^{34}S as sodium sulfate and using the incorporation of ^{34}S-containing amino acids to determine kinetics of production, degradation and secretion of a recombinant antibody fragment in *P. pastoris*. In mammalian cell culture bioprocesses, proper glycosylation is crucial and kinetic models were developed with the aim of predicting product glycosylation. A dynamic model of N-glycosylation was used to describe the effect of increasing expression of a target glycoprotein on the product glycoform distribution and to evaluate appropriate metabolic engineering strategies [77] Jimenez et al. [61] presented a dynamic mathematical model describing glycosylation and other processes of protein maturation in the Golgi apparatus that could be directly applied for engineering of protein production and secretion. Glycosylation network modeling was recently reviewed by Puri and Neelamegham [116].

Metabolic burden and bottlenecks

Production and secretion of a protein consume both carbon and energy. Thus, recombinant production of proteins, often driven by strong promoters, creates a constant drain of

cellular resources and results in a burden on metabolism and in case of secretion also on the secretory machinery. This burden has a strong impact on the central carbon metabolism of the cell, affecting amino acid metabolism, energy and redox metabolism and provision of precursor molecules [10, 53, 63, 74, 80]. Increase in RNA content and interestingly also lipid content has been observed in a human cell line upon production of a recombinant protein [102].

Often, cell growth and the macromolecular composition of the cell already reflect the metabolic changes in response to recombinant protein secretion. For *P. pastoris* and *S. cerevisiae*, a decrease of the maximum specific growth rate μ_{max} by 43 and 35 %, respectively, has been attributed to the intracellular production of a recombinant protein [46, 53]. Recombinant protein production caused an increase in the cellular protein and RNA content of *S. cerevisiae* and *S. pombe* [74, 81, 134]. Limitations can already occur at the stage of transcription and translation due to a drain of tRNA pools, limited mRNA availability by increased degradation or inhibition of its formation and suppression of ribosome biosynthesis [16, 17, 123, 134]. Additionally, strains of *S. cerevisiae* and *S. pombe* secreting recombinant proteins at a high level additionally showed an elevated cellular lipid content most likely correlated to an increasing number of intracellular membranes and transport vesicles, which can effectively limit the rate of protein secretion [56, 74].

Finally, an increased level of protein production is obviously a constant drain on amino acid biosyntheses, resulting in decreased pool sizes of free amino acids in recombinant protein producing *P. pastoris* [19, 64]. The increased amino acid demand in turn causes a higher demand for precursors and energy supplied from the central carbon metabolism, causing strong rearrangements of metabolic fluxes [10, 46, 74, 134].

Limitations of metabolic pathway activities do finally limit the secretion of the protein-of-interest. The TCA cycle, as a main source of precursors and energy represents a common bottleneck in recombinant protein secretion in yeast. For *P. pastoris* producing intracellular recombinant proteins, constant TCA cycle fluxes were described with increasing level of product formation to cope with an increased precursors demand, although glucose uptake was lowered at the same time [53, 99]. In contrast, intracellular production of recombinant superoxide dismutase in *S. cerevisiae* resulted in a decrease of TCA cycle fluxes [46]. Decreased TCA cycle fluxes were also described for *S. pombe* and strains of the filamentous fungus *Aspergillus* which were secreting recombinant proteins [36, 74, 114]. Different from that, strains of *P. pastoris* showed no impact on TCA cycle activity [10] or even an increase in activity [62] when secreting recombinant proteins. It appears that different yeasts (and fungi) regulate TCA cycle activity very differently to cope with an increased metabolic burden caused by recombinant protein production. Especially *P. pastoris* cells seem to be able to counteract the metabolic burden by upholding or even increasing a certain carbon flux through the TCA cycle. However, for other yeasts, the additional demand for energy, redox equivalents and carbon associated with secretion appears to limit metabolic fluxes toward the TCA cycle, resulting in the observed decreased TCA cycle activity and thereby limiting protein secretion.

Redox and energy metabolism represent the second major bottleneck in yeasts. Protein production is associated with elevated activities of the PPP, which is the major source for cytosolic NADPH [10, 46, 74]. Cytosolic NADPH is mainly consumed in the biosynthesis of amino acids and the increased formation of lipids associated with elevated levels of protein secretion [56, 74]. Additionally, electrons for folding and refolding of disulfide bonds during protein secretion are supplied by cytosolic NADPH via, e.g., glutathione in yeast [71, 134]. Controlling the redox state of the cytosol and the endoplasmic reticulum is a prerequisite for efficient folding and secretion of proteins in yeast [30, 148]. Increased protein production has been associated with increased oxygen uptake and NADPH formation in α-amylase producing strains of *S. cerevisiae* and in *S. pombe* high-level maltase producers [74, 134]. Protein production and secretion are also associated with ATP consumption during biosynthesis of amino acids and peptide bond formation as well as during active transport out of the cell. The TCA cycle is the main source of ATP, and limitations in its activity have been shown to hamper secretion in *P. pastoris* and *S. pombe* cultivated under respiratory conditions [53, 74]. In response to recombinant protein production, the Crabtree positive yeast *P. pastoris* reduces the formation of fermentative byproducts [53, 99]. This indicates more efficient utilization of the available carbon source, which was also observed for protein-secreting strains of *S. pombe* when grown under respiratory conditions [74]. Under fermentative growth conditions, TCA cycle activities are very low in Crabtree negative yeasts. To meet the increased demand for ATP, increased glycolytic fluxes and increased NAD^+ regeneration via fermentative pathways have been reported for *S. cerevisiae* [46, 82]. Thus, the metabolic bottleneck, namely ATP supply, is the same but depending on the growth conditions, the cells will pursue different routes of metabolic flux rearrangements to cope with this limitation.

In mammalian cells, different aspects of varying productivity and burden of protein production have been studied. This includes not only studies in which more or less non-targeted omics technologies were applied to understand the system [3, 51, 78, 129, 131], but also studies in which genetic and metabolic optimization strategies were already proposed [8, 35, 97, 138]. Particularly proteomic and transcriptomic analyses pointed to alterations in translation/

protein synthesis, cell growth controlling networks, chaperones, energy metabolism and cytoskeletal proteins [3, 129]. Yee et al. [147] partly supported these findings by again studying the transcriptome and concluding that particularly cytoskeletal elements, vesicle trafficking and endocytosis go along with increased specific productivity. In another study, the metabolic burden of protein production on human AGE1.HN cells was studied by comparing a high producing cell line with its parental cell line [102]. It was observed that cellular RNA, lipid and phosphatidylcholine fractions were increased in the producer, which also caused metabolic changes, particularly increased glycine and glutamate production. The results were additionally verified by setting up a detailed model of intracellular protein production and simulating the theoretical changes on metabolite demand upon increased production of a particular protein. It could be shown that the differences observed in the metabolic profile upon protein production match with the increased nucleotide, lipid precursor and C1 unit demand.

Engineering media for reducing the metabolic burden of protein production

A straightforward approach to improve recombinant protein production in yeasts is the supplementation of media with amino acids, as shown for *P. pastoris* and *S. cerevisiae* [53, 136]. Also, the use of different carbon sources besides glucose can be beneficial. Recently, Liu et al. [82] reported improved productivity of α-amylase with *S. cerevisiae* in batch cultures after the diauxic shift from glucose to ethanol consumption. Applying mixtures of glucose and acetate or glycerol and acetate during respiratory growth of *S. pombe* could improve production of homologous maltase [74] and of two recombinant proteins (manuscript submitted) by overcoming limitations in the TCA cycle and lipid biosynthesis. Metabolic flux analysis of *P. pastoris* grown on mixtures of glycerol and methanol showed that this substrate mix may be generally applicable to reduce the metabolic burden of recombinant protein production [62]. However, increasing uptake rates of methanol lead to a complete metabolization of methanol to CO_2, as shown by ^{13}C metabolic flux analysis. Therefore, a waste of substrate results. Thus, besides optimization of the media, also smart feeding strategies are important during application of substrate mixtures [5]. The addition of vitamins and precursors can improve the production of a recombinant protein as well. A prominent case is the feeding of δ-aminolevulinic acid, a precursor of heme biosynthesis for the functional expression of horseradish peroxidase (HRP) in *P. pastoris* and *S. cerevisiae* [76, 92].

Besides the main carbon sources, also oxygen is an important substrate for the cell. Oxygen limitation results in an increased activity of fermentative pathways and therefore increased formation of byproducts. Surprisingly, recent studies with *P. pastoris* and *S. cerevisiae* described an improved specific recombinant protein production under oxygen limiting conditions [10, 81]. In case of *P. pastoris*, among others, membrane composition was affected by oxygen limitation and addition of subtoxic concentrations of fluconazole, which is an inhibitor of ergosterol biosynthesis, improved recombinant protein secretion in *P. pastoris* [9]. For *S. cerevisiae*, growth and production under anaerobic cultivation conditions could be further increased by addition of fumarate, which was not used as carbon source but served instead as final electron acceptor [81].

Media optimization for mammalian cells in general is a topic which cannot be dealt with in a short paragraph. Along the value chain of biopharmaceutical production, media development is an important issue and almost each company has its own strategy, processes and materials to deal with that. A large part of the biopharmaceutical production process is focused on product yield, which drives among others drug costs and manufacturing capacity. Besides manufacturing equipment, process parameters and downstream process, the yield is mainly influenced, as discussed before, not only by characteristics of the cell and of the product but also by the media and feed regime applied. Therefore, media optimization is an important issue. In the media optimization process, components such as amino acids, vitamins, ions and other substrates are adjusted in media and feed solutions such that the cell culture process is perfectly supported. However, the main goal of media development is to support cell growth having a robust process with high integral viable cell density, high product titer and high product quality. In the end, this "objective function" drives the development of media, which should reduce the metabolic burden since media components are adjusted such that limitation/accumulation of metabolites is avoided, and cell growth and product quality are supported.

Engineering cells for improved production

Genetic engineering of yeast cells to improve recombinant protein secretion has been performed for decades and was recently expanded into so-called synthetic biology [70]. Most of these studies focus directly on the secretory pathway by overexpressing chaperones [58], engineering of the UPR [88] or vacuolar sorting pathways [56, 59, 118]. While early studies dealt with overexpression of single chaperones to improve transport and folding, new approaches target transcriptional regulators to activate global cellular stress responses [54, 55, 135]. However, little literature is available which employs genetic engineering of central metabolic pathways to improve

recombinant protein production. In a specialized case, the production of human hemoglobin was significantly improved by metabolic engineering of the heme biosynthetic pathway in *S. cerevisiae* [80]. Engineering of substrate uptake is a target with apparently strong impact on the central carbon metabolism. For *S. cerevisiae*, the construction of a strain with a chimeric hexose transporter resulted in respiratory growth even under excess glucose conditions, which can be beneficial to avoid formation of ethanol and improve product yields [39, 107]. Mutant strains of *P. pastoris* were described where methanol assimilating pathways were downregulated to restrain methanol metabolization (Mut^s) [76]. Co-overexpression of formaldehyde dehydrogenase resulted in a strain with improved product per substrate yield and rates comparable to strains without an engineered methanol utilization pathway [76].

Recent publications describe model-based approaches for the identification of metabolic targets [38, 105]. Genome-scale metabolic models, which also consider metabolic demand for precursors, energy and transport reactions, were presented for *S. cerevisiae* and *P. pastoris*. The *P. pastoris* model has been applied for the development of strategies to improve production of an intracellular protein, human superoxide dismutase (hSOD) [105]. Predicted targets for gene overexpression were located in the PPP and TCA cycle, and included deletion of fermentative pathways. Construction of these strains for model validation actually proved that most predictions had the desired effect, thus showing the high benefit such models create for the rational engineering of the central carbon metabolism for recombinant protein production. In a protein secretion study in *A. niger*, ^{13}C metabolic flux analysis and in silico network analysis using elementary modes revealed several interesting targets for improved protein production [36]. The *S. cerevisiae* model developed by the Nielsen group [38] comprises a whole-genome approach to describe the energetic and metabolic demand of the complete secretory machinery to predict the cost, and thus the efficiency of secretion for a variety of recombinant proteins in yeast. To our knowledge, this is the first whole-genome attempt to model the complete yeast secretory machinery. However, verification of predicted targets to improve recombinant protein secretion is still pending at this point. In the future, isolated genetic engineering strategies will be further replaced by more systematic approaches to achieve further significant optimization of yeast recombinant protein production. In this context, the need for multi-omics studies to gain a systematic understanding of the cell and the power of genome-scale models to predict engineering targets in both the metabolism and the secretory machinery is evident.

In contrast to yeast, rational genetic engineering approaches for mammalian cell lines have been hampered for years since high-quality genome sequences were not available. This changed since sequences of the genomes of different CHO cell lines have been published and are available now [18, 48, 49, 79, 124]. Additionally, companies started to apply next-generation sequencing to get the genome sequences of their proprietary cell lines. Host cell engineering for biopharmaceutical production will be boosted by next-generation sequencing techniques and also new gene editing techniques allowing understanding and modifying cells conveniently [84, 121]. An interesting option for the modification of regulatory processes are microRNAs: similar to transcription factors, microRNAs can regulate expression of ~100 proteins allowing global changes in the cellular protein expression pattern and thereby metabolism and cellular behavior [94]. While exogenous supply of small non-coding RNA by transfection seems difficult but possible [41], endogenous production of mature forms require careful design of DNA to achieve efficient transcription and functionality [72].

Importantly, genetic engineering is strongly supported by the increasing number and the improvement of synthetic biology tools streamlining design and optimization of cellular processes [149]. Time and costs of metabolic/genetic engineering can be significantly reduced by relying on appropriate in silico solutions increasing the success of projects [68]. A list of free online tools is provided, e.g., at http://syntheticbiology.org/Tools.html. Generally, there are not only different synthetic biology online tools, e.g., for vector editing, primer search, or sequence alignment or for simulating cloning operations such as primer design or codon optimization (e.g., Geneious, Genome Compiler, Vector NTI or Benchling), but also comprehensive software packages which integrate many functionalities in one platform (e.g., Genedata Selector) [112]. One of the most prominent examples showing how to improve protein production in a mammalian cell line is the overexpression of X-box binding protein 1 (XBP-1). It was shown that overexpression of this important regulator, which is normally functional in plasma cells controlling differentiation and UPR, resulted in an expansion of the cellular secretion machinery, i.e., endoplasmic reticulum and Golgi apparatus and finally in enhanced productivity of Chinese Hamster Ovary cells [133]. Another very interesting genetic engineering strategy focused on modifying the expression of the regulator cdc42. It was found particularly that fast cycling cdc42 GTPase enhanced protein production yield and accelerated trafficking of proteins from endoplasmic reticulum to the Golgi [122]. In this context, Selvarasu et al. [128] could recently demonstrate the impact of lipid precursor availability limiting intracellular protein trafficking during a CHO fed-batch process performing metabolomics-based metabolic flux

analysis. Important processes in mammalian cells are protein refolding primarily carried out by chaperone systems and protein foldases as well as the UPR that can be engineered to improve productivity and product quality [69]. In the future, we envisage more studies concerning the influence of cell cycle that is, however, still lacking powerful methods for investigation [60]. Generally, system biology methods will be increasingly important for designing producer strains or cell lines as has been reviewed for glycosylation and related pathways [96].

Concluding remarks

Engineering the supply chain, i.e., the supply of building blocks and energy not only for the protein synthesis and secretion itself but also for the machinery that is required for the synthesis and secretion, is gaining increasing interest, both in industry and academia, particularly because of the high demand created by high rates and high titers attempted. Systems biology-oriented methods increasingly support the detailed study of function and control of the supply chain. The successful application of powerful new genetic engineering tools must go hand-in-hand with the development of novel and efficient computational tools for rational design of new strains and cell lines.

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