Detection and elimination of cellular bottlenecks in protein producing yeasts

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Abstract

Yeasts are efficient cell factories and are commonly used for the production of recombinant proteins for biopharmaceutical and industrial purposes. For such products high levels of correctly folded proteins are needed, which sometimes requires improvement and engineering of the expression system. The article summarizes major breakthroughs that led to the efficient use of yeasts as production platforms and reviews bottlenecks occurring during protein production. Special focus is given to the metabolic impact of protein production. Furthermore, strategies that were shown to enhance secretion of recombinant proteins in different yeast species, are presented.

Keywords:

Yeasts, protein production, secretion, chaperones, protein degradation, metabolism, promoters

Introduction

Yeasts are efficient hosts for the production of recombinant proteins. Since the first product was approved in the 1980s, the repertoire of yeasts used as production hosts has expanded. Today, in addition to *Saccharomyces cerevisiae*, research and industry also applies other species including the methylotrophic yeasts *Pichia pastoris* (syn. *Komagataella* spp) and *Hansenula polymorpha* (syn. *Ogataea polymorpha* and *O. parapolymorpha*), the oleagineous yeast *Yarrowia lipolytica*, *Kluyveromyces lactis* and fission yeast *Schizosaccharomyces pombe*.

Protein production is regulated at several cellular levels, starting with transcription of the gene of interest and subsequent translation of the mRNA. After translation, post-translational steps, most importantly protein folding and secretion, are taking place. Furthermore a sufficient supply of metabolic precursors and energy is required. Over the years several aspects of protein production have been improved, which are presented in the following.

Early bottlenecks in yeast protein production: transcription and translation

Transcription

With the development of recombinant protein production in general, and in bakers' yeast [1] it has been recognized that transcriptional efficiency, or in other words promoter strength, is a central issue for efficient production. Isolation of promoters from yeast genomes however was difficult at times

when no genome sequence information was available. Therefore in the 1980ies and 1990ies only few promoters were in use, mainly deriving from genes encoding metabolic enzymes, such as alcohol dehydrogenase [1], glyceraldehyde 3-phosphate dehydrogenase [2] or enolase [3]. These promoters are not strictly controllable in a bioprocess, so that regulated promoters were sought after. Two concepts of regulated promoters dominated in the early time of protein production with *S. cerevisiae*, being copper regulated promoters of metallothionein genes (*CUP1-1*, *CUP1-2*, *CRS5*) [4], and promoters regulated by galactose (*GAL1*, *GAL10*) [5]. Both types of promoters are not ideal for efficient protein production. Firstly, they are not among the strongest yeast promoters, and secondly, their regulation and deregulation mechanisms are not ideal for large scale production. *CUP* promoters require the addition of heavy metal ions to the fermentation broth which is not favored on a large scale due to obvious concerns of water pollution. *GAL* promoters, on the other hand, are repressed by glucose so that another, non-repressing carbon source (mostly raffinose) is used for the production phase, which increases media costs considerably.

The isolation of methanol inducible promoters of the alcohol oxidase and dihydroxyacetone synthase genes was a major breakthrough for the development of the *Pichia pastoris* protein production platform [6]. However, even with these strong promoters the achievable recombinant protein levels lag behind the levels of the natively produced proteins. This fact, although party curable by increasing the gene copy number, indicates the consecutive appearance of several bottlenecks in the cellular protein production process, as discussed below.

With the availability of genome sequence data for the production hosts it has become way easier to directly access promoter regions and test them for recombinant protein production. Transcriptome data, providing information on the expression levels and regulation of nearly all genes are a great resource for the identification of new promoters with desired features. Based on DNA microarray data, Stadlmayr et al. [7] identified 24 promoters of *P. pastoris* with distinctly different expression levels. Prielhofer et al. [8] used transcriptomics to identify *P. pastoris* promoters of genes that were both highly expressed in glucose limited fed-batch cultures and strongly downregulated in carbon-rich batch cultures. Later, Prielhofer et al. [9] exploited transcriptional data from 4 different carbon sources [10] to generate a library of promoters and transcriptional terminators that can be used for protein production or for strain engineering purposes (available as Golden*Pi*CS kit at Addgene #1000000133). Vogl et al. [11] used transcriptome data of *P. pastoris* cultures in glucose rich conditions and upon glucose depletion, methanol addition and glucose re-addition, respectively, to identify promoters with different regulatory properties (however with the disadvantage of sampling all consecutively from the same batch cultures). In *S. cerevisiae*, transcriptomics was applied to identify strong and upregulated promoters active on glycerol [12].

Engineering of promoters, that is the modification and/or addition of transcription factor binding sites has been proposed for bacteria and yeasts [13], potentially increasing or decreasing expression strength, as well as a potentially modifying or removing of regulation (reviewed by [14]). Hartner et al. [15] created a library of engineered *P. pastoris AOX1* promoter variants with partly stronger, mostly lower strength and a gradual loss of regulation. Transcriptional engineering was introduced by Ata et al. [16] as a concept to engineer promoter strength both at the level of transcription factor binding sites and tuning the expression of the respective transcription factors, as illustrated with the P. pastoris *TDH3* promoter (PGAP).

Synthetic promoters use many of the elements introduced above for engineered promoters. Leavitt et al. [17] described a hybrid *S. cerevisiae* promoter combined with an engineered transcription factor. The same group advanced this concept by creating synthetic promoters of *Yarrowia lipolytica* by combining disparate upstream activating sequences to a core promoter [18]. Similarly, more synthetic promoters inducible by alternative carbon sources such as erythritol and erythrulose were described for *Y. lipolytica* [19]. By using orthogonal transcription factor binding site/DNA binding domains of bacterial origin, Rantasalo et al. [20] developed a synthetic promoter concept that is nearly independent of the physiology of the yeast cell. Thus, many new yeast promoters have been described in the recent years. It should be noted, however, that most of them have not been tested yet for larger scale production in a bioreactor.

It is well established that increased gene copy numbers can contribute to higher expression levels. In *S. cerevisiae* high copy numbers are usually achieved by using high copy episomal vectors (such as 2µ-derived plasmids), while in many other yeasts genome integration is the method of choice due to the lack of stable episomal plasmids. Methods to increase gene copy number include post-transformational vector amplification (cultivation on increasing selection pressure, commonly used in *P. pastoris* and *H. polymorpha*, [21,22], or integration in native multicopy-loci such as the *Y. lipolytica* Ylt1 retrotransposon or the “zeta” sequences [23,24], or the ribosomal DNA NTS loci for several yeasts [25]. However there are reports that with the increase of gene copy numbers the relative expression levels per copy may decrease, which is probably due to a titration effect of transcription factors which are required to activate the respective promoter [26,27].

Translation

Different to bacteria, where translation initiation signals are rather clearly defined, there are no such distinct signals in yeasts. One obvious translation signal is the Kozak sequence that has a (species dependent) consensus sequence [28,9], and is typically added 5’ to a coding sequence in gene

constructs for recombinant expression. Depending on the expression vector used either the native Kozak sequence of the applied promoter or a predetermined well performing Kozak sequence are used.

The three dimensional structure of the target gene's mRNA can potentially influence translation, especially when double strand loops can form at the 5' end of the mRNA. The chance of double strand formation can be lowered during sequence design for synthesis of codon optimized genes.

The impact of transcriptional terminators (TTs) on recombinant gene expression has been discussed [29]. In *S. cerevisiae* terminator activity was shown to vary on a genome scale by 70-fold [30]. Expression enhancing terminators increased mRNA and protein levels more than ten-fold in *S. cerevisiae* especially when combined with weaker promoters [31]. So far, such a marked difference has not been observed for different TTs in *P. pastoris* where all tested TTs displayed almost equal efficiency [11,9]. Notably, the commonly used *CYC1*-TT is by far not the best TT in *S. cerevisiae*, *Y. lipolytica* and *P. pastoris*. Apparently, transcription terminators have very conserved features and can be readily transferred from one yeast species to another as exemplified by the fact that synthetic TTs designed for *S. cerevisiae* can also be applied for *Y. lipolytica* [29].

Metabolic limitations in recombinant protein production in yeast

It has long been recognized that high level expression of heterologous proteins has a direct impact on host cells metabolism (also known as metabolic burden, [32]), often negatively affecting growth parameters such as growth rate, biomass yield, and specific substrate consumption rate [33-37], or accumulation of less- or non-producing cell populations [38], thereby limiting the amount of foreign protein that can be produced from the organism. In principle, foreign gene expression leads to an increase in specific transcription and translation, which may become limiting at very high levels due to depletion of precursors and energy. Producing strains may not cope with the additional demand for ATP, NADPH and precursors for *de novo* biosynthesis of amino acids, thus leading to a suboptimal cell fitness and reduced production yields [39]. Amino acid supplementation of growth media has been reported to be beneficial for high level heterologous protein production in yeast, supporting *a priori* the hypothesis of limited supply of precursors [40-42]. Nonetheless, the specific productivities – particularly for secreted proteins – often achieved in yeast systems are rather low (relative to the total cell protein), therefore suggesting that limitations in amino acid synthesis would not be the major bottleneck. Still, the energy demand can be significantly higher for secreted proteins, as folding, glycosylation and secretion are energy-intensive pathways, particularly in terms of NADPH, which is required for disulfide bond formation and alleviating ER oxidative stress [43]. Moreover, cellular stress

responses to unfolded proteins (unfolded protein response, UPR), which are often triggered upon overexpression of a secreted recombinant protein, further increase the metabolic demand of the folding and secretory processes. Indeed, a metabolic burden related to protein secretion has been observed in yeast, even at low to medium expression levels [44-46]. In this context, it is also important to note that large-scale production of heterologous proteins in yeast is often carried out in high-cell density cultivations operated in fed-batch mode. Such processes are typically performed at low growth rate when operated under substrate limiting conditions. The effects of a low growth rate have a big impact on the cell physiology and, consequently, on the specific productivity [47,48], as growth rate regulates core processes such as protein synthesis and secretion, as well as stress response [49]. Cellular responses such as nutrient starvation may also be elicited, thereby contributing to the metabolic stress of the host cells. Notably, a substantial part of the substrate carbon is expended to meet maintenance-energy requirements under growth-limiting conditions such as those found in fed-batch processes. High maintenance requirements go at the expense of biomass and product formation and therefore are not desired in heterologous protein production [47].

Over the past 15 years, several physiological studies have brought new insights on the impact of recombinant protein production on the metabolic network operation of yeast. Global analysis of the host cell metabolism by means of omics analytical platforms such as transcriptomics, proteomics, metabolomics and fluxomics can now be used to investigate the physiological effect of both environmental stresses and recombinant biosynthesis, guiding the identification of potential metabolic targets for cell engineering, selection of growth conditions and cultivation strategies favouring recombinant protein production. In particular, ^{13}C-based metabolic flux analysis (^{13}C-MFA) and metabolomics studies have revealed a significant impact of synthesis and secretion of heterologous proteins on energy metabolism, resulting in altered metabolic flux distributions through the central carbon metabolism of yeast (reviewed in Ferrer and Albiol, [50]; Klein et al., [51]).

The effect of different recombinant protein production levels was assessed with ^{13}C-MFA using a series of *P. pastoris* strains producing a model protein intracellularly (a bacterial β-aminopeptidase) in glucose-based fed-batch cultures, achieving up to 2 g/L of β-aminopeptidase [35,40]. Using a strain expressing an intracellular recombinant protein enabled to discard a potential metabolic burden related to protein secretion. The recombinant strain showed an increased relative flux through the TCA cycle compared to the reference strain, resulting in significantly increased NADH and ATP regeneration rates. In addition, the recombinant strain showed a slight decrease in the biomass yield compared to the reference strain, which correlated with a lower pentose phosphate pathway (PPP) activity. These studies provided direct evidence for a direct response of *P. pastoris*' metabolic network to recombinant protein production, which could not be explained by the direct resources (in terms of

building blocks) necessary to produce it. This became even more evident in the light of other ^{13}C-based metabolic flux analysis and metabolomics studies on the metabolic burden caused by secretion of a recombinant lipase from *Rhizopus oryzae* (Rol) in *P. pastoris* growing on glucose/methanol mixtures [44,45]. Even though Rol is produced at low levels (mg/L range), MFA allowed the identification of a limited but significant metabolic flux redistribution. Specifically, the flux through the glycolysis, TCA cycle and methanol dissimilatory pathway (generating NADH) were increased in the Rol-producing strains in relation to the reference strain. Remarkably, although the biomass yield of the Rol-producing strains was somewhat lower compared to the reference strain, the flux through the oxidative branch of the PPP appeared to be constant in all strains. This points at the hypothesis of increased NADPH supply through this pathway in the Rol-producing strains. Such effect could be the indirect consequence of methanol co-assimilation, acting as an auxiliary substrate [52].

Overall, these studies strongly suggest that yeasts compensate the additional resources required for recombinant protein production by redirection of intracellular fluxes resulting in increased energy supply (NADH, ATP) [44,45,35,40]. Nonetheless, it also results in altered redox cofactor state and, specifically, a reduction in NADPH availability, reflected in reduced biomass yields [35,40]. Interestingly, supplementing acetate to glucose or glycerol minimal media of recombinant *Schizosaccharomyces pombe* secreting a model protein (maltase) in aerobic chemostat cultures improved protein secretion. ^{13}C-based MFA revealed that acetate co-feeding allowed for an increased carbon flux through the TCA cycle as well as increased mitochondrial NADPH production [46], i.e. provoking an effect similar as methanol co-feeding in *P. pastoris* [52]. Moreover, a model-based study using a genome-scale metabolic model identified NADPH generating reactions as a major cell engineering target for improved protein production [53,54]. Nocon et al. [53,55] further validated such *in silico* predictions by overexpressing genes coding for enzymes of the oxidative branch of the PPP, obtaining higher productivities in heterologous protein secretion. More recently, Tomàs-Gamisans [56] has demonstrated that the ATP-mediated conversion of NADH to NADPH using a heterologous cytosolic NADH kinase in a recombinant *P. pastoris* strain leads to increased recombinant protein secretion. Notably, such an effect was boosted under hypoxic conditions, where the reduced oxygen availability for electron transport chain leads to cytosolic NADH excess (reflected in a higher NADH/NAD+ ratio, [57]), increasing the flux to NADPH.

Besides NADPH requirements to cope with the ER-stress resulting from protein processing, NADPH is particularly required for biosynthesis of amino acids as building blocks of proteins. This suggests that increased NADPH levels may meet the extra demand for the synthesis of recombinant protein. Heyland et al. [40] showed that metabolically costly (in terms of NADPH requirements) amino acids constitute a bottleneck in the production of β-aminopeptidase in *P. pastoris*. Nonetheless, other

metabolomics studies of *P. pastoris* secreting different model proteins do not provide clear supporting data for this hypothesis [45,58,59].

Interestingly, ^{13}C-based studies of *Aspergillus niger* revealed that production of a recombinant fructofuranosidase induced a significant redistribution of metabolic fluxes enabling an elevated supply of NADPH via activation of the cytosolic pentose phosphate pathway and the mitochondrial malic enzyme, whereas the flux through the TCA cycle was reduced [60]. This common finding (the changing contribution of the PPP and the TCA cycle) in yeast and filamentous fungi, although in opposite directions, points at a general feature of the underlying carbon metabolism, i.e. the metabolic flexibility of fungi to cope with different cellular burdens and environmental perturbations by modulating the fluxes through these pathways.

Enhancing protein folding and secretion

Recombinant proteins can be either produced in the cytosol or secreted to the cell exterior. Secretory production has several benefits including easy purification of the product from the supernatant. For natively secreted products, which constitute a majority of pharmaceutical proteins and industrial enzymes, a correctly processed N-terminus, disulfide bond formation and post-translational modifications can be achieved in yeast production platforms. On their way to the cell exterior, secreted proteins have to traverse the secretory pathway, which poses further possible obstacles to the desired product. Thus, many studies aim at customizing the secretory machinery for high level production. High-level overexpression of heterologous secretory proteins has repeatedly been shown to activate cellular stress response pathways, including the unfolded protein response (UPR) and ER-associated protein degradation (ERAD) (e.g. [61-64]).

Right after synthesis, the recombinant protein may be exposed to the cytosolic environment of the cell. The heat shock response (HSR) is a major cellular process regulating the expression of chaperones and other proteins assisting in protein folding or degradation of heat-denatured proteins in the cytosol. Targeted induction of the HSR by overexpression of its constitutively activated transcriptional regulator Hsf1 led to improved secretion of recombinant proteins in S. *cerevisiae* [65], with larger benefits for the larger of the two tested recombinant proteins (insulin precursor vs alpha-amylase). One possible explanation for the observed secretion-enhancing phenotype was that induction of the HSR reduced ER stress of the producing cells [65]. On the other hand, induction of the UPR by overexpression of the induced version of its transcriptional activator Hac1^{i} enhanced secretion of several recombinant proteins in *S. cerevisiae*, *P. pastoris*, *Y. lipolytica* and other fungal hosts [66-70], which is somehow counterintuitive to the previous study. One possible explanation might be that UPR induction does not

only impact the levels of ER-resident chaperones and foldases [71-73], but also leads to an enlarged ER size [74], thereby diminishing the possibility of protein aggregation in the ER. Indeed, expanding the ER size by deleting the lipid regulator Opi1 in *S. cerevisiae* led to 4-fold higher secretion levels of a recombinant antibody [75]. Strikingly, this positive effect was only observed for complex glycosylated mammalian proteins, while the secretion of yeast endogenous proteins was rather decreased in the Δ*opi1* strain [76]. ER size and membrane composition were also reported to be a bottleneck for membrane protein production, and could be overcome by redirecting the flux from storage lipids to (ER) membrane proliferation in *Y. lipolytica* [77] or humanization of the lipid composition in *P. pastoris* [78,79].

For secretory proteins, the first step on the secretory pathway is translocation of the nascent protein into the ER. Recently, insufficient translocation has been described as a potential bottleneck during secretion of a recombinant fluorescent reporter protein in *S. cerevisiae* and *P. pastoris* [80]. A further study in *P. pastoris* revealed that heterologous Fab fragments accumulated prior to translocation and might be degraded by a specific form of ERAD [81]. This specific form of ERAD, termed pre-insertional ERAD, was shown to clears proteins clogging the translocon channel at the cytosolic side [82]. Attempts to engineer protein translocation in *S. cerevisiae* showed that overexpressing folding and translocation aiding factors improved recombinant protein secretion [83], yet the effect depended on both, signal peptides and product proteins [84].

Once inside the ER, the nascent proteins need to be correctly folded and post-translationally modified. Several ER resident chaperones and foldases involved in disulfide bond formation and peptidyl-prolyl isomerization are acting in close interplay with the glycosylation machinery to achieve these tasks. Especially disulfide bond formation has been reported as rate limiting step, thus many attempts to improve secretory protein production relied on overexpression of protein disulfide isomerase Pdi1, either alone or together with its oxidase Ero1 (reviewed by [85-88]). By this approach higher titers could be obtained for several different recombinant proteins in several different yeast species including *S. cerevisiae*, *P. pastoris* and *K. lactis*. Pdi1 overexpression was also combined with the overexpression of ER chaperones such as binding protein Kar2 (reviewed by [85-88]). Interestingly, there was no evidence that beneficial combinatorial or even synergistic effects can be discovered by simultaneous overexpression of two or more chaperone genes [75,76].

More recently, overexpression peptidyl-prolyl isomerase Cpr5 (either from *S. cerevisiae* or from human origin) was reported to increase secretion of IgG, which are proline-rich recombinant proteins and

require cis-trans isomerization of at least one of these prolines in order to attain the correct Ig-folds [75].

The expression of secretory recombinant proteins often triggers the UPR [89] and this, in turn, is supposed to be linked to the ERAD system [90]. The significance of intracellular degradation was shown repeatedly, and Pfeffer et al. [91] determined the amount of antibody fragment lost in this way to be 58%. Due to this and the major role of the UPR, the ERAD complex emerged as strain engineering target. ERAD was disrupted in antibody producing *S. cerevisiae* by deleting the genes *HTM1*, *YOS9*, *HRD1*, *HRD3*, or *UBC7* [92]. The increase in antibody secretion was, if at all, only very minor compared to e.g. folding helper overexpression. Similar effects were observed in *P. pastoris* when disrupting *HRD3*, *DER1*, *RPN4* or *DOA1* [81]. Together these results indicate that removal of the recombinant protein from the ER does not seem to be a major limitation.

After successful folding and ER-quality control, the recombinant protein travels to the Golgi apparatus in COPII vesicles. Stimulating ER exit and COPII formation by overexpression of Sec16 (but not other factors) enhanced the secretion of 3 model proteins in *S. cerevisiae*, but also led to a depletion of ER membrane [93]. The latter could be rescued by simultaneously overexpressing a component of the retrograde transport process, Glo3, which is involved in trafficking of COPI vesicles from the Golgi to the ER [94]. In contrast to *S. cerevisiae*, overexpression of *SEC16* in *P. pastoris* does not increase the number of ER exit sites [95]. Another approach to increase the rate of anterograde transport is to overexpress components of the vesicle fusion machinery including SNAREs and Sec1/Munc18 (SM) proteins which are required for membrane-specific fusion events during protein trafficking [87,96,97]. Based on several studies in *S. cerevisiae* it can be concluded that the effects of overexpressing ER-to-Golgi SNAREs and SM proteins seems to be rather specific for the recombinant protein to be produced. Besides ER-to-Golgi transport, strengthening Golgi-to-plasma membrane (PM) transport may have a more general positive effect on secretion of several model proteins. For example, overexpression of ER-to-Golgi SM Sly1 increased only the production of an α-amylase, but not insulin precursor, whereas the Golgi-to-PM SM Sec1 increased secretion of both recombinant proteins and also the secretion of the endogenous enzyme invertase [98]. Similarly, the overexpression of exocytic SNAREs such as Sso1/2 or Snc2 enhanced the secretion of α-amylase and several cellulolytic enzymes [99-102], whereas the single or concerted overexpression of ER-to-Golgi SNARE components had contrary effects on their secretion [102,103]. Apart from the mostly beneficial impact of syntaxin (Sso1/2) overexpression (which enhances secretion on average by 10-50%), there is no clear picture which component of the vesicle fusion machinery is most rate-limiting based on these studies.

In the Golgi, further post-translational modifications (such as elongation of glycans and proteolytic processing) take place, and the proteins are sorted towards their final destination. For recombinant

proteins, especially the cleavage of the secretion leader by trans-Golgi endoprotease Kex2 was described as rate-limiting step that could be overcome by overexpression of Kex2 or a truncated variant thereof [104-106]. Another interesting aspect relates to the Golgi localized Ca^{2+}/Mn^{2+}-ATPase Pmr1, which is responsible for Ca^{2+} and Mn^{2+} import into the secretory pathway [107]. Disruption of Pmr1 alters calcium homeostasis in the secretory organelles and leads to incomplete outer-chain glycosylation. Nevertheless, *pmr1Δ* mutants have been described to display a "super-secretory" phenotype for several heterologous proteins in *S. cerevisiae* and *K. lactis* [108,109,100,110,111]. The deletion likely causes a Ca^{2+} decrease in the ER, which in turn facilitates an ATP-dependent dissociation of BiP (Kar2) from the substrate. However, also several other cellular functions are affected by *pmr1Δ*. To overcome the reduced growth rate and low viability of the *pmr1Δ* strains, extracellular Ca^{2+} should be added to the growth media. Extracellular Ca^{2+} addition also (at least partially) rescues the glycosylation defect in *S. cerevisiae pmr1Δ*, but not in *K. lactis pmr1Δ* [111,107], which makes the latter an interesting alternative when hyper-glycosylation is supposed to be prevented. In contrast, alternating results regarding protein secretion were obtained in *H. polymorpha, P. pastoris* and *Y. lipolytica* disrupted for *PMR1*, which might be partly attributed to a reduced viability of the strains thus eventually masking the positive effects on secretion [112-115]. Furthermore, it was recently demonstrated that there are also Pmr1-independent routes of Ca^{2+} delivery to the secretory organelles operating in *H. polymorpha* [116].

Even though the default pathway for recombinant proteins should be towards the cell exterior, the next possible major branch-off on the secretory pathway is missorting from the Golgi to the vacuole and subsequent degradation. Yeast cells have a quality control system in which the vacuolar sorting receptor Vps10 targets misfolded proteins from the Golgi to the vacuole. By mutating Vps10, Fitzgerald and Glick [80] could prevent accumulation of msGFP in the vacuole of *S. cerevisiae*. *VPS10* deletion also increased the secretion of other recombinant proteins in several yeast species (*H. polymorpha* [117], *S. cerevisiae* [100] and *S. pombe* [118]. Kitagawa et al. found that the deletion of genes encoding subunits of vacuole protein sorting complexes such as *VPS3*, *VPS16*, *YPT7* and *VPS41* enhanced the secretion of recombinant endoglucanase in *S. cerevisiae*. Especially, the *VPS3* deletion increased secretion of all tested reporter proteins, highlighting its general importance [119]. High-throughput screening for enhanced IgG secretion revealed *VPS30* as potential bottleneck, its disruption enhanced IgG secretion or the secretion of acid phosphatase about 2-fold [120]. Also in the methylotrophic yeasts *H. polymorpha* and *P. pastoris*, disruption of *VPS* genes proved to be beneficial for secretory protein production [117,121]. In fission yeast *S. pombe*, the knockout of several vacuolar protease genes was required to enhance productivities REF [118]. Yapsins are a family of proteases located in the late

secretory pathway or at the cell surface of yeasts [122] and can thus harm even correctly folded recombinant proteins during the late steps of secretion and excretion. Disruption of yapsins has been successfully attempted to reduce proteolysis of degradation prone products such as collagen-polymers, human parathyroid hormone hPTH, human pre-elafin, or human serum albumin (HSA)-fusion proteins, while no effect was observed for other more stable recombinant proteins [123-128].

While there are many reports indicating that vacuolar protein sorting is posing a limitation in recombinant protein secretion, there is no clear teaching to which of the *VPS* genes should be deleted. While some explanations exist why deletion of the receptor Vps10 prevents missorting of specific recombinant proteins, the beneficial effects of most other vps mutants were found by high-throughput screening for enhanced secretion [120,119,129,117]. Furthermore, it should be considered that some vps mutants also over-secrete vacuolar proteases, which can be harmful to the secreted product. Proteolysis caused by the mis-sorted vacuolar proteases may even mask the positive effect of preventing vacuolar transport of the recombinant protein, as reported by Marsalek et al. [121]. Only the combined disruption of Vps8 or Vps21 with vacuolar proteases such as Pep4 or Prb1 led to increased titers of secreted product.

The final step on the secretory pathway is the exocytic fusion of secretory vesicles with the plasma membrane, representing another possible bottleneck and therefore strain engineering target. Early work indicated the beneficial effect of overexpression of Sso1/2 on secretion of heterologous α-amylase and endogenous invertase in *S. cerevisiae* [101] and antibody Fab fragments in *P. pastoris* [130]. Also overexpression of Sec4, which is required for vesicle-mediated exocytic secretion, yielded enhanced secretion in both yeast species [131,132]. The overexpression of components of the exocytic SNARE complex (Snc1/2, Sso1/2 and Sec9) in *S. cerevisiae* could improve the secretion of cellulolytic reporter proteins [99]. However, not all single gene overexpressions could improve secretion and the simultaneous overexpression of several components turned out to be product specific, yielding different best performing combinations. Along with SNARE component overexpression, Xu et al. obtained their best performing quadruple-modified strain (*vps10Δ*/*pmr1Δ*/*SSO1*/*PDI1*/*cel7AF*), by combing several targets in the secretory pathway [100]. Interestingly, while all their *pmr1Δ* strains showed enhanced secretion characteristics for the recombinant cellulase, no impact on invertase secretion was observed.

After being secreted from the plasma membrane, the recombinant protein will still have to diffuse through the cell wall, which may or may not be a barrier [133]. Especially, in the case of accumulation of the recombinant protein in this cell wall-bounded periplasmic space, endocytosis may significantly

decrease productivity. Along this line of evidence, the concentration of α-amylase in the supernatant could be increased by conditional knock-down of *RVS161* and *END3*. However, no effect could be observed on the secretion of recombinant insulin [134].

Another approach to lessen the cell wall barrier is to disrupt cell-wall crosslinking proteins. Knock out of the major cell wall beta-1,3-glucanosyltransferase Gas1 has been shown to enhance the secretion of some recombinant proteins in several different yeasts [135-138]. Furthermore, Larsen et al. reported the positive impact of disruption of cell-wall related genes when screening for enhanced β-galactosidase secretion in *P. pastoris* [139].

Interestingly, some bottlenecks seem to be host-related rather than protein-specific. It was reported that human parathyroid hormone (hPTH) production required the inhibition or disruption of extracellular proteases in *S. cerevisiae* [140], with disruption of all five yapsins (quintuple disruptant *yps1*Δ *yps2*Δ *yps3*Δ *yps6*Δ *yps7*Δ) giving the best effects in fed batch cultivations [124]. In contrast, reducing proteolytic activity was not required for hPTH production in *P. pastoris* [141], but overexpression of *PDI1* proved to be beneficial.

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