

# Cell Factory Engineering

Anne Mathilde Davy,<sup>1</sup> Helene Fastrup Kildegaard,<sup>2</sup> and Mikael Rørdam Andersen<sup>1,\*</sup>

<sup>1</sup>Department of Biotechnology and Biomedicine

<sup>2</sup>The Novo Nordisk Foundation Center for Biosustainability

Technical University of Denmark, 2800 Kgs. Lyngby, Denmark

\*Correspondence: [mr@bio.dtu.dk](mailto:mr@bio.dtu.dk)

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Rational approaches to modifying cells to make molecules of interest are of substantial economic and scientific interest. Most of these efforts aim 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 meta-review 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 meta-review 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 USD/year on the global markets: pharmaceutical proteins have been estimated at 140 billion USD in 2013 (Walsh, 2014); industrial enzymes in the range of 1.8 billion USD in 2009 (Waegeman and Soetaert, 2011); bio-derived non-protein pharmaceuticals ~100 billion USD (Chemier et al., 2009); and bulk biochemicals (excluding biofuels) 58 billion USD (Nieuwenhuizen and 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 (1) inverse metabolic engineering (Bailey et al., 2002), in which one starts with the desired phenotype and works toward that goal by directed genetic or environmental manipulation, and (2) systems metabolic engineering as coined by the group of Sang-Yup Lee (Lee et al., 2007; Lee and 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 meta-review defines cell factory engineering, encompassing all rational approaches to improve a cell factory.

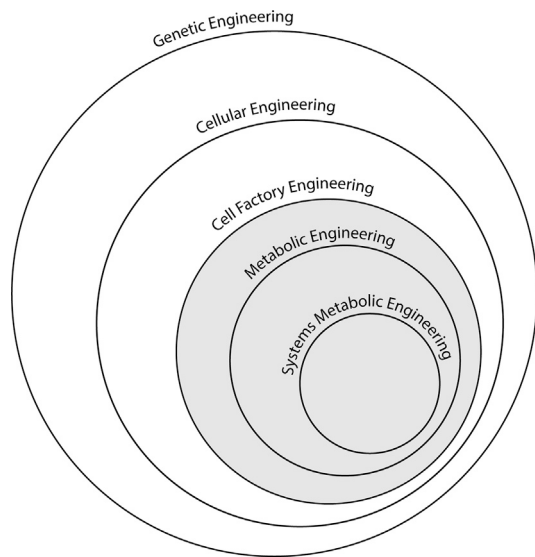
The objective of this meta-review is specifically not to present a comprehensive list of examples within the individual strategies, nor is it to present direct strategies for target identification, such as modeling in tandem with predictive algorithms (Ranganathan et al., 2010; Burgard et al., 2003; Pharkya and 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 and Kim, 2015). In this text, we 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 Chinese hamster ovary [CHO] cells), as well as some higher fungi. See also Box 1 for an overview of cell factory engineering methods in other fields. This meta-review provides 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





**Figure 1. Ontology of Different Types of Cellular Engineering Covered in This Review**

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 and 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 and Vallino, 1991; Keasling, 2008, 2012; Hwang et al., 2014; Wu et al., 2014; Weber et al., 2015; Kiel et al., 2010; Xiao and Zhong, 2016). Here, we 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–J).

**1A<sub>1-2</sub>. 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<sub>1</sub>). 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 anal-

ysis (Nielsen, 1998) should be employed. It has also been seen that heterologous expression of ortholog enzymes from related species (1A<sub>2</sub>) 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 tricarboxylic acid cycle enzymes from *Saccharomyces cerevisiae* and *Rhizopus oryzae* (de Jongh and Nielsen, 2008), or improved ganoderic acid accumulation in *Ganoderma lucidum* (Xu et al., 2012).

**1B<sub>1-2</sub>. 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<sub>1</sub>) (Dunlop et al., 2011; Wu et al., 2014; Lee et al., 2012; Lee and 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<sub>1</sub>, gene knockouts of uptake transporters specific for the product (1B<sub>2</sub>) can also be effective (Lee et al., 2012).

**1C. De-branching:** Branching or competing pathways can decrease the overall flux toward the product (Pickens et al., 2011; Lee et al., 2012; Pfeleger 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 knockdown or, e.g., tunable promoters can be an alternative option. This is a common strategy; one comprehensive example includes the knockout 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 that convert 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. (2007), where threonine dehydrogenase was deleted in *E. coli* to increase the production of L-threonine.

**1E<sub>1-3</sub>. Co-factor engineering:** In some cases, it has been shown that a major limitation is the availability of co-factors (NADH/NAD<sup>+</sup>, NADPH<sub>2</sub>, NADP<sup>+</sup>, acetyl-CoA, etc.) (Lee and Kim, 2015; Ghosh et al., 2011; Lee et al., 2012; Pfeleger 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<sub>1</sub>). 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<sub>2</sub>). An example of this is substitution of a native NADPH-dependent glutamate dehydrogenase with an overexpressed 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 NADPH (1E<sub>3</sub>).

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 and Kim, 2015). In some cases, analogs of the product, which bind 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 and 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 by-products. Often these by-products, 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 and 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 acetyl-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 de-repression 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 pathway that shares intermediates or precursors 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, co-factors, 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 in several systems, in particular in 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



**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 function, 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 often applied in generating reagents for research. Examples include 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 nuclear magnetic resonance spectroscopists (Edwards et al., 2000). In addition, the products from cell factory engineering can be applied in screening for drug activity or as a potential drug target for medical applications (Trosset and 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).

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 by-products), 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 outweighs 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 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 the following studies: Pickens et al. (2011), Pfeifer and Khosla (2001), Lee et al. (2012), and Xiao and 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 the case where 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 in both *E. coli* (Dueber et al., 2009) and *S. cerevisiae* (Wang and Yu, 2012).

**2B<sub>1-2</sub>. 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<sub>1</sub>), as shown, e.g., in *Streptomyces coelicolor* (Borodina et al., 2008). This may be specific to the compartment (2B<sub>2</sub>). Alternatively, transhydrogenases may be engineered as described in 1E<sub>1-2</sub>.

**2C<sub>1-2</sub>. 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<sub>1</sub>). 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, 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 amounts, it is necessary to insert or overexpress biosynthetic genes for these as well (2C<sub>2</sub>). 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<sub>1-2</sub>. 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 and Khosla, 2001). Passive transport or unspecific transporters may be available, but if this is not the case a specific transporter must be added (2D<sub>1</sub>) 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<sub>2</sub>), 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,



**Table 1. Overview of Reviews Covered in This Text**

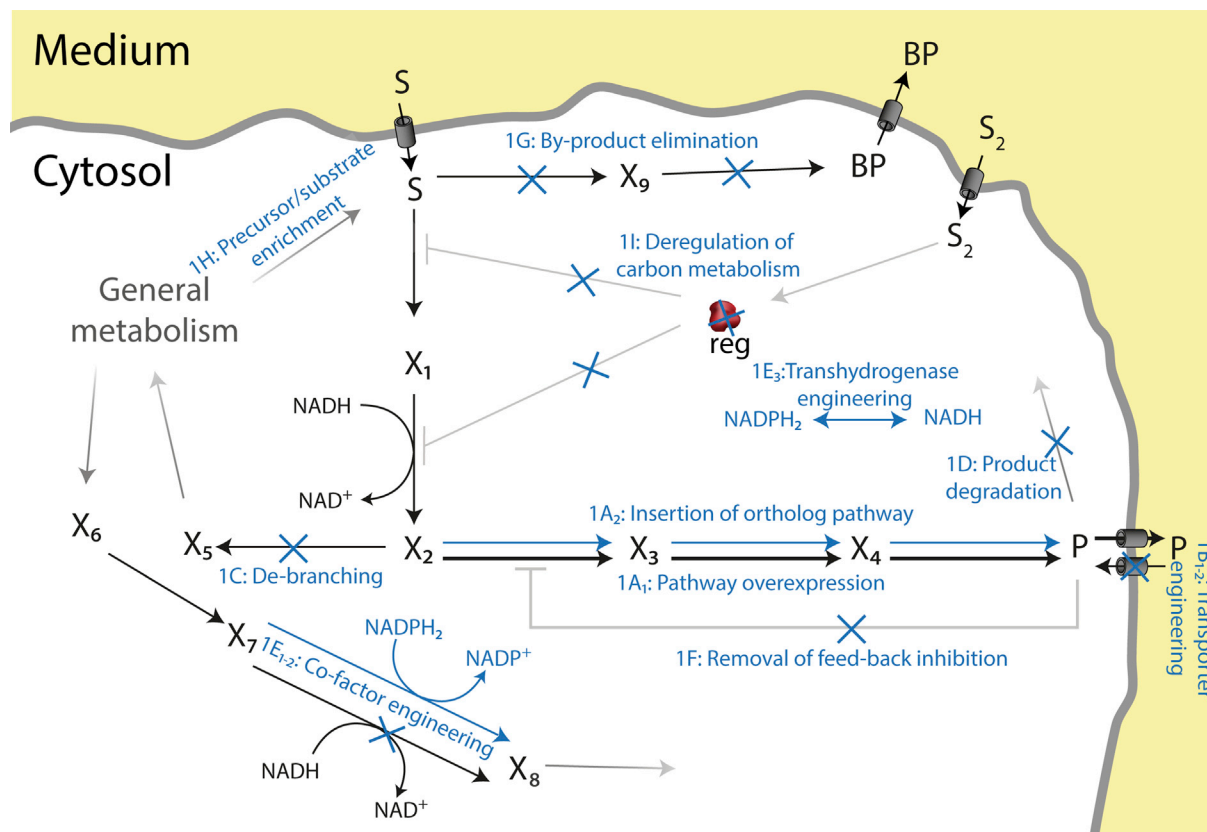
Reference	Species	Strategies	Products	
Walsh (2014)	bacteria, yeasts, fungi, mammalian cells	protein expression	biopharma	*
Waegeman and Soetaert (2011)	<i>E. coli</i>	protein expression	enzymes and biopharma	*
Chemier et al. (2009)	<i>E. coli</i> , <i>S. cerevisiae</i>	native and heterologous pathways	bioactives, biofuels	**
Lee and Kim (2015)	bacteria, yeasts	native and heterologous pathways	any	***
Pickens et al. (2011)	bacteria, fungi	heterologous pathways	bioactives	***
Hwang et al. (2014)	<i>Streptomyces</i> sp.	native pathways	bioactives	**
Wu et al. (2014)	<i>E. coli</i> , <i>S. cerevisiae</i>	heterologous pathways	flavonoids	**
Anyagou and Mortensen (2015)	fungi	heterologous pathways	bioactives	*
Xiao and Zhong (2016)	basidiomycetes	heterologous pathways	terpenoids	*
Nielsen (1998)	bacteria, yeasts, fungi, mammalian cells	all	any	**
van Rossum et al. (2016)	<i>S. cerevisiae</i>	native and heterologous pathways	acetyl-CoA-derived compounds	**
Lee et al. (2012)	<i>E. coli</i>	native and heterologous pathways	small molecules	***
Nielsen (2014)	<i>S. cerevisiae</i>	native and heterologous pathways	acetyl-CoA-derived compounds	*
Pfleger et al. (2015)	<i>E. coli</i> , <i>S. cerevisiae</i>	native and heterologous pathways	oleochemicals	**
Pfeifer and Khosla (2001)	<i>S. coelicolor</i> , <i>E. coli</i> , yeasts, fungi	native and heterologous pathways	polyketides	**
Siddiqui et al. (2012)	yeasts	heterologous pathways	bioactives	***
Franken et al. (2011)	fungi	heterologous pathways	heme	*
Baltz (2016)	actinomycetes	heterologous pathways	bioactives	**
Bekiesch et al. (2016)	actinomycetes	heterologous pathways	bioactives	*
Fisher et al. (2014)	bacteria, yeasts, fungi	heterologous pathways	any	**
Mattanovich et al. (2012)	yeasts	protein expression	biopharma, enzymes	**
Hossler (2012); Hossler et al. (2009)	mammalian cells	protein expression	biopharma	**
Andersen et al. (2011)	mammalian cells	protein expression	biopharma	**
Damasceno et al. (2012)	<i>P. pastoris</i>	protein expression	proteins	***
Celik and Calik (2011)	yeasts	protein expression	proteins	***
Ward (2011)	fungi	protein expression	proteins	***
Wurm (2004)	mammalian cells	protein expression	biopharma	*
Berkmen (2012)	<i>E. coli</i>	protein expression	proteins with disulfide bonds	*
de Marco (2009)	<i>E. coli</i>	protein expression	proteins with disulfide bonds	*
Schröder (2008)	eukaryotes	protein expression	biopharma	**
Fleissner and Dersch (2010)	<i>Aspergillus</i>	protein expression	enzymes	**
Lubertozzi and Keasling (2009)	<i>Aspergillus</i>	native and heterologous pathways, protein expression	small molecules, proteins	*
Vogl et al. (2013)	<i>P. pastoris</i>	protein expression	biopharma	*
Rosano and Ceccarelli (2014)	<i>E. coli</i>	protein expression	proteins	**
Liu et al. (2013)	Gram-positives	protein expression	proteins	**
Gasser et al. (2008)	bacteria, yeasts, fungi	protein expression	proteins	**

Asterisks on the right denote the general relevance of the text for cell factory engineering.

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





**Figure 2. Engineering Strategies for Optimizing the Production of Native Metabolites**

Metabolites are denoted: S, substrate; S<sub>2</sub>, alternative substrate; X<sub>1-9</sub>, 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).

to be functional, it may be required to express the native biosynthesis pathway heterologously (2E). A specific type of Fe-S proteins (ferredoxins) mediates 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 *Aspergillus terreus* into *Aspergillus 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 path-

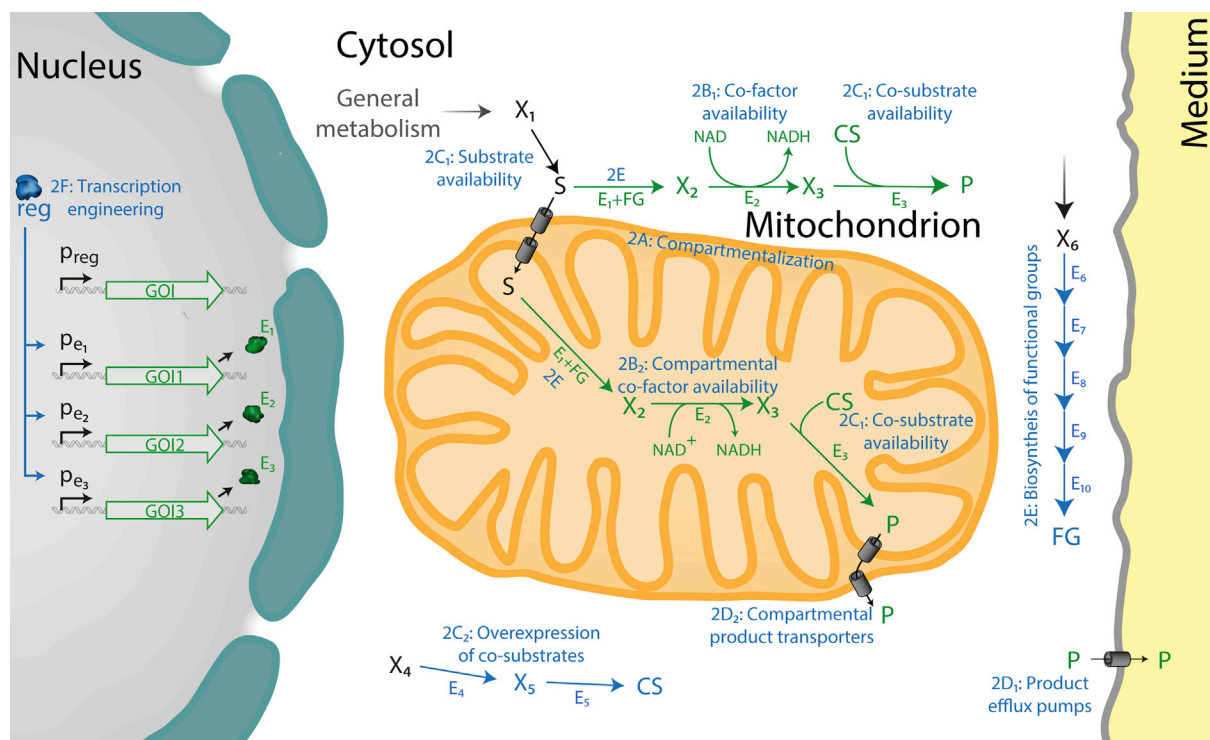
ways, and issues arriving from interaction with the new host. Roughly, the considerations can be sorted into two major steps.

**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 phase to remove or minimize the problems (for a few reviews on host selection, see, e.g., Fisher et al., 2014; Lee and 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:

- Compartmentalization (2A). Spatial co-localization of the inserted enzymes as well as availability of co-factors and precursors in the compartment(s) of choice.
- Functional group biosynthesis (2E). Ensuring functionality of all enzymes.
- 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.





**Figure 3. Engineering Strategies for Heterologous Expression of Biosynthetic Pathways in a Eukaryotic Host Cell**

Metabolites are denoted: S, Substrate;  $X_{1-6}$ , pathway intermediates; P, product of interest; CS, co-substrate;  $E_{1-10}$ , 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).

- Application of transcription engineering where possible (2B). Increased transcription of all enzymatic steps is an efficient way to increase enzyme levels.
- Pathway overexpression strategies (1A<sub>1</sub>) and removal of feedback inhibition (1F) are equally applicable to heterologous pathways.
- 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).
- Improving the product efflux by transporter engineering (2D and 1B).
- Removal of by-products (1G) can possibly be considered last, as the strategies above are more direct toward 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 *Ba-*

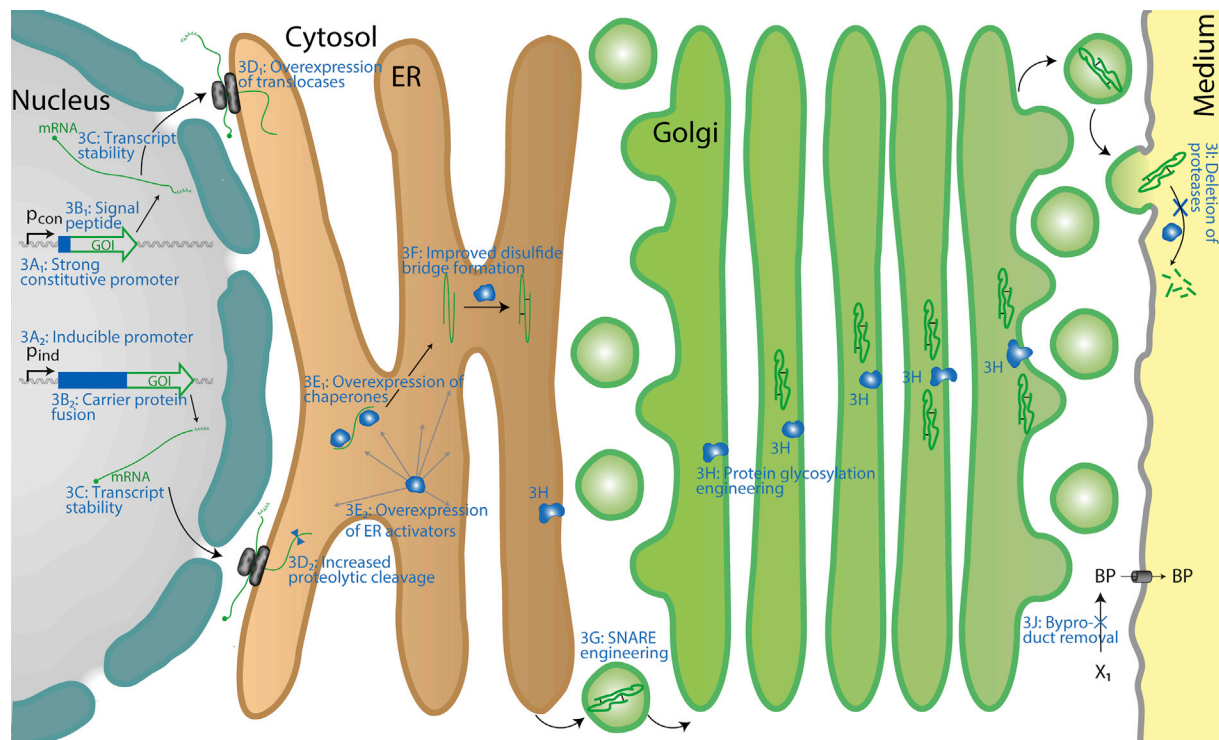
*cillus subtilis*, over yeasts, e.g., *Kluyveromyces lactis*, *Pichia pastoris*, and *S. cerevisiae*, through filamentous fungi such as *A. niger*, to cells derived from multicellular organisms such as mammals and insects. The variety of proteins of commercial interest is great, ranging from bulk enzymes 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 and Soetaert (2011) for a 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 and Soetaert, 2011).

Overall, the successful high-yield process for production of a given protein requires high transcription and translation of the





**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).

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, 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.

**3A<sub>1-2</sub>. 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 (3A<sub>1</sub>). 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 and 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 and Dersch, 2010). Alternatively, one can employ strong inducible promoters (3A<sub>2</sub>) to have a biphasic process (Waegeman and Soetaert, 2011; Fleissner and 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 and Calik, 2011; Fleissner and Dersch, 2010). A complementary strategy to the use of strong promoters is the expression from high-copy plasmids (Rosano and 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 and Dersch, 2010; Westwood et al., 2010).

**3B<sub>1-2</sub>. 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 (3B<sub>1</sub>). 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 and 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<sub>2</sub>), 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 and 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 and 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 a 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<sub>1-2</sub>. Improved Translocation to the Endoplasmic Reticulum:** 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<sub>1</sub>). A successful approach for several systems is overexpression of signal peptidases cleaving the signal peptide by entrance to the endoplasmic reticulum (ER) (Meta et al., 2009; van Dijk et al., 1991; Ailor et al., 1999). Insufficient amount of proteolytic cleavage enzymes may also be limiting for secreted proteins with precursor domains (3D<sub>2</sub>). 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 (Sathiyamurthy et al., 2012).

**3E<sub>1-2</sub>. 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 and Keasling, 2009; Gasser et al., 2008; Schröder, 2008). One generally applied strategy is the overexpression of chaperones (3E<sub>1</sub>). This strategy has been proven to be successful in several studies in a multitude of systems: *E. coli* (Waegeman and Soetaert, 2011; Gasser et al., 2008; Rosano and Ceccarelli, 2014), other bacteria (Gasser et al., 2008), yeasts (Mattanovich et al., 2012; Gasser et al., 2008), fungi (Ward, 2011; Fleissner and Dersch, 2010), and CHO cells (Ailor and Betenbaugh, 1998; Jossé et al., 2012; Pybus et al., 2013). It has also been broadly successful in regulating global activators of the ER or the unfolded protein response (3E<sub>2</sub>), in bacteria (Gasser et al., 2008), *S. cerevisiae* (Valkonen et al., 2003b; Mattanovich et al., 2012; Calfon et al., 2002), in *A. niger* var. *awamori* (Valkonen et al., 2003a; Carvalho et al., 2012; Fleissner and Dersch, 2010), and in mammalian cells (Ohya et al., 2008; Tigges and 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 in many other organisms, such as several yeasts, *Aspergillus* (Gasser et al., 2008; Fleissner and 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 stimulate 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 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 and 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 (Walsh, 2010).

**3I. Protease Deletions:** The deletion of extracellular proteases has been pursued in many systems with significant effects (Ward, 2011; Fleissner and Dersch, 2010). Examples include the deletion of all 25 known proteases in *E. coli* (Meerman and 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 and Dersch, 2010).

**3J. By-product Removal:** A final general strategy, in all species, is the removal of by-products with negative effect on protein production. Examples include removal of acetate biosynthesis in *E. coli* (Waegeman and Soetaert, 2011), oxalic acid production in *Aspergilli* (Li et al., 2013), or lactate production in CHO cells (Kim and 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 degrade 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 severalfold.
- iv. Protein secretion stress reduction (3E), in particular regarding the formation of disulfide bridges, generally increases product formation.
- v. 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, this is in only 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 meta-review. Once it has been established, one can apply one or more of the following three engineering types:

- Protein glycosylation engineering (3H) is generally very attractive for glycosylated biopharmaceuticals (Walsh, 2014; Ratner, 2014).
- Engineering disulfide 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 bio-based nylon through large-scale engineering in *C. glutamicum* (Kind et al., 2014). We are also now seeing engineering of a 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 improvements in synthetic promoters (Vogl et al., 2013) and, at larger scale, the building of artificial pathways rather than using “simple” heterologous expression. Examples here include the biosynthesis of gastrin (Bai et al., 2016) and the impressive feat of the Smolke Lab to biosynthesize opioids 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 activities and synergies from using multiple species. A recent example employs bacterial consortia for desulfurization of oil-based fuels (Martinez et al., 2016), thus improving the quality.

Even so, there are still significant problems that 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 is 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 toward 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 (Jakociūnas 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.

## AUTHOR CONTRIBUTIONS

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



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