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# Cell Factory Engineering

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**Abstract**

Rational approaches to modifying cells to make molecules of interest are of substantial economic and scientific interest. Most of these efforts are aimed at the production of native metabolites, expression of heterologous biosynthetic pathways, or protein expression. Reviews of these topics have largely focused on individual strategies or cell types, but collectively they fall under the broad umbrella of a growing field known as cell factory engineering. Here we condense >130 reviews and key studies in the art into a metareview of cell factory engineering. We identified 33 generic strategies in the field, all applicable to multiple types of cells and products, and proven successful in multiple major cell types. These apply to three major categories: Production of native metabolites and/or bioactives, heterologous expression of biosynthetic pathways, and protein expression This metareview provides general strategy guides for the broad range of applications of rational engineering of cell factories.

## Introduction

Cells engineered for the enhanced production of native compounds, or production of heterologous products is an established and economically important discipline. Serving as the basis of all product-oriented industrial biotechnology, the economic footprint of these cell factories ranges in the hundreds of billions of US$/year on the global markets: Pharmaceutical proteins have been estimated at 140 billion US$ in 2013 (Walsh 2014); Industrial enzymes in the range of 1.8 billion US$ in 2009 (Waegeman & Soetaert 2011); Bio-derived non-protein pharmaceuticals ~100 billion US$ (Chemier et al. 2009); and bulk biochemicals (excluding biofuels) 58 billion USD (Nieuwenhuizen & Lyon 2011). For comparison, the petrochemical industry is >3 trillion USD/year (2015), so there is still a large market to expand into.

While industrial biotechnology has a long history, it was not until the arrival of *genetic engineering* that it became possible to modify the DNA of the cell factories to improve production (Figure 1), a process that hitherto had been based on clonal selection. Such developments gave rise to the discipline *cellular engineering* (Nerem 1991), which covers both basic and applied cell research . The same year, Bailey defined *metabolic engineering* as a rational and directed process of engineering metabolism, rather than a cycle of trial and error (Bailey 1991). Since then, the field of engineering cell factories has expanded in outlook and scope to include several "flavors" of cellular engineering specific to industrial biotechnology (Figure 1). Two examples are (i) inverse metabolic engineering (Bailey et al. 2002), in which one starts with the desired phenotype

and works towards that goal by directed genetic or environmental manipulation, and (ii) *systems metabolic engineering* as coined by the group of Sang-Yup Lee (Lee et al. 2007; Lee & Kim 2015) as a term for large-scale holistic metabolic engineering. However, in many applications, the engineering efforts are not limited to the metabolic network of the cell, and therefore "metabolic engineering" does not fully encompass all activities. This is particularly true for the large sector of expression of protein, native and heterologous, which covers the range from bulk enzymes to formulated pharmaceutical proteins. Here, engineering targets can be within cellular machinery such as the protein secretion pathway. To include all of these activities, this metareview defines *Cell Factory Engineering*, encompassing all rational approaches to improve a cell factory.

The objective of this metareview is specifically *not* to present a comprehensive list of examples within the individual strategies, nor is it to present direct strategies for target identification, such as modeling in tandem with predictive algorithms (Ranganathan et al. 2010; Burgard et al. 2003; Pharkya & Maranas 2006). For this, specialized reviews of high quality and information content already exist (See e.g. the excellent and recent work of the group of Sang Yup Lee (Lee & Kim 2015)). In this text, we will provide a meta-review summarizing several years of cell engineering efforts, in essence an applicable list of strategies generally applicable across species and products suitable for the experienced scientist. In this, we have focused on strategies applied reproducibly across multiple cell factories, and chosen the most applied microbial cell factories from the entire tree of life, spanning bacteria, yeast, filamentous fungi, and mammalian cells (in particular CHO cells), as well as some higher fungi. See also Box 1 for an overview of cell factory engineering methods in other fields. The metareview will furthermore provide representative examples of applications of the strategies for illustration.

## Meta-review Overview

The analysis here draws on a long list of reviews supplemented by primary literature to provide an overview of cell factory engineering. Table 1 lists the reviews cited in this text and annotations on which types of strategies and organisms these reviews are most relevant for.

It is the reductionist argument of this meta-review that nearly all cell factory engineering efforts can be classified in one of the following three categories or as combinations of them: (1) optimization of the production of a metabolite in the native host; (2) production of a non-native metabolite by expression of a heterologous biosynthetic pathway; and (3) expression of a heterologous protein. Here, we condense the strategies of the reviews in Table 1 into these three generic categories, examine each in detail, and provide guidance on choosing and applying individual strategies.

## Production of native metabolites

Native metabolites are here compounds naturally produced by the cell factory, either intracellularly or (preferably) a secreted compound. Examples are amino

and nucleic acids, antibiotics, vitamins, enzymes, bioactive compounds and proteins produced from anabolic pathways of cells (see details for protein products further below). Common for these are that they cannot be synthetically produced or for which it is not economical to do so (Stephanopoulos & Vallino 1991). This has been examined for specific cells or products in a multitude of excellent reviews (see e.g. (Bailey et al. 2002; Pickens et al. 2011; Stephanopoulos & Vallino 1991; Keasling 2008; Keasling 2012; Hwang et al. 2014; Wu et al. 2014; Weber et al. 2015; Kiel et al. 2010; Xiao & Zhong 2016)). Here, we will provide an overview of *general* strategies to increase the formation of native metabolites (Figure 2).

The strategies one would apply to this problem can be reduced to ten types (**Figure 2, 1A-1J**).

**1A$_{1\text{-}2}$. Pathway overexpression:** Using this strategy, one would typically overexpress one or more enzymes in the biosynthetic pathway. It is a common strategy and is often achieved by overexpressing the native genes (**1A$_1$**). As an alternative to normal overexpression, enzymes could be engineered to have higher activity. In either case, it can be advantageous to identify enzymatic steps with particular control of the flux to the product, such as irreversible reactions, or the first steps in the pathway. Some steps in the pathway (often the latter) may have very little control over the flux, so multiple targets should be engineered and/or metabolic control analysis (Nielsen 1998) should be employed. It has also been seen that heterologous expression of ortholog enzymes from related species (**1A$_2$**) can have a larger effect than the native enzymes. The reason for this remains speculative, but one hypothesis could be a lower regulatory effect on the heterologous proteins. One example of the latter is enhanced citrate production in *Aspergillus niger* by heterologous expression of TCA cycle enzymes from *Saccharomyces cerevisiae* and *Rhizopus oryzae* (de Jongh & Nielsen 2008), or improved ganoderic acid accumulation in *Ganoderma lucidum* (Xu et al. 2012).

**1B$_{1\text{-}2}$. Transporter engineering:** Accumulation of the product in the cell can decrease the carbon flux by affecting enzyme kinetics, thus decreasing production rates and yields. Furthermore, accumulation of product can trigger feedback inhibition, which will severely limit the flux through the pathway. In some cases, the product may even be toxic. Overexpressing product efflux pumps can thus be an efficient way of increasing the flux (**1B$_1$**) (Dunlop et al. 2011; Wu et al. 2014; Lee et al. 2012; Lee & Kim 2015). One example is the improved production of biofuels in *Escherichia coli* by systems engineering of 43 efflux pumps (Dunlop et al. 2011). This strategy both increased production and lowered product toxicity. Alternatively, or in combination with **1B$_1$**, gene knock-outs of uptake transporters specific for the product (**1B$_2$**) can also be effective (Lee et al. 2012).

**1C. De-branching:** Branching or competing pathways can decrease the overall flux towards the product (Pickens et al. 2011; Lee et al. 2012; Pfleger et al. 2015). If these pathways are not lethal, deleting the first branching step may improve product formation. With essential pathways, decreasing the

activity by knock-down or e.g. tunable promoters can be an alternative option. This is a common strategy; one comprehensive example includes the knock-out of L-lysine, L-methionine, and L-glycine biosynthesis for improved isoleucine production in *E. coli* (Park et al. 2012).

**1D. Product degradation:** Any non-essential reactions which converts the product to unwanted metabolites, should be deleted, as these may degrade the product and decrease yields and titers. Such an example is the work of Lee *et al*, where threonine dehydrogenase was deleted in *E. coli* to increase the production of L-threonine (Lee et al. 2007).

**1E$_{1\text{-}3}$. Co-factor engineering:** In some cases, it has been shown that a major limitation is the availability of co-factors (NADH/$NAD^+$, $NADPH_2$, $NADP^+$, Ac-CoA, etc) (Lee & Kim 2015; Ghosh et al. 2011; Lee et al. 2012; Pfleger et al. 2015; van Rossum et al. 2016). In these cases, one must make more co-factors available by engineering other pathways. Ideally, the deletion of a non-essential enzyme, which catabolizes large amounts of the co-factor, is preferred (**1E$_1$**). In cases where this is not possible, an alternative might be replacing such an enzyme with a native or heterologous enzyme with the same function, but specific for another co-factor (**1E$_2$**). An example of this is substitution of a native NADPH-dependent glutamate dehydrogenase with an over-expressed NADH-dependent glutamate dehydrogenase to enhance sesquiterpene production in *S. cerevisiae* (Asadollahi et al. 2009). A third option is the insertion (Yamauchi et al. 2014) or overexpression (Cui et al. 2014) of an *E. coli* transhydrogenase for interconversion of NADH and NAPDH (**1E$_3$**).

**1F. Removal of feedback inhibition:** In many cases, especially with products that are a part of standard growth metabolism (e.g. amino acids), strong feedback inhibition exists to tightly regulate the concentrations of the product. When one wishes to produce such compounds in large amounts, it can be necessary to disable feedback inhibition. Often this is achieved by random or targeted mutagenesis of enzymes in the pathway known to be feedback inhibited (Lee & Kim 2015). In some cases, analogs of the product, which binds tightly/near irreversibly to the regulated enzymes, can be used to screen for feedback deregulated mutants. This has been used e.g. for L-threonine (Lee et al. 2003), and L-tryptophan and L-serine (Rodrigues et al. 2013), both in *E. coli*. This strategy was also efficient for engineering acid production in *A. niger* (de Jongh & Nielsen 2008) and for production of fatty acids in *E. coli* (Pfleger et al. 2015).

**1G. By-product elimination:** Several species produce varying amounts of byproducts. Often these byproducts – while not directly linked to the metabolic pathway of the product – compete with the product for available carbon and/or co-factors (Lee et al. 2012). If possible without making lethal deletions, the enzymatic activities producing such compounds should be deleted or reduced. Numerous successful examples of this strategy can be found, for instance removal of glycerol biosynthesis in *S. cerevisiae* for increased ethanol-production (Wang et al. 2013).

**1H. Precursor/substrate enrichment:** It will often be advantageous to increase the availability of the substrate for the product biosynthesis (Lee & Kim 2015; Pickens et al. 2011; Lee et al. 2012). This can be achieved by a multitude of strategies, essentially by considering the substrate as an intermediate product, and applying one or more of strategies **1A-1J** to increase substrate formation. When considering substrates, one should also remember to take co-substrates such as acetyl-CoA in account (See e.g. a recent review of Ac-CoA engineering in *S. cerevisiae* (Nielsen 2014)). Other carbon donors can also become limiting, e.g. malonyl-CoA and glucose-1-phosphate in the production of an anti-cancer compound in *Streptomyces argillaceus* (Zabala et al. 2013).

**1I. De-regulation of carbon catabolism:** In some cases, the pathway of interest may be subject to general metabolic regulation of the cell e.g. general regulators of carbon catabolism or nitrogen source induced regulation. Examples of this is de-regulation of galactose metabolism in *S. cerevisiae* by deletion of negative regulators, leading to derepression and increased galactose utilization (Ostergaard et al. 2000) or disruption of a global regulator in *Pichia guilliermondii* to trigger aerobic glucose catabolism for ethanol production (Qi et al. 2014).

**1J. Signal transduction engineering:** In some cases, the production of specific metabolites is not regulated by carbon or nitrogen sources **(1I),** but may be subject to signals from e.g. micronutrients, or from other steps in the pathway. In these cases, engineering signal transduction can be a strong strategy (Kiel et al. 2010).

**Choosing a strategy for producing native metabolites**

Generally, there is a logical order in which to apply strategies **1A-1J.** The strategies can be sorted into three categories, which we suggest to apply in progression.

*Step 1: Direct optimization of the pathway in any way possible*. The main goal of this step is to ensure that neither enzymes nor intermediates of the pathway are limiting production. If this is not achieved, the other strategies may not be effective. This can be addressed by the following actions in roughly this order:

i. Overexpression of the biosynthetic pathway using the strategies of **1A**. This ensures that the concentrations of the enzymes are not limiting.
ii. Enrichment for the substrates (**1H**) and for the co-factors (**1E**), thus ensuring that the required metabolites, precursors and co-factors do not become limiting.
iii. Ensuring that the product is removed from the cell by transporter engineering (**1B**) if possible. Accumulation of the product can seriously decrease product formation as enzyme kinetics are dependent on concentrations of the product. Furthermore, product accumulation can in some cases lead to feedback inhibition of the entire pathway.
iv. If feedback inhibition is known for the pathway, this should be engineered out if possible, or removed by mutagenesis, screening and reverse genetics (**1F**). Again, this may not be a problem if actions i–iii are limiting.

*Step 2: Remove competing activities*. Once the pathway itself is optimized, the next steps is to ensure that no other pathways are impairing the product formation, either directly by sharing metabolites or co-factors, or by using carbon which could be converted to product. The three main strategies here are as follows:

i. De-branching (**1C**). Any pathways that share intermediates or pre-cursors with the pathway of interest should be deleted if possible.
ii. Product degradation (**1D**). A particular case of **1C** is pathways converting/degrading the product of interest. These should also be deleted if possible.
iii. Removal of by-products (**1G**). While by-products are not often directly associated with the product pathway, by-products will use carbon, cofactors, and energy which could be converted into product.

*Step 3*: *Application of global regulation engineering.* This does not seem to be common strategies, as it will often be highly effective to perform the actions above. However, should this be in place, engineering carbon repression (**1I**) or similar signal transduction pathways (**1J**) can be a final approach.

Clearly, the actions above can - and should - be combined for increased effects. Prime examples of this are large-scale rational design of metabolic pathways, which has been applied to great effect several systems, in particular bacterial hosts (Lee et al. 2007; Rodrigues et al. 2013; Becker et al. 2013) and yeast (Wu et al. 2014; Lee et al. 2012).

## Heterologous expression of biosynthetic pathways

When trying to produce an interesting compound, one of the most important decisions is the choice of production in the native host, and optimize this host, or transfer of the pathway to another well-known host. If the original host can be adapted to an industrial fermentation process, and there are no health-related risks in doing so (e.g. production of toxic byproducts), this can be a preferred strategy (as was the case e.g. for penicillin). However, in many modern cases, the potential of using an industrially preferred cell factory and related platform processes out-weighs the difficulty of transferring the pathway. In some cases, transplanting a pathway also removes metabolic inhibition found in the original host (Martin et al. 2003).

In this section we will examine how one may need to adapt the cell factory in order to accommodate production of a heterologous product. In general, several excellent specialized reviews exist within this area, and for additional details on specific cases for particular groups of compounds, we recommend these for further studies (See e.g. (Pickens et al. 2011; Pfeifer & Khosla 2001; Lee et al. 2012; Xiao & Zhong 2016)). Here, we give an overview of common and general problems regarding heterologous expression of pathways.

**Innate differences between the native host and the cell factory of choice are major challenges in expressing a pathway in a new host. In general, the compatibility of the enzymes and metabolites with the new host should be considered. The more complicated the pathway, the larger the advantage of choosing a more closely related host. Major types of challenges are shown in**

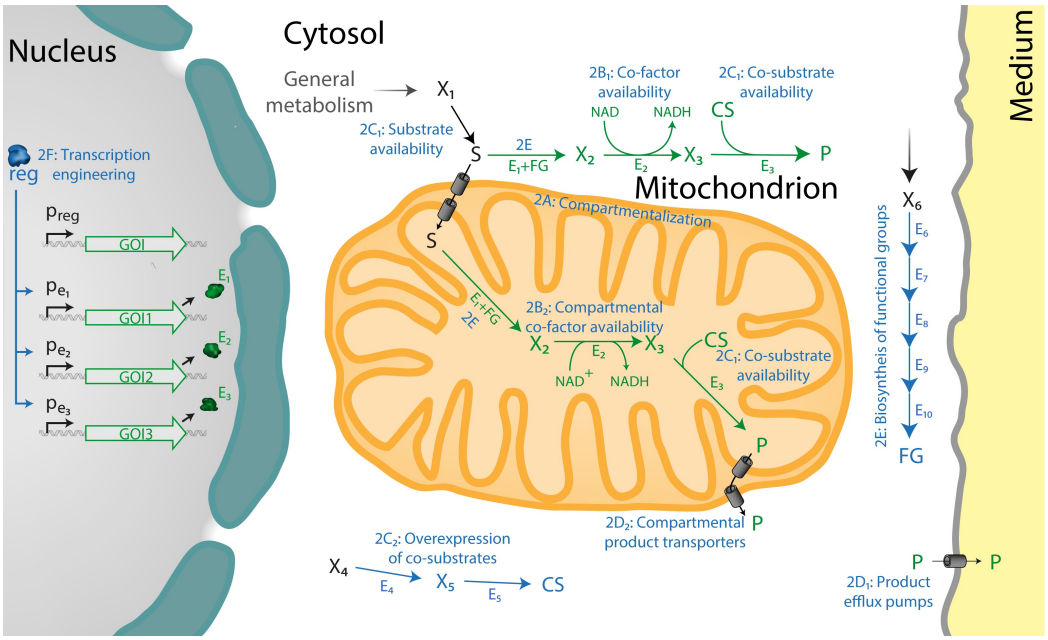


Figure 3.

These challenges can be condensed into points **2A-2F** below. Note that these cover both eukaryotic and prokaryotic hosts and donors in combination, meaning that some of these are specific to certain types of cells (e.g. intracellular compartmentalization is seldom a problem in prokaryotes).

**2A. Compartmentalization or steric proximity:** In heterologous expression, a common pitfall is not making sure that the pathway is expressed in the same compartment as the substrate metabolite. If the heterologous enzymes do not contain targeting signals, in a eukaryotic host, they will be expressed in the cytosol. In case the substrate is in another compartment, targeting sequences or gene fusions can be applied to direct the heterologous enzyme(s) to the correct compartment (Siddiqui et al. 2012). As an alternative, synthetic scaffolds have been made to bring biosynthetic enzymes together with great effect both in *E. coli* (Dueber et al. 2009) and *S. cerevisiae* (Wang & Yu 2012).

**2B$_{1\text{-}2}$. Co-factor availability:** Any overexpressed pathway will present a significant drain on available co-factors (van Rossum et al. 2016). It is advantageous to ensure that these are present in sufficient amounts in the host (**2B$_1$**), as shown e.g. in *Streptomyces coelicolor* (Borodina et al. 2008). This may be specific to the compartment (**2B$_2$**). Alternatively, transhydrogenases may be engineered as described in **1E$_{1\text{-}2}$**.

**2C$_{1\text{-}2}$. Substrate and co-substrate availability:** In addition to co-factors, one must also ensure that the host produces all substrates and co-substrates/precursors required for the pathway (**2C$_1$**). It may also be the case that the host produces similar compounds, which may be competing for the substrate or precursors. In these cases, it can be advantageous to delete the competing pathways (Baltz 2016). It has been demonstrated in e.g. *E. coli* (Rodrigues et al. 2013; Rodrigues et al. 2014) and

*Corynebacterium glutamicum* (Becker et al. 2013) that high availability of the substrate in the heterologous host improves productivity. If all (co-)substrates are not available or present in low amount, it is necessary to insert or overexpress biosynthetic genes for these as well (**2C$_2$**), Examples of this are seen for e.g. amino acids or oxaloacetate (Kind et al. 2010; Rodrigues et al. 2013) or adipic acid (Yu et al. 2014).

**2D$_{1\text{-}2}$. Product efflux pumps:** When adding biosynthesis of a new compound to a cell, specific transporters for that compound may not exist. Accumulation of the product in the cell will decrease the flux through the biosynthetic pathway (Lee et al. 2012) and may also have toxic effects on the cell (Pfeifer & Khosla 2001). Passive transport or unspecific transporters may be available, but if this is not the case, a specific transporter must be added (**2D$_1$**) as seen for e.g. flavonoids (Wu et al. 2014) or cadaverine (Qian et al. 2011). Should the pathway be compartmentalized, this also needs to be accounted for, possibly by expressing an organelle-specific transporter (**2D$_2$**), e.g. with mitochondrial products (Chen et al. 2015).

**2E. Biosynthesis of functional groups.** For a number of proteins, all functional groups are not encoded by the gene, but require separate biosynthesis. One example is heme groups, found in multiple types of enzymes requiring oxygen as a co-factor. Heme groups are not found in all prokaryotes (Cavallaro et al. 2008), and may be limiting in some fungal systems (Franken et al. 2011). Another functional group is Fe-S clusters, which have several different biosynthetic pathways specific to the type of host organism. Fe-S clusters are synthesized in the cytoplasm of bacteria and in the mitochondrion of eukaryotic microbes, from where they are transported into the cytosol. In order for the heterologous pathway to be functional, it may be required to express the native biosynthesis pathway heterologously **(2E)**. A specific type of Fe-S proteins (ferredoxins) mediate electron transfer. Cases exist where the expression of specific ferredoxins from the native host were necessary for optimal expression of the pathway (Molnár et al. 2006).

**2F. Transcription engineering.** With many secondary metabolites, several genes are required to act in concert to form the product. If only one or a few genes are active, the product may be absent or different from the expected product. For many of these pathways, one regulatory protein exists, which transcriptionally activates the entire pathway. If one uses native promoters to express the genes, it can be advantageous to overexpress the regulatory protein (if it can be identified), and thereby induce the entire set of genes (Pickens et al. 2011; Baltz 2016; Bekiesch et al. 2016). Examples of this include the transplant of the geodin gene cluster from *A. terreus* into *A. nidulans* and substitution of the native promoter for the transcription factor for a strong constitutive promoter, thus allowing heterologous expression of geodin (Nielsen et al. 2013).

## Choosing a strategy for heterologous pathway expression

For heterologous pathways, the strategy is a combination of the issues encountered in the expression of native pathways, and issues arriving from

interaction with the new host. Roughly, the considerations can be sorted into two major steps:

*Step 1: Compatibility of the pathway to the host.* The actions listed in this step are interesting in that they may not be needed, dependent on the interaction with the host. Appropriate host selection can thus be used already in the design fase to remove or minimize the problems (For a few reviews on host selection, see e.g. (Fisher et al. 2014; Lee & Kim 2015; Bekiesch et al. 2016)). However, if these are not considered, no other engineering strategies may be effective. The three main things to consider are thus:

i. Compartmentalization (**2A**). Spatial co-localization of the inserted enzymes as well as availability of co-factors and precursors in the compartment(s) of choice.

ii. Functional group biosynthesis (**2E**). Ensuring functionality of all enzymes.

iii. Substrates, co-substrates (**2C**), and co-factors (**2B**). Ensuring that all required precursors are available in the host.

*Step 2: Optimization of the pathway*. Once it is ensured that the pathway is functional in the host, one can apply strategies to increase flux through the pathway. Here the following five steps should be investigated, sorted in order of perceived importance.

i. Application of transcription engineering where possible (**2B**). Increased transcription of all enzymatic steps is an efficient way to increase enzyme levels.

ii. Pathway overexpression strategies (**1A$_1$)** and removal of feedback inhibition (**1F**) are equally applicable to heterologous pathways.

iii. Removal of competing activities as described in **1C** and **1D**. This is particularly interesting when producing a compound, where the host produces several similar compounds competing for the precursors, e.g. within microbial bioactive compounds (Pickens et al. 2011).

iv. Improving the product efflux by transporter engineering (**2D** and **1B**).

v. Removal of by-products (**1G)** can possibly be considered last, as the strategies above are more direct towards improving the pathway. However, by-products removal has been seen to have importance here (Wu et al. 2014; Pickens et al. 2011).

In summary, the overview above provides a strategy guide for heterologous pathway expression encompassing many different reviews and studies. However, it is important to note, that this does not cover host-specific or donor-specific problems. In these cases, we direct the reader to Table 1 to find suggestions for additional species-specific engineering challenges.

## Protein expression

The expression of proteins, both homologous and heterologous, is presently done in a wide variety of hosts from *E. coli* and *Bacillis subtilis,* over yeasts, e.g. *Klyuveromyces lactis, Pichia pastoris* and *S. cerevisiae*, through filamentous fungi such as *A. niger,* to cells derived from multicellular organisms such as mammals and insects. The variety of proteins of commercial interest is great, ranging from bulk enzymes to complex biopharmaceuticals (Association of Manufacturers and Formulators of Enzyme Products 2009; Walsh 2014).

Due to the diverse properties of proteins, it is currently not possible to use one platform organism for expression of all proteins. The scientist must thus choose the cell factory based on the properties and applications of the desired protein. The advantages and disadvantages of applying different cell factories are discussed in several excellent reviews specialized to particular expression systems (See Table 1). In particular we recommend the review of (Waegeman & Soetaert 2011) for an very clear comparison of expression systems in addition to a thorough overview of *E. coli* expression.

Despite the variety of employed systems, there are generic strategies applicable to high-yield expression of proteins. Not all of the strategies presented here are applicable in every host, but we focus on strategies, which are applicable in several hosts. For this reason, the present review does not discuss strategies related to the accumulation of protein in inclusion bodies, a feature encountered in some bacterial hosts such as *E. coli* for some proteins with particular folding. For this, we again direct the reader to specialized overviews (de Marco 2009; Waegeman & Soetaert 2011).

**Overall, the successful high-yield process for production of a given protein requires high transcription and translation of the gene, successful targeting of the protein to the secretion pathway (if secretion is desired), correct folding and limited induction of secretion stress, the desired post-translational modifications (PTMs), efficient secretion, and limited or no degradation of the product in the medium. In general, the major strategies for engineering increased protein expression can be found in**

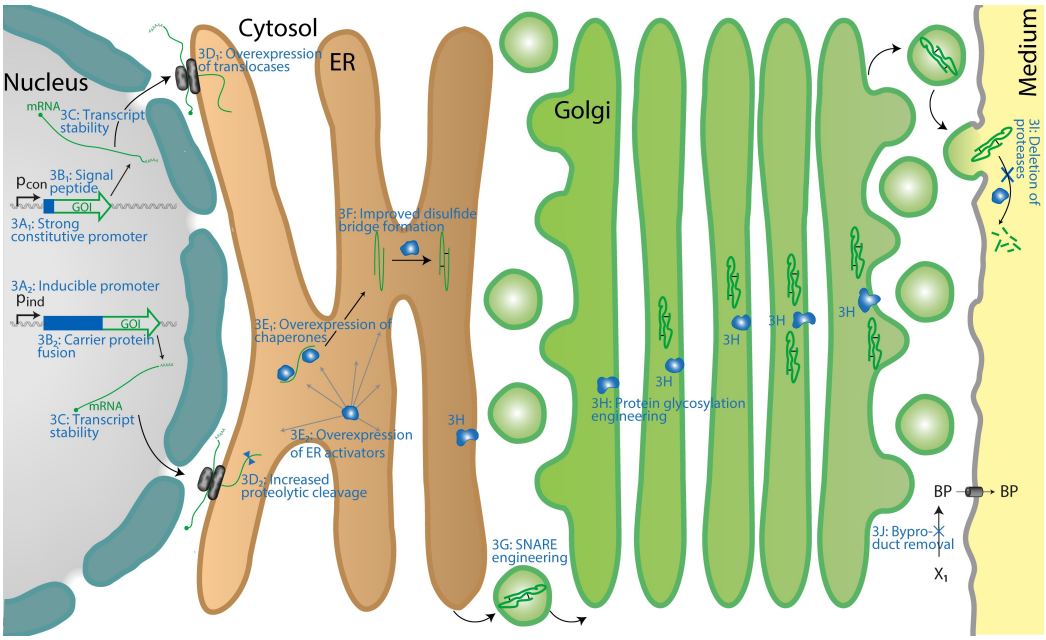


Figure 4, and are summarized in points **3A-3F** below:

**3A1-2. Promoter engineering:** Nearly all systems aim at ensuring maximal availability of recombinant mRNA so that this is not a bottle neck for protein expression. The major strategy employed is addition of a highly expressed constitutive promoter (**3A1**). A selection of these are known for most hosts such as the native GAPDH-promoter in yeasts (Mattanovich et al. 2012), the heterologous *gdhA* promoter in *Aspergillus* species (Fleissner & Dersch 2010), or viral promoters in mammalian hosts (Wurm 2004). It is also a common strategy to develop synthetic promoters (Dehli et al. 2012; Vogl et al. 2013; Fleissner & Dersch 2010). Alternatively, one can employ strong inducible promoters (**3A2**) to have a bi-phasic process (Waegeman & Soetaert 2011; Fleissner & Dersch 2010), for instance methanol-inducible gene expression in the methylotrophic yeast *P. pastoris* (Mattanovich et al. 2012; Damasceno et al. 2012). Reviews with particularly good overviews of promoters for specific systems are available (Celik & Calık 2011; Fleissner & Dersch 2010). A complementary strategy to the use of strong promoters is the expression from high-copy plasmids (Rosano & Ceccarelli 2014), or multigene insertions (Westwood et al. 2010; Wurm 2004; Damasceno et al. 2012). Other transcriptional elements such as enhancers, transcription factor binding, and chromosomal elements should be considered dependent on expression systems (Liu et al. 2013; Fleissner & Dersch 2010; Westwood et al. 2010).

**3B1-2. Gene fusion for enhanced secretion:** For proteins with no inherent secretion signal, the gene sequence requires engineering to facilitate secretion of the protein. The predominant way is the addition of a signal peptide/secretion leader signal (**3B1**). This can also be applied to substitute the original signal peptide for improved secretion in the host. For the major hosts, efficient signal peptides are known from native secreted proteins, e.g. alpha-mating factor or acid phosphatase in yeasts (Mattanovich et al. 2012;

Damasceno et al. 2012) or leader sequences from secreted proteins in *Aspergillus* (Fleissner & Dersch 2010) or bacteria (Liu et al. 2013). In some combinations of host and protein, this may not be sufficient; in which case, the gene of interest is fused with the sequence for a carrier protein (**3B$_2$**), which then has the effect of ushering the protein out of the cell. One example is the production of animal proteins in *Aspergillus* species, where a successful strategy for bovine chymosin production was fusion with the glucoamylase gene. This approach has since then become a preferred method (Ward et al. 1990; Ward 2011; Fleissner & Dersch 2010).

**3C. Stability of heterologous gene transcripts:** Most eukaryotic genes contain introns. In many cases, their removal from the transcript is necessary to generate a functional gene product due to differences in (or absence of) splicing machinery between species (Hamann & Lange 2006; Innis et al. 1985). In higher eukaryotic systems a single intron early in the transcript or in the promoter can however successfully enhance stability of mRNA and increase the final product titer (Borkovich et al. 2004)**.** In many cases, codon optimization of heterologous transcripts are often needed due to incompatibility between the host and the protein codons, e.g. use of rare codons or difference in stop codons. In general, the half-life of a heterologous transcript might be different from related transcripts of the host. In bacterial system, the importance of terminators and 3'UTR regions to transcript stability has been well established (Cambray et al. 2013; Pfleger et al. 2006; Curran et al. 2013). Often changing natural or adding new structures, e.g. hairpin structures, to the ends of transcripts, have been shown in bacteria to accumulate mRNA and increase product formation (Hienonen et al. 2007). In yeast and fungal systems, recent studies show that changing a terminator can effectively optimize the transcript stability and increase the product titer (Curran et al. 2013)**.**

**3D$_{1\text{-}2}$. Improved translocation to the ER:** The induced pressure on the secretion machinery creates numerous rate-limiting steps. The first is already at the entrance of the secretion pathway, through translocation (**3D$_1$**). A successful approach for several systems is overexpression of signal peptidases cleaving the signal peptide by entrance to the ER (Meta et al. 2009; Dijl et al. 1991; Ailor et al. 1999). Insufficient amount of proteolytic cleavage enzymes may also be limiting for secreted proteins with pre-cursor domains (**3D$_2$**). An example is for therapeutic protein produced in CHO cells, where overexpression of the cleaving enzyme PACE, increase the secretion capability for several different proteins (Sathyamurthy et al. 2012).

**3E$_{1\text{-}2}$. Protein secretion stress engineering:** It is generally found that overexpression of proteins induces protein secretion stress to some degree, which decreases productivity and overall cell fitness (Lubertozzi & Keasling 2009; Gasser et al. 2008; Schröder 2008). One generally applied strategy is the overexpression of chaperones (**3E$_1$**). This strategy has been proven to be successful in several studies in a multitude of systems: *E. coli* (Waegeman & Soetaert 2011; Gasser et al. 2008; Rosano & Ceccarelli 2014), other bacteria (Gasser et al. 2008), yeasts (Mattanovich et al. 2012; Gasser

et al. 2008), fungi (Ward 2011; Fleissner & Dersch 2010), and CHO cells (Ailor & Betenbaugh 1998; Jossé et al. 2012; Pybus et al. 2013). It has also been broadly successful to regulate global activators of the ER or the unfolded protein response (**3E2**), in bacteria (Gasser et al. 2008), *S. cerevisiae* (Valkonen, Penttilä, et al. 2003; Mattanovich et al. 2012; Calfon et al. 2002), in *A. niger* var. *awamori* (Valkonen, Ward, et al. 2003; Carvalho et al. 2012; Fleissner & Dersch 2010) and in mammalian cells (Ohya et al. 2008; Tigges & Fussenegger 2006; Ku et al. 2008).

**3F. Engineering the post-translational modification machinery:** In some cases, the bottlenecks are in the formation of disulfide bridges (Schröder 2008). This has been a problem in *E. coli* in particular (de Marco 2009), but proteins involved in disulfide bridge formation have been seen to be limiting in many cases, as seen by the positive effect of protein disulfide isomerase (PDI) in many other organisms, such as several yeasts, *Aspergillus* (Gasser et al. 2008; Fleissner & Dersch 2010) and CHO (Borth et al. 2005; Davis et al. 2000; Mohan et al. 2007).

**3G. Improved vesicle trafficking.** Another rate-limiting step is the vesicle trafficking between ER-Golgi and Golgi-membrane**.** Overexpression of SNAREs and their key regulators can stimulates vesicular trafficking in yeast and enhance heterologous protein secretion (Hou et al. 2012; Ruohonen et al. 1997). Vacuolar protein sorting is complex, illustrated by disruption of the vacuolar protein sorting receptor, Vsp10p, which has a positive impact on secreted protein in both filamentous fungi and yeast (Yoon et al. 2010; Idiris et al. 2010).

**3H. Protein glycosylation engineering.** This discipline does not directly aim to improve to production rate or titer of the product, but instead addresses protein quality, in the form of protein glycosylation. This has two branches, one where it is sought to optimize the native protein glycosylation, and one where the host organism does not have the required protein glycosylation features, and these are engineered into the cell factory (Mattanovich et al. 2012; Hossler 2012; Hossler et al. 2009; Andersen et al. 2011; Vogl et al. 2013). *E. coli*, like most prokaryotes, does not have native protein glycosylation, but genes from other prokaryotes with protein glycosylation have successfully been engineered into the host (Waegeman & Soetaert 2011). Protein glycosylation has also been engineered in filamentous fungi (Ward 2011). A very ambitious example is the expression of major parts of the human glycosylation pathway in *P. pastoris* (Li et al. 2006; Hamilton et al. 2006; Choi et al. 2003; Damasceno et al. 2012), a technology later acquired by Merck Inc. (Walsh 2010).

**3I. Protease deletions:** The deletion of extracellular proteases has been pursued in many systems with significant effects (Ward 2011; Fleissner & Dersch 2010). Examples include the deletion of all 25 known proteases in *E. coli* (Meerman & Georgiou 1994), *S. cerevisiae* (Tyo et al. 2014), and the deletion of five proteases *in A. oryzae* (Jin et al. 2007). Another strategy, with effects in several *Aspergillus* species, has been the identification and deletion of a global regulator of protease expression, PrtT. Deletion of this

gene eliminates nearly all protease activity (Punt et al. 2008; Fleissner & Dersch 2010).

**3J. By-product removal:** A final general strategy, in all species, is the removal of byproducts with negative effect on protein production. Examples include removal of acetate biosynthesis in *E. coli* (Waegeman & Soetaert 2011), oxalic acid production in Aspergilli (Li et al. 2013), or lactate production in CHO cells (Kim & Lee 2007). All of these have been shown to improve product formation, growth characteristics or both.

## Choosing a strategy for protein expression

Contrary to the strategies for production of smaller compounds, where the yield and titer of the product is the primary optimization criterion, it is more difficult to define a generalized order of engineering strategies for protein products. The main reason is, that for some proteins – in particular pharmaceuticals – quality is more important than quantity. In some cases, quantity even has a detrimental effect on quality, as it may elicit stress responses in the cell which degrades the product (Wurm 2004; Damasceno et al. 2012; Hossler 2012; Hossler et al. 2009). Therefore, we propose two strategies, one for optimizing titers (e.g. for enzymes and bulk products), and one for products focused on quality (i.e. pharmaceuticals):

*Strategy A: Optimal expression of the heterologous gene.* Here, multiple initiatives can be used separately, sequentially or in parallel, to find the strategy that is the most efficient. The following six actions are thus applicable only in the cases where that factor is limiting. In general, actions 3A-3C in particular are relatively consistently applied in successful studies.

i. Selection and engineering of optimal promoters (**3A**) are vital for high levels of transcript, so this does not become a limiting factor.

ii. Engineering of the heterologous gene in regard to codon compatibility and optimality and removal or adaptation of introns (**3C**) are also found in nearly all studies.

iii. Selection and/or engineering of the secretion signal (**3B**) is required to ensure secretion of the product, and appropriate trafficking of the peptide chain to the ER. This can affect the production by several fold.

iv. Protein secretion stress reduction (**3E**), in particular regarding the formation of di-sulfide bridges, generally increases product formation.

v. Similar to as is seen for small molecules (**1D**), removal of product degradation improves productivity. For proteins, this is solved by protease deletions (**3I**).

vi. Finally, it has been shown that engineering vesicle trafficking (**3G**) and translocation to the ER (**3D**) increases productivity. However, it is quite few cases, possibly due to the complexity of engineering these processes. It thus becomes difficult to evaluate how applicable this is in general.

*Strategy B: Protein quality.* For optimization of protein quality, the strategy depends on which quality criterion is suboptimal in the production process, and a first step should thus be the determination of this. Here, analytical biochemistry will be the primary tool, and thus not within the scope of this metareview. Once it has been established, one can apply one or more of the following four engineering types:

- Protein glycosylation engineering (**3H**) is generally very attractive for glycosylated biopharmaceuticals(Walsh 2014; Ratner 2014).

- Engineering di-sulfide bridge formation (**3F**) and protein folding (**3E**) in general can help remove erroneously folded protein and decrease protein folding-associated stress.

- Protease deletions (**3I**) are just as important for maintaining protein quality as quantity.

In addition to the strategies of this section, one can also consider adding strategies of the previous sections where appropriate. In particular by-product removal (**3J**) has been demonstrated to be efficient.

## Conclusions

Considering the breadth and depth of the strategies discussed above, it is clear that the field of cell factory engineering as a whole has come a long way. Through tens of thousands of studies, a multitude of individual challenges have been solved across a broad range of expression systems and diverse types of compounds. New and interesting avenues are being opened, such as expansion of the substrate range of *E. coli* turning it into a synthetic methylotroph (Müller et al. 2015), or achieving the biosynthesis of caffeine and other methylxanthines in yeast from plant biosynthetic genes (McKeague et al. 2016), or achieving biobased nylon through large-scale engineering in *C. glutamicum* (Kind et al. 2014). We are also now seeing engineering of central metabolism for increased protein production (Nocon et al. 2014). It seems like there is no obvious limit to the possibilities in sight.

Furthermore, increasingly advanced work is being published, opening the field up into the applications of synthetic biology. This impacts small parts of the cell factory engineering, such as the improvements in synthetic promoters (Vogl et al. 2013), and at larger scale such as building artificial pathways rather than using "simple" heterologous expression. Examples here include the biosynthesis of gastrodin (Bai et al. 2016), and the impressive feat of the Smolke lab to biosynthesize opiods in *S. cerevisiae* (Thodey et al. 2014; Galanie et al. 2015).

Another interesting development is the use of engineered consortia of species for achieving particular activites and synergies from using multiple species. A recent example employs bacterial consortia for desulfurisation of oil-based fuels (Martínez et al. 2016), thus improving the quality.

Even so, there are still significant problems, which need to be addressed. Despite the extensive size of the toolbox of strategies outlined above, it is still difficult to

know *a priori* which modifications are required for a specific combination of product and cell factory. This is one of the main reasons why the development time for new cell factories remains the largest bottleneck for new bioproducts.

In order to move the field forward, these challenges must be addressed in multiple ways. Currently we see a next major step to predict how cells change dynamically over the course of cultivation. At the moment, the most successful modeling of biological systems has been for steady state; which is often not representative of the conditions in a bioreactor in a long production process. Another brick in the wall will be the new conceptual frameworks (e.g. systems biology or system metabolic engineering), which are moving towards holistic design of cell factories and biological networks (Nocon et al. 2014).

To achieve these goals, the importance of efficient genetic engineering and genome editing tools cannot be overstated. Every time genetic engineering technologies have improved, so has the sophistication of cell factory engineering. Synthetic biology and genome editing technologies such as CRISPR will accelerate cell factory engineering as we know it(Jakočiūnas et al. 2016), and they also promise to facilitate more-rapid tests of new theories, permutations of solutions, and generally cell engineering at a systems level.

In tandem, dynamic modeling, holistic design, synthetic biology, and genome editing hold great promises for rational design of biological systems.

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## Author contributions

All authors were a part of the literature analysis and wrote the manuscript.

## Box 1. Research applications of cell factory engineering

The focus of the current review is on cell factory engineering for biotechnological applications. However, there are many other applications of cell factory engineering in life sciences and medicine.

Besides industrial applications, a main application of cell factory engineering is to study the biological function of genes and proteins in basic research. For this, genome engineering and synthetic biology tools can be applied to regulate and remove current gene function or introduce new followed by analyzing the effect on cellular functions including biochemical reactions, regulatory networks or cell phenotypes (Hsu et al. 2014; Bashor et al. 2010). An example is engineering of genes involved in glycosylation to study their function in generating certain glycoforms that can be applied to achieve homogenized glycoforms on

recombinant proteins for comparative studies of their biological effect (Yang et al. 2015).

Cell factory engineering is also highly applied in generating reagents for research. Examples includes expression of antibodies to obtain reagents for genetics studies (Barnstable et al. 1978), hormones to obtain reagents for immunoassays (Ribela et al. 1996), and purified proteins for structural analysis by crystallographers and NMR spectroscopists (Edwards et al. 2000). In addition, the produced products from cell factory engineering can be applied in screening for drug activity or as potential drug target for medical applications (Trosset & Carbonell 2015). This also includes engineering of natural probiotics to produce valuable compounds for enhancement of their benefit to the host (Behnsen et al. 2013).

## References


Ailor, E. et al., 1999. A bacterial signal peptidase enhances processing of a recombinant single chain antibody fragment in insect cells. *Biochemical and biophysical research communications*, 255(2), pp.444–50.

Ailor, E. & Betenbaugh, M.J., 1998. Overexpression of a cytosolic chaperone to improve solubility and secretion of a recombinant IgG protein in insect cells. *Biotechnol Bioeng*, 58(2–3), pp.196–203.

Andersen, M.R., Nam, J.H. & Sharfstein, S.T., 2011. Protein glycosylation: Analysis, Characterization, and Engineering. In *Encyclopedia of Industrial Biotechnology, Bioprocess, Bioseparation, and Cell Technology*. John Wiley & Sons Ltd, pp. 1–50.

Anyaogu, D.C. & Mortensen, U.H., 2015. Heterologous production of fungal secondary metabolites in Aspergilli. *Frontiers in Microbiology*, 6. Available at: http://journal.frontiersin.org/Article/10.3389/fmicb.2015.00077/abstract.

Asadollahi, M.A. et al., 2009. Enhancing sesquiterpene production in Saccharomyces cerevisiae through in silico driven metabolic engineering. *Metabolic engineering*, 11(6), pp.328–34. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19619667 [Accessed October 18, 2013].

Association of Manufacturers and Formulators of Enzyme Products, 2009. List of Commercial Enzymes.

Bai, Y. et al., 2016. De novo biosynthesis of Gastrodin in Escherichia coli. *Metabolic Engineering*, 35, pp.138–147.

Bailey, J.E. et al., 2002. Inverse metabolic engineering: a strategy for directed genetic engineering of useful phenotypes. *Biotechnol Bioeng*, 79(5), pp.568–579. Available at: http://dx.doi.org/10.1002/bit.10441.

Bailey, J.E., 1991. Toward a science of metabolic engineering. *Science (New York, N.Y.)*, 252(5013), pp.1668–75. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2047876 [Accessed October 16, 2013].

703 Baltz, R.H., 2016. Genetic manipulation of secondary metabolite biosynthesis for
704 improved production in Streptomyces and other actinomycetes. *Journal of*
705 *Industrial Microbiology & Biotechnology*, 43(2–3), pp.343–370. Available at:
706 http://link.springer.com/10.1007/s10295-015-1682-x.

707 Barnstable, C.J. et al., 1978. Production of monoclonal antibodies to group A
708 erythrocytes, HLA and other human cell surface antigen-new tools for
709 genetic analysis. *Cell*, 14(May), pp.9–18.

710 Bashor, C.J. et al., 2010. NIH Public Access. *October*, pp.515–537.

711 Becker, J. et al., 2013. Systems metabolic engineering of Corynebacterium
712 glutamicum for production of the chemical chaperone ectoine. *Microbial Cell*
713 *Factories*, 12(1), p.110. Available at:
714 http://www.microbialcellfactories.com/content/12/1/110.

715 Behnsen, J. et al., 2013. Probiotics: Properties, examples, and specific
716 applications. *Cold Spring Harbor Perspectives in Medicine*, 3(3).

717 Bekiesch, P., Basitta, P. & Apel, A.K., 2016. Challenges in the Heterologous
718 Production of Antibiotics in Streptomyces. *Archiv der Pharmazie*, 349(8),
719 pp.594–601. Available at: http://www.ncbi.nlm.nih.gov/pubmed/27258165.

720 Berkmen, M., 2012. Production of disulfide-bonded proteins in Escherichia coli.
721 *Protein expression and purification*, 82(1), pp.240–51. Available at:
722 http://www.ncbi.nlm.nih.gov/pubmed/22085722 [Accessed October 21,
723 2013].

724 Borkovich, K.A. et al., 2004. Lessons from the genome sequence of Neurospora
725 crassa: tracing the path from genomic blueprint to multicellular organism.
726 *Microbiology and molecular biology reviews : MMBR*, 68(1), pp.1–108.

727 Borodina, I. et al., 2008. Antibiotic overproduction in Streptomyces coelicolor
728 A3(2) mediated by phosphofructokinase deletion. *Journal of Biological*
729 *Chemistry*, 283(37), pp.25186–25199. Available at:
730 http://www.jbc.org/cgi/doi/10.1074/jbc.M803105200.

731 Borth, N. et al., 2005. Effect of increased expression of protein disulfide
732 isomerase and heavy chain binding protein on antibody secretion in a
733 recombinant CHO cell line. *Biotechnol Prog*, 21(1), pp.106–111. Available at:
734 http://dx.doi.org/10.1021/bp0498241.

735 Burgard, A.P., Pharkya, P. & Maranas, C.D., 2003. Optknock: a bilevel
736 programming framework for identifying gene knockout strategies for
737 microbial strain optimization. *Biotechnology and bioengineering*, 84(6),
738 pp.647–57. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14595777
739 [Accessed October 17, 2013].

740 Calfon, M. et al., 2002. IRE1 couples endoplasmic reticulum load to secretory
741 capacity by processing the XBP-1 mRNA. *Nature*, 415(6867), pp.92–96.
742 Available at: http://dx.doi.org/10.1038/415092a.

743 Cambray, G. et al., 2013. Measurement and modeling of intrinsic transcription
744 terminators. *Nucleic acids research*, 41(9), pp.5139–48.

745 Carvalho, N.D. et al., 2012. Genome-wide expression analysis upon constitutive

activation of the HacA bZIP transcription factor in Aspergillus niger reveals a coordinated cellular response to counteract ER stress. *BMC Genomics*, 13, p.350. Available at: http://dx.doi.org/10.1186/1471-2164-13-350.

Cavallaro, G., Decaria, L. & Rosato, A., 2008. Genome-based analysis of heme biosynthesis and uptake in prokaryotic systems. *Journal of proteome research*, 7(11), pp.4946–54. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18808173 [Accessed October 18, 2013].

Celik, E. & Calık, P., 2011. Production of recombinant proteins by yeast cells. *Biotechnology advances*, 30(5), pp.1108–18. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21964262 [Accessed October 21, 2013].

Chemier, J.A. et al., 2009. Trends in microbial synthesis of natural products and biofuels. *Advances in enzymology and related areas of molecular biology*, 76, pp.151–217.

Chen, X. et al., 2015. Mitochondrial engineering of the TCA cycle for fumarate production. *Metabolic engineering*. Available at: http://www.sciencedirect.com/science/article/pii/S1096717615000130.

Choi, B.-K. et al., 2003. Use of combinatorial genetic libraries to humanize N-linked glycosylation in the yeast Pichia pastoris. *Proc Natl Acad Sci U S A*, 100(9), pp.5022–5027. Available at: http://dx.doi.org/10.1073/pnas.0931263100.

Cui, Y.-Y. et al., 2014. Production of shikimic acid from Escherichia coli through chemically inducible chromosomal evolution and cofactor metabolic engineering. *Microbial cell factories*, 13(1), p.21. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3923554&tool=pmcentrez&rendertype=abstract.

Curran, K. a et al., 2013. Use of expression-enhancing terminators in Saccharomyces cerevisiae to increase mRNA half-life and improve gene expression control for metabolic engineering applications. *Metabolic engineering*, 19, pp.88–97.

Damasceno, L.M., Huang, C.-J.J. & Batt, C.A., 2012. Protein secretion in Pichia pastoris and advances in protein production. *Applied microbiology and biotechnology*, 93(1), pp.31–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22057543 [Accessed October 21, 2013].

Davis, R. et al., 2000. Effect of PDI overexpression on recombinant protein secretion in CHO cells. *Biotechnol Prog*, 16(5), pp.736–743. Available at: http://dx.doi.org/10.1021/bp000107q.

Dehli, T., Solem, C. & Jensen, P.R., 2012. Tunable promoters in synthetic and systems biology. *Sub-cellular biochemistry*, 64, pp.181–201.

Dijl, J.M. Van et al., 1991. Signal peptidase I overproduction results in increased efficiencies of export and maturation of hybrid secretory proteins in Escherichia coli. , pp.40–48.

790 Dueber, J.E. et al., 2009. Synthetic protein scaffolds provide modular control over
791 metabolic flux. *Nature Biotechnology*, 27(8), pp.753–759. Available at:
792 http://www.nature.com/doifinder/10.1038/nbt.1557.

793 Dunlop, M.J. et al., 2011. Engineering microbial biofuel tolerance and export
794 using efflux pumps. *Molecular systems biology*, 7, p.487.

795 Edwards, a M. et al., 2000. Protein production: feeding the crystallographers and
796 NMR spectroscopists. *Nature structural biology*, 7 Suppl(november),
797 pp.970–972.

798 Fisher, A.K. et al., 2014. A review of metabolic and enzymatic engineering
799 strategies for designing and optimizing performance of microbial cell
800 factories. *Computational and Structural Biotechnology Journal*, 11(18),
801 pp.91–99.

802 Fleissner, A. & Dersch, P., 2010. Expression and export: recombinant protein
803 production systems for Aspergillus. *Applied microbiology and biotechnology*,
804 87(4), pp.1255–70. Available at:
805 http://link.springer.com/article/10.1007/s00253-010-2672-6 [Accessed
806 October 21, 2013].

807 Franken, A.C.W. et al., 2011. Heme biosynthesis and its regulation: towards
808 understanding and improvement of heme biosynthesis in filamentous fungi.
809 *Appl Microbiol Biotechnol*, 91(3), pp.447–460. Available at:
810 http://dx.doi.org/10.1007/s00253-011-3391-3.

811 Galanie, S. et al., 2015. Complete biosynthesis of opioids in yeast. *Science (New*
812 *York, N.Y.)*, 349(6252), pp.1095–100. Available at:
813 http://www.ncbi.nlm.nih.gov/pubmed/26272907.

814 Gasser, B. et al., 2008. Protein folding and conformational stress in microbial
815 cells producing recombinant proteins: a host comparative overview.
816 *Microbial cell factories*, 7, p.11. Available at:
817 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2322954&tool
818 =pmcentrez&rendertype=abstract [Accessed October 25, 2013].

819 Ghosh, A., Zhao, H. & Price, N.D., 2011. Genome-scale consequences of cofactor
820 balancing in engineered pentose utilization pathways in Saccharomyces
821 cerevisiae. *PLoS ONE*, 6(11).

822 Hamann, T. & Lange, L., 2006. Discovery, cloning and heterologous expression of
823 secreted potato proteins reveal erroneous pre-mRNA splicing in Aspergillus
824 oryzae. *Journal of biotechnology*, 126(3), pp.265–76.

825 Hamilton, S.R. et al., 2006. Humanization of yeast to produce complex terminally
826 sialylated glycoproteins. *Science (New York, N.Y.)*, 313(5792), pp.1441–1443.

827 Hienonen, E. et al., 2007. Transcript stabilization by mRNA sequences from hrpA
828 of Pseudomonas syringae. *Journal of biotechnology*, 128(2), pp.258–67.

829 Hossler, P., 2012. Protein glycosylation control in Mammalian cell culture: past
830 precedents and contemporary prospects. *Advances in Biochemical*
831 *Engineering/Biotechnology*, 127, pp.187–219. Available at:
832 http://link.springer.com/chapter/10.1007/10_2011_113%5Cnhttp://www

.ncbi.nlm.nih.gov/pubmed/22015728.

Hossler, P., Khattak, S.F. & Li, Z.J., 2009. {O}ptimal and consistent protein glycosylation in mammalian cell culture. *Glycobiology*, 19(9), pp.936–949. Available at: http://dx.doi.org/10.1093/glycob/cwp079.

Hou, J. et al., 2012. Engineering of vesicle trafficking improves heterologous protein secretion in Saccharomyces cerevisiae. *Metabolic engineering*, 14(2), pp.120–7.

Hsu, P.D., Lander, E.S. & Zhang, F., 2014. Development and applications of CRISPR-Cas9 for genome engineering. *Cell*, 157(6), pp.1262–1278. Available at: http://dx.doi.org/10.1016/j.cell.2014.05.010.

Hwang, K.-S. et al., 2014. Systems biology and biotechnology of Streptomyces species for the production of secondary metabolites. *Biotechnology Advances*, 32(2), pp.255–268. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0734975013001857.

Idiris, A. et al., 2010. Enhanced protein secretion from multiprotease-deficient fission yeast by modification of its vacuolar protein sorting pathway. *Applied microbiology and biotechnology*, 85(3), pp.667–77.

Innis, M.A. et al., 1985. Expression, Glycosylation, and Secretion of an Aspergillus Glucoamylase by Saccharomyces cerevisiae. *Science*, 228(4695), pp.21–26.

Jakočiūnas, T., Jensen, M.K. & Keasling, J.D., 2016. CRISPR/Cas9 advances engineering of microbial cell factories. *Metabolic Engineering*, 34, pp.44–59. Available at: http://www.sciencedirect.com/science/article/pii/S1096717615001597%5Cnhttp://linkinghub.elsevier.com/retrieve/pii/S1096717615001597.

Jin, F.J. et al., 2007. Double disruption of the proteinase genes, tppA and pepE, increases the production level of human lysozyme by Aspergillus oryzae. *Applied microbiology and biotechnology*, 76(5), pp.1059–68. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17622525 [Accessed October 21, 2013].

de Jongh, W.A. & Nielsen, J., 2008. Enhanced citrate production through gene insertion in Aspergillus niger. *Metabolic engineering*, 10(2), pp.87–96. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18162426 [Accessed October 17, 2013].

Jossé, L., Smales, C.M. & Tuite, M.F., 2012. Engineering the chaperone network of CHO cells for optimal recombinant protein production and authenticity. *Methods in molecular biology (Clifton, N.J.)*, 824, pp.595–608. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22160922 [Accessed October 25, 2013].

Keasling, J.D., 2012. Synthetic biology and the development of tools for metabolic engineering. *Metabolic engineering*, 14(3), pp.189–95. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22314049 [Accessed September 16, 2013].

Keasling, J.D., 2008. Synthetic biology for synthetic chemistry. *ACS chemical*

*biology*, 3(1), pp.64–76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18205292 [Accessed September 17, 2013].

Kiel, C., Yus, E. & Serrano, L., 2010. Engineering Signal Transduction Pathways. *Cell*, 140(1), pp.33–47. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0092867409016146.

Kim, S.H. & Lee, G.M., 2007. Down-regulation of lactate dehydrogenase-A by siRNAs for reduced lactic acid formation of Chinese hamster ovary cells producing thrombopoietin. *Appl Microbiol Biotechnol*, 74(1), pp.152–159. Available at: http://dx.doi.org/10.1007/s00253-006-0654-5.

Kind, S. et al., 2014. From zero to hero - Production of bio-based nylon from renewable resources using engineered Corynebacterium glutamicum. *Metabolic Engineering*, 25, pp.113–123.

Kind, S. et al., 2010. Systems-wide metabolic pathway engineering in Corynebacterium glutamicum for bio-based production of diaminopentane. *Metabolic Engineering*, 12(4), pp.341–351.

Ku, S.C.Y. et al., 2008. Effects of overexpression of X-box binding protein 1 on recombinant protein production in Chinese hamster ovary and NS0 myeloma cells. *Biotechnol Bioeng*, 99(1), pp.155–164. Available at: http://dx.doi.org/10.1002/bit.21562.

Lee, J.-H. et al., 2003. Global Analyses of Transcriptomes and Proteomes of a Parent Strain and an L-Threonine-Overproducing Mutant Strain. *Journal of Bacteriology*, 185(18), pp.5442–5451. Available at: http://jb.asm.org/content/185/18/5442?related-urls=yes&legid=jb;185/18/5442 [Accessed October 18, 2013].

Lee, J.W. et al., 2012. Systems metabolic engineering of microorganisms for natural and non-natural chemicals. *Nature Chemical Biology*, 8(6), pp.536–546. Available at: http://www.nature.com/doifinder/10.1038/nchembio.970.

Lee, K.H. et al., 2007. Systems metabolic engineering of Escherichia coli for L-threonine production. *Molecular systems biology*, 3, p.149. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2174629&tool=pmcentrez&rendertype=abstract [Accessed October 17, 2013].

Lee, S.Y. & Kim, H.U., 2015. Systems strategies for developing industrial microbial strains. *Nature biotechnology*, 33(10), pp.1061–72. Available at: http://www.nature.com/doifinder/10.1038/nbt.3365.

Li, A. et al., 2013. Reduced by-product formation and modified oxygen availability improve itaconic acid production in Aspergillus niger. *Appl Microbiol Biotechnol*, 97(9), pp.3901–3911. Available at: http://dx.doi.org/10.1007/s00253-012-4684-x.

Li, H. et al., 2006. Optimization of humanized IgGs in glycoengineered Pichia pastoris. *Nat Biotechnol*, 24(2), pp.210–215. Available at: http://dx.doi.org/10.1038/nbt1178.

Liu, L. et al., 2013. Recent advances in recombinant protein expression by Corynebacterium, Brevibacterium, and Streptomyces: From transcription and translation regulation to secretion pathway selection. *Applied Microbiology and Biotechnology*, 97(22), pp.9597–9608.

Lubertozzi, D. & Keasling, J.D., 2009. Developing Aspergillus as a host for heterologous expression. *Biotechnology advances*, 27(1), pp.53–75. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18840517 [Accessed October 21, 2013].

de Marco, A., 2009. Strategies for successful recombinant expression of disulfide bond-dependent proteins in Escherichia coli. *Microbial cell factories*, 8, p.26. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2689190&tool=pmcentrez&rendertype=abstract [Accessed October 21, 2013].

Martin, V.J.J. et al., 2003. Engineering a mevalonate pathway in Escherichia coli for production of terpenoids. *Nature Biotechnology*, 21(7), pp.796–802. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12778056 [Accessed September 25, 2013].

Martínez, I. et al., 2016. Engineering synthetic bacterial consortia for enhanced desulfurization and revalorization of oil sulfur compounds. *Metabolic Engineering*, 35, pp.46–54.

Mattanovich, D. et al., 2012. Recombinant protein production in yeasts. *Methods in molecular biology (Clifton, N.J.)*, 824, pp.329–58. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22160907 [Accessed October 21, 2013].

McKeague, M. et al., 2016. Engineering a microbial platform for de novo biosynthesis of diverse methylxanthines. *Metabolic Engineering*, 38, pp.191–203.

Meerman, H.J. & Georgiou, G., 1994. Construction and characterization of a set of E. coli strains deficient in all known loci affecting the proteolytic stability of secreted recombinant proteins. *Bio/technology (Nature Publishing Company)*, 12(11), pp.1107–10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7765553 [Accessed October 21, 2013].

Meta, A. et al., 2009. High-yield production and characterization of biologically active recombinant aprotinin expressed in Saccharomyces cerevisiae. *Protein expression and purification*, 66(1), pp.22–7.

Mohan, C. et al., 2007. Effect of doxycycline-regulated protein disulfide isomerase expression on the specific productivity of recombinant CHO cells: thrombopoietin and antibody. *Biotechnol Bioeng*, 98(3), pp.611–615. Available at: http://dx.doi.org/10.1002/bit.21453.

Molnár, I. et al., 2006. Biocatalytic conversion of avermectin into 4''-oxo-avermectin: discovery, characterization, heterologous expression and specificity improvement of the cytochrome P450 enzyme. *Biochemical Society transactions*, 34(Pt 6), pp.1236–40. Available at:

963 http://www.ncbi.nlm.nih.gov/pubmed/17073793 [Accessed October 18,
964 2013].

965 Müller, J.E.N. et al., 2015. Engineering Escherichia coli for methanol conversion.
966 *Metabolic Engineering*, 28, pp.190–201.

967 Nerem, R.M., 1991. Cellular engineering. *Annals of biomedical engineering*, 19(5),
968 pp.529–45. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1741530.

969 Nielsen, J., 1998. Metabolic engineering: techniques for analysis of targets for
970 genetic manipulations. *Biotechnology and bioengineering*, 58(2–3), pp.125–
971 132. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10191381.

972 Nielsen, J., 2014. Synthetic Biology for Engineering Acetyl Coenzyme A
973 Metabolism in Yeast: FIG 1. *mBio*, 5(6), pp.e02153-14. Available at:
974 http://mbio.asm.org/lookup/doi/10.1128/mBio.02153-14.

975 Nielsen, M.T. et al., 2013. Heterologous reconstitution of the intact geodin gene
976 cluster in Aspergillus nidulans through a simple and versatile PCR based
977 approach. M.-J. Virolle, ed. *PloS one*, 8(8), p.e72871. Available at:
978 http://dx.plos.org/10.1371/journal.pone.0072871 [Accessed October 18,
979 2013].

980 Nieuwenhuizen, P.J. & Lyon, D., 2011. Anticipating opportunities in industrial
981 biotechnology: Sizing the market and growth scenarios. *Journal of*
982 *Commercial Biotechnology*, 17(2), pp.159–164.

983 Nocon, J. et al., 2014. Model based engineering of Pichia pastoris central
984 metabolism enhances recombinant protein production. *Metabolic*
985 *Engineering*, 24, pp.129–138. Available at:
986 https://www.scopus.com/inward/record.uri?eid=2-s2.0-
987 84901621407&partnerID=40&md5=a2386fa67e20d4b9508ca30c5d49207
988 2.

989 Ohya, T. et al., 2008. Improved production of recombinant human antithrombin
990 III in Chinese hamster ovary cells by {ATF}4 overexpression. *Biotechnol*
991 *Bioeng*, 100(2), pp.317–324. Available at:
992 http://dx.doi.org/10.1002/bit.21758.

993 Ostergaard, S. et al., 2000. Increasing galactose consumption by Saccharomyces
994 cerevisiae through metabolic engineering of the GAL gene regulatory
995 network. *Nature biotechnology*, 18(12), pp.1283–6. Available at:
996 http://www.ncbi.nlm.nih.gov/pubmed/11101808 [Accessed October 18,
997 2013].

998 Park, J.H. et al., 2012. Rational design of Escherichia coli for L-isoleucine
999 production. *ACS synthetic biology*, 1(11), pp.532–40.

1000 Pfeifer, B.A. & Khosla, C., 2001. Biosynthesis of polyketides in heterologous hosts.
1001 *Microbiology and molecular biology reviews : MMBR*, 65(1), pp.106–18.
1002 Available at:
1003 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=99020&tool=p
1004 mcentrez&rendertype=abstract [Accessed October 18, 2013].

1005 Pfleger, B.F. et al., 2006. Combinatorial engineering of intergenic regions in

operons tunes expression of multiple genes. *Nature biotechnology*, 24(8), pp.1027–32.

Pfleger, B.F., Gossing, M. & Nielsen, J., 2015. Metabolic engineering strategies for microbial synthesis of oleochemicals. *Metabolic Engineering*, 29, pp.1–11.

Pharkya, P. & Maranas, C.D., 2006. An optimization framework for identifying reaction activation/inhibition or elimination candidates for overproduction in microbial systems. *Metabolic engineering*, 8(1), pp.1–13. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16199194 [Accessed October 17, 2013].

Pickens, L.B., Tang, Y. & Chooi, Y.-H., 2011. Metabolic engineering for the production of natural products. *Annual review of chemical and biomolecular engineering*, 2, pp.211–36. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22432617 [Accessed September 26, 2013].

Punt, P.J. et al., 2008. Characterization of the Aspergillus niger prtT, a unique regulator of extracellular protease encoding genes. *Fungal genetics and biology : FG & B*, 45(12), pp.1591–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18930158 [Accessed October 25, 2013].

Pybus, L.P. et al., 2013. Model-directed engineering of "difficult-to-express" monoclonal antibody production by Chinese hamster ovary cells. *Biotechnology and bioengineering*. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24081924 [Accessed October 25, 2013].

Qi, K., Zhong, J.J. & Xia, X.X., 2014. Triggering respirofermentative metabolism in the crabtree-negative yeast pichia guilliermondii by disrupting the CAT8 gene. *Applied and Environmental Microbiology*, 80(13), pp.3879–3887.

Qian, Z.-G., Xia, X.-X. & Lee, S.Y., 2011. Metabolic engineering of Escherichia coli for the production of cadaverine: A five carbon diamine. *Biotechnology and Bioengineering*, 108(1), pp.93–103. Available at: http://doi.wiley.com/10.1002/bit.22918.

Ranganathan, S., Suthers, P.F. & Maranas, C.D., 2010. OptForce: an optimization procedure for identifying all genetic manipulations leading to targeted overproductions. *PLoS computational biology*, 6(4), p.e1000744. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2855329&tool=pmcentrez&rendertype=abstract [Accessed October 17, 2013].

Ratner, M., 2014. Genentech's glyco-engineered antibody to succeed Rituxan. *Nature biotechnology*, (December), pp.2013–2014. Available at: http://www.nature.com/nbt/journal/v32/n1/full/nbt0114-6b.html.

Ribela, M.T., Bianco, A.C. & Bartolini, P., 1996. The use of recombinant human thyrotropin produced by Chinese hamster ovary cells for the preparation of immunoassay reagents. *The Journal of Clinical Endocrinology & Metabolism*, 81(1), pp.249–256. Available at:

http://press.endocrine.org/doi/10.1210/jcem.81.1.8550760.

Rodrigues, A.L. et al., 2014. Systems metabolic engineering of Escherichia coli for gram scale production of the antitumor drug deoxyviolacein from glycerol. *Biotechnology and Bioengineering*, 111(11), pp.2280–2289. Available at: http://doi.wiley.com/10.1002/bit.25297.

Rodrigues, A.L. et al., 2013. Systems metabolic engineering of Escherichia coli for production of the antitumor drugs violacein and deoxyviolacein. *Metabolic Engineering*, 20, pp.29–41. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1096717613000827.

Rosano, G.L. & Ceccarelli, E.A., 2014. Recombinant protein expression in Escherichia coli: Advances and challenges. *Frontiers in Microbiology*, 5(APR).

van Rossum, H.M. et al., 2016. Engineering cytosolic acetyl-coenzyme A supply in Saccharomyces cerevisiae: Pathway stoichiometry, free-energy conservation and redox-cofactor balancing. *Metabolic Engineering*, 36, pp.99–115.

Ruohonen, L. et al., 1997. Enhancement of Protein Secretion in Saccharomyces cerevisiae by Overproduction of Sso Protein, a Late-acting Component of the Secretory Machinery. *Yeast*, 13(4), pp.337–351.

Sathyamurthy, M. et al., 2012. Overexpression of PACEsol improves BMP-7 processing in recombinant CHO cells. *Journal of biotechnology*, 164(2), pp.336–9.

Schröder, M., 2008. Engineering eukaryotic protein factories. *Biotechnology Letters*, 30(2), pp.187–196.

Siddiqui, M.S. et al., 2012. Advancing secondary metabolite biosynthesis in yeast with synthetic biology tools. *FEMS Yeast Research*, 12(2), pp.144–170. Available at: http://femsyr.oxfordjournals.org/cgi/doi/10.1111/j.1567-1364.2011.00774.x.

Stephanopoulos, G. & Vallino, J.J., 1991. Network rigidity and metabolic engineering in metabolite overproduction. *Science (New York, N.Y.)*, 252(5013), pp.1675–81.

Thodey, K., Galanie, S. & Smolke, C.D., 2014. A microbial biomanufacturing platform for natural and semisynthetic opioids. *Nature Chemical Biology*, 10(August), pp.1–10. Available at: http://www.nature.com/doifinder/10.1038/nchembio.1613.

Tigges, M. & Fussenegger, M., 2006. Xbp1-based engineering of secretory capacity enhances the productivity of Chinese hamster ovary cells. *Metab Eng*, 8, pp.264–272. Available at: http://dx.doi.org/10.1016/j.ymben.2006.01.006.

Trosset, J.Y. & Carbonell, P., 2015. Synthetic biology for pharmaceutical drug discovery. *Drug Design, Development and Therapy*, 9, pp.6285–6302. Available at: https://www.dovepress.com/articles.php?article_id=24873#.

Tyo, K.E.J. et al., 2014. Impact of protein uptake and degradation on recombinant protein secretion in yeast. *Applied Microbiology and Biotechnology*, 98(16), pp.7149–7159.

Valkonen, M., Ward, M., et al., 2003. Improvement of foreign-protein production in Aspergillus niger var. awamori by constitutive induction of the unfolded-protein response. *Applied and environmental microbiology*, 69(12), pp.6979–86. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=309985&tool=pmcentrez&rendertype=abstract [Accessed October 25, 2013].

Valkonen, M., Penttilä, M. & Saloheimo, M., 2003. Effects of inactivation and constitutive expression of the unfolded- protein response pathway on protein production in the yeast Saccharomyces cerevisiae. *Applied and environmental microbiology*, 69(4), pp.2065–72. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=154816&tool=pmcentrez&rendertype=abstract [Accessed October 25, 2013].

Vogl, T., Hartner, F.S. & Glieder, A., 2013. New opportunities by synthetic biology for biopharmaceutical production in Pichia pastoris. *Current Opinion in Biotechnology*, 24(6), pp.1094–1101. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0958166913000384.

Waegeman, H. & Soetaert, W., 2011. Increasing recombinant protein production in Escherichia coli through metabolic and genetic engineering. *Journal of industrial microbiology & biotechnology*, 38(12), pp.1891–910. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21901404 [Accessed October 21, 2013].

Walsh, G., 2010. Biopharmaceutical benchmarks 2010. *Nature biotechnology*, 28(9), pp.917–24. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20829826.

Walsh, G., 2014. Biopharmaceutical benchmarks 2014. *Nature biotechnology*, 32(10), pp.992–1000. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25299917.

Wang, J. et al., 2013. Increasing ethanol titer and yield in a gpd1Δ gpd2Δ strain by simultaneous overexpression of GLT1 and STL1 in Saccharomyces cerevisiae. *Biotechnology letters*, 35(11), pp.1859–1864. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23801122 [Accessed October 15, 2013].

Wang, Y. & Yu, O., 2012. Synthetic scaffolds increased resveratrol biosynthesis in engineered yeast cells. *Journal of Biotechnology*, 157(1), pp.258–260. Available at: http://dx.doi.org/10.1016/j.jbiotec.2011.11.003.

Ward, M. et al., 1990. Improved production of chymosin in Aspergillus by expression as a glucoamylase-chymosin fusion. *Bio/technology (Nature Publishing Company)*, 8(5), pp.435–40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1366537 [Accessed October 25, 2013].

Ward, O.P., 2011. Production of recombinant proteins by filamentous fungi. *Biotechnology advances*, 30(5), pp.1119–39. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21968147 [Accessed October 21, 2013].

Weber, T. et al., 2015. antiSMASH 3.0--a comprehensive resource for the genome mining of biosynthetic gene clusters. *Nucleic Acids Research*, pp.1–7. Available at: http://nar.oxfordjournals.org/lookup/doi/10.1093/nar/gkv437.

Westwood, A.D., Rowe, D.A. & Clarke, H.R.G., 2010. Improved recombinant protein yield using a codon deoptimized DHFR selectable marker in a CHEF1 expression plasmid. *Biotechnology Progress*, 26(6), pp.1558–1566.

Wu, J. et al., 2014. Systems metabolic engineering of microorganisms to achieve large-scale production of flavonoid scaffolds. *Journal of Biotechnology*, 188, pp.72–80. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0168165614008219.

Wurm, F.M., 2004. Production of recombinant protein therapeutics in cultivated mammalian cells. *Nat Biotechnol*, 22(11), pp.1393–1398. Available at: http://dx.doi.org/10.1038/nbt1026.

Xiao, H. & Zhong, J.-J., 2016. Production of Useful Terpenoids by Higher-Fungus Cell Factory and Synthetic Biology Approaches. *Trends in Biotechnology*, 34(3), pp.242–255. Available at: http://linkinghub.elsevier.com/retrieve/pii/S016777991500267X.

Xu, J.W., Xu, Y.N. & Zhong, J.J., 2012. Enhancement of ganoderic acid accumulation by overexpression of an n-terminally truncated 3-hydroxy-3-methylglutaryl coenzyme a reductase gene in the basidiomycete Ganoderma lucidum. *Applied and Environmental Microbiology*, 78(22), pp.7968–7976.

Yamauchi, Y. et al., 2014. Enhanced acetic acid and succinic acid production under microaerobic conditions by Corynebacterium glutamicum harboring Escherichia coli transhydrogenase gene pntAB. *The Journal of general and applied microbiology*, 60(3), pp.112–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25008167.

Yang, Z. et al., 2015. Engineered CHO cells for production of diverse, homogeneous glycoproteins. *Nature Biotechnology*, 33(8), pp.842–844. Available at: http://www.nature.com/doifinder/10.1038/nbt.3280.

Yoon, J. et al., 2010. Enhanced production and secretion of heterologous proteins by the filamentous fungus Aspergillus oryzae via disruption of vacuolar protein sorting receptor gene Aovps10. *Applied and environmental microbiology*, 76(17), pp.5718–27.

Yu, J. Le et al., 2014. Direct biosynthesis of adipic acid from a synthetic pathway in recombinant escherichia coli. *Biotechnology and Bioengineering*, 111(12), pp.2580–2586.

Zabala, D. et al., 2013. Engineering precursor metabolite pools for increasing production of antitumor mithramycins in Streptomyces argillaceus. *Metabolic engineering*, 20, pp.187–97.

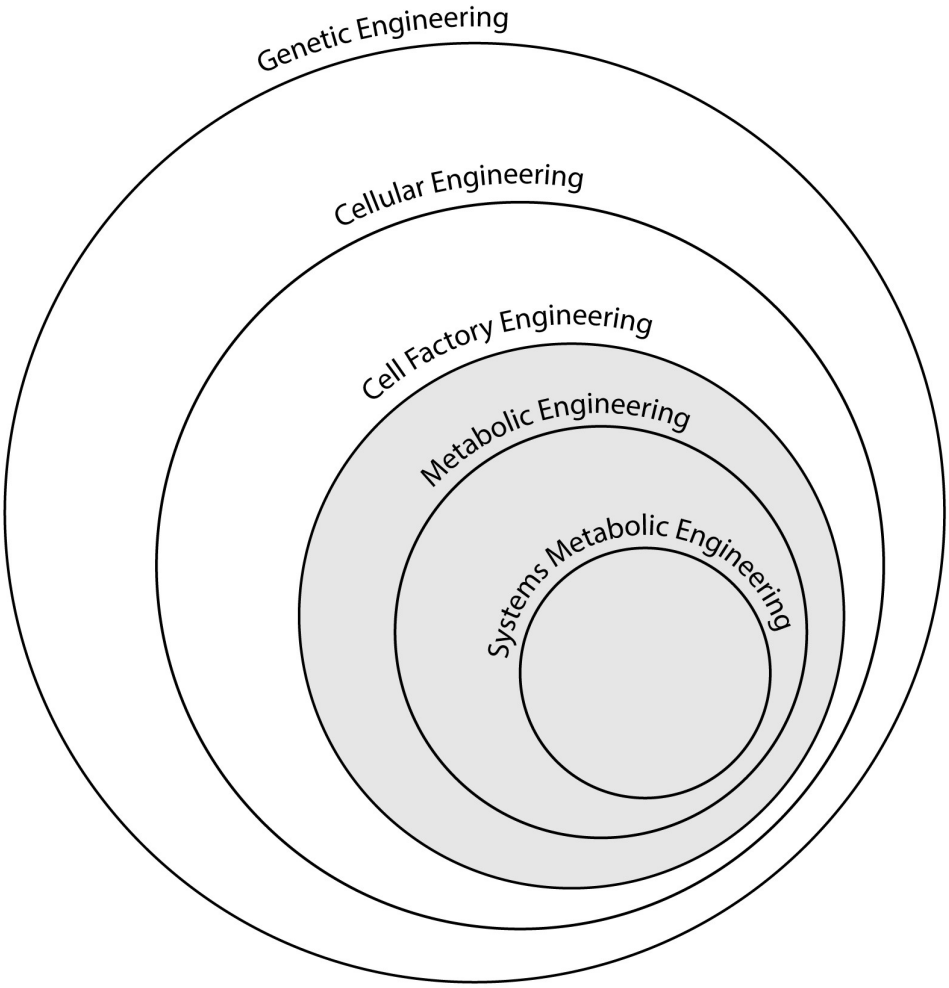
**Table 1** – Overview of reviews covered in this text. Stars on the right denote the general relevance of the text for cell factory engineering

| Reference | Species* | Strategies | Products | |
|---|---|---|---|---|
| (Walsh 2014) | Bac, Yeasts, Fungi, Mam | Protein expression | Biopharma | * |
| (Waegeman & Soetaert 2011) | *E. coli* | Protein expression | Enzymes & Biopharma | * |
| (Chemier et al. 2009) | *E. coli, S. cerevisiae* | Native and heterologous PWs | Bioactives, biofuels | ** |
| (Lee & Kim 2015) | Bac, Yeasts | Native and heterologous PWs | Any | *** |
| (Pickens et al. 2011) | Bac, Fungi | Heterologous PWs | Bioactives | *** |
| (Hwang et al. 2014) | *Streptomyces* sp. | Native PWs | Bioactives | ** |
| (Wu et al. 2014) | *E. coli, S. cerevisiae* | Heterologous PWs | Flavenoids | ** |
| (Anyaogu & Mortensen 2015) | Fungi | Heterologous PWs | Bioactives | * |
| (Xiao & Zhong 2016) | Basidiomycetes | Heterologous PWs | Terpenoids | * |
| (Nielsen 1998) | Bac, Yeasts, Fungi, Mam | All | Any | ** |
| (van Rossum et al. 2016) | *S. cerevisiae* | Native and heterologous PWs | Ac-CoA-derived compounds | ** |
| (Lee et al. 2012) | *E. coli* | Native and heterologous PWs | Small molecules | *** |
| (Nielsen 2014) | *S. cerevisiae* | Native and heterologous PWs | Ac-CoA-derived compounds | * |
| (Pfleger et al. 2015) | *E. coli, S. cerevisiae* | Native and heterologous PWs | Oleochemicals | ** |
| (Pfeifer & Khosla 2001) | *S. coelicolor, E. coli*, Yeasts, Fungi | Native and heterologous PWs | Polyketides | ** |
| (Siddiqui et al. 2012) | Yeasts | Heterologous PWs | Bioactives | *** |
| (Franken et al. 2011) | Fungi | Heterologous PWs | Heme | * |
| (Baltz 2016) | Actinomycetes | Heterologous PWs | Bioactives | ** |
| (Bekiesch et al. 2016) | Actinomycetes | Heterologous PWs | Bioactives | * |
| (Fisher et al. 2014) | Bac, Yeasts, Fungi | Heterologous PWs | Any | ** |

| | | | | |
|---|---|---|---|---|
| (Mattanovich et al. 2012) | Yeasts | Protein expression | Biopharma, Enzymes | ** |
| (Hossler 2012; Hossler et al. 2009) | Mam | Protein expression | Biopharma | ** |
| (Andersen et al. 2011) | Mam | Protein expression | Biopharma | ** |
| (Damasceno et al. 2012) | *P. pastoris* | Protein expression | Proteins | *** |
| (Celik & Calık 2011) | Yeasts | Protein expression | Proteins | *** |
| (Ward 2011) | Fungi | Protein expression | Proteins | *** |
| (Wurm 2004) | Mam | Protein expression | Biopharma | * |
| (Berkmen 2012) | *E. coli* | Protein expression | Proteins with disulfide bonds | * |
| (de Marco 2009) | *E. coli* | Protein expression | Proteins with disulfide bonds | * |
| (Schröder 2008) | Eukaryotes | Protein expression | Biopharma | ** |
| (Fleissner & Dersch 2010) | *Aspergillus* | Protein expression | Enzymes | ** |
| (Lubertozzi & Keasling 2009) | *Aspergillus* | Native and heterologous PWs, protein expression | Small molecules, proteins | * |
| (Vogl et al. 2013) | *P. pastoris* | Protein expression | Biopharma | * |
| (Rosano & Ceccarelli 2014) | *E. coli* | Protein expression | Proteins | ** |
| (Liu et al. 2013) | Gram-positives | Protein expression | Proteins | ** |
| (Gasser et al. 2008) | Bac, Yeasts, Fungi | Protein expression | Proteins | ** |

1181 *Abbreviations: Bac: Bacteria. Mam: Mammalian Cells.

1182




1183

1184 **Figure 1**. Ontology of different types of cellular engineering covered in this review.

1185

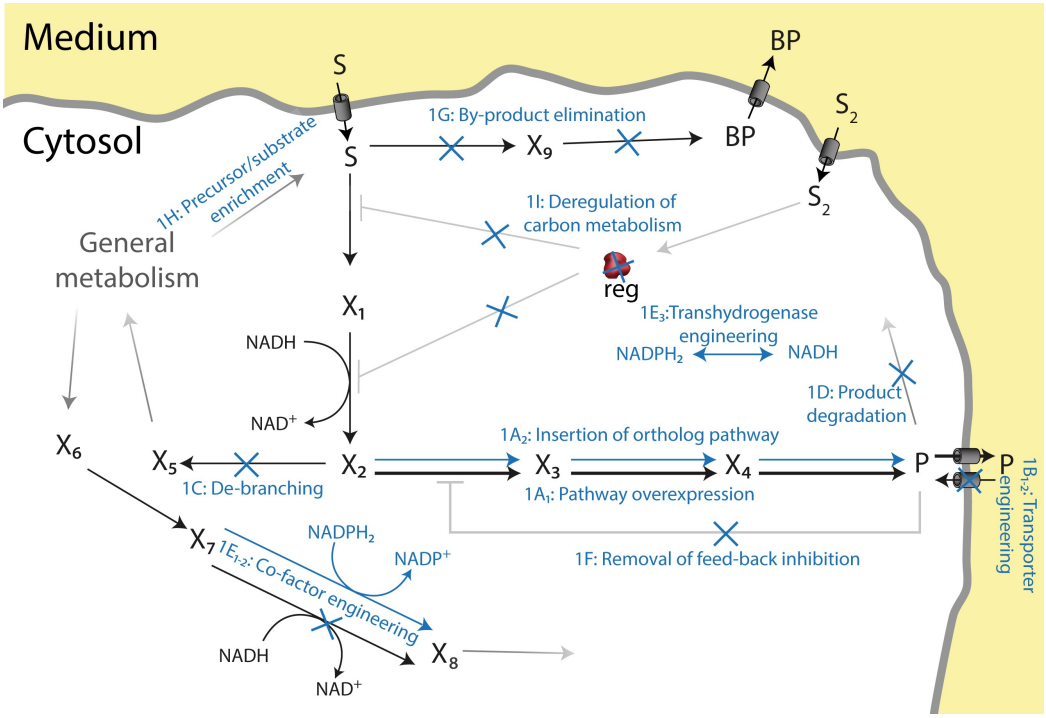



**Figure 2**. Engineering strategies for optimizing the production of native metabolites. Metabolites are denoted S: Substrate. $S_2$: Alternative substrate, $X_{1\text{-}9}$: Pathway intermediates, P: Product of interest, BP: By-product. Generic engineering strategies are marked in blue and annotated as **1A-1I** (described in the main text).

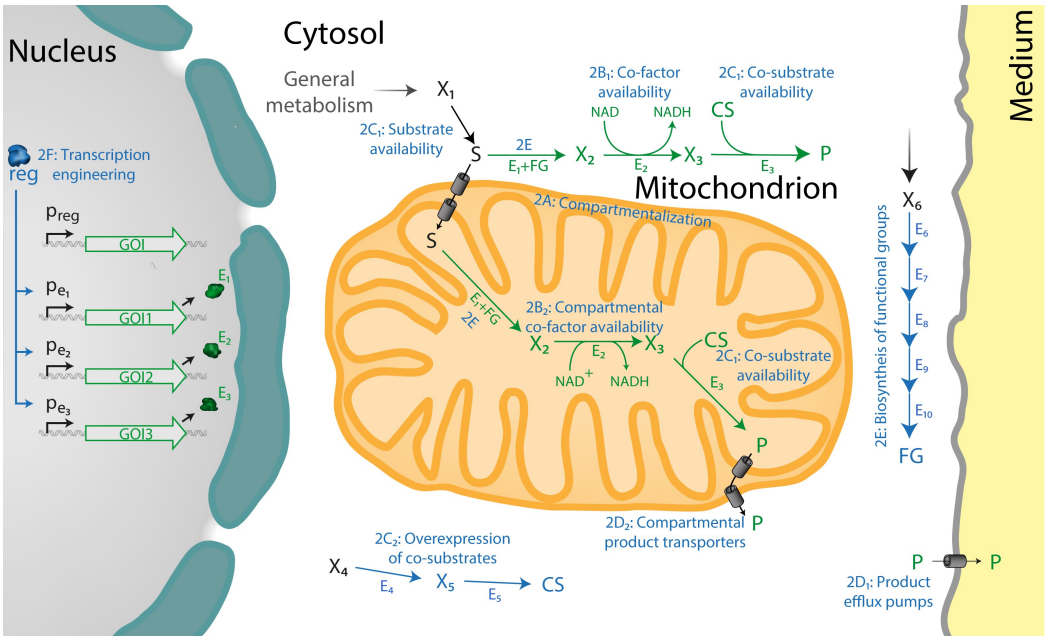



**Figure 3. E**ngineering strategies for heterologous expression of biosynthetic pathways in a (eukaryotic) host cell. Metabolites are denoted S: Substrate, $X_{1\text{-}6}$: Pathway intermediates, P: Product of interest, CS: Co-substrate, $E_{1\text{-}10}$: Enzymes, FG: Functional group. The inserted heterologous pathway is marked in green. Generic engineering strategies are boxed in blue and annotated as **2A-2F** (described in the main text).

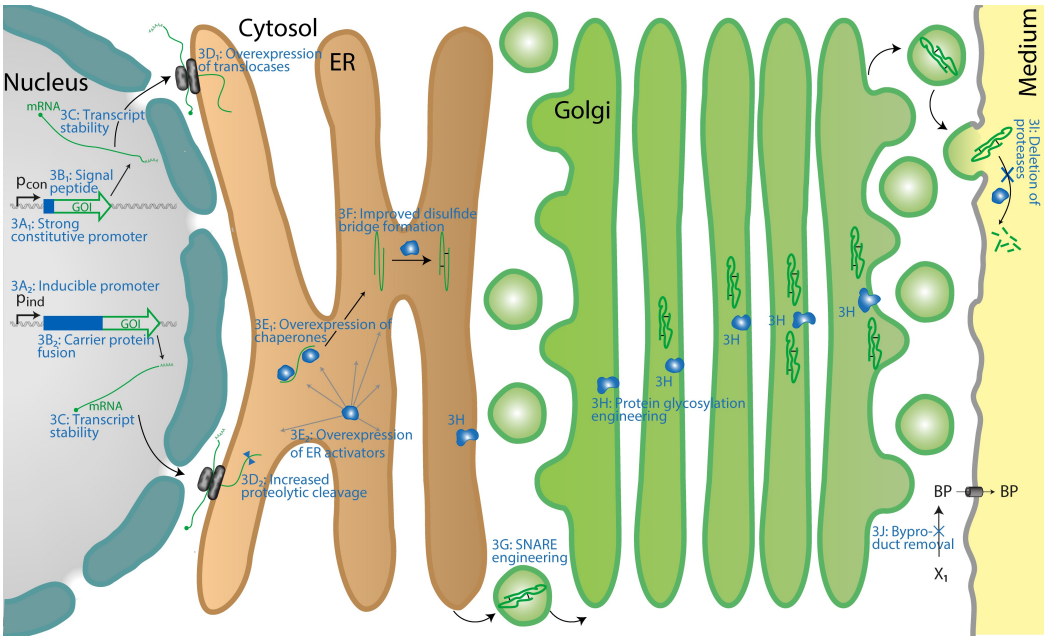



**Figure 4.** Overview of generic engineering strategies for expression of proteins in a (eukaryotic) host cell. The inserted gene and its derived mRNA and polypeptide are marked in green. Generic engineering strategies are marked in blue and annotated as **3A-3J** (described in the main text).