THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Engineering Yeast for the Production of Biologicals

Veronica Johanna Marie Gast

Department of Biology and Biological Engineering

CHALMERS UNIVERSITY OF TECHNOLOGY

Gothenburg, Sweden 2022

Engineering Yeast for the Production of Biologicals

Veronica Johanna Marie Gast
ISBN 978-91-7905-638-4

Doktorsavhandlingar vid Chalmers tekniska högskola
Ny serie nr : 5104

ISSN 0346-718X

Department of Biology and Biological Engineering
Chalmers University of Technology
SE-412 96 Gothenburg
Sweden
Telephone + 46 (0)31-772 1000

Cover design: Three strategies employed to improve the production of biologicals by *Saccharomyces cerevisiae.*

Printed by Chalmers Reproservice
Gothenburg, Sweden 2022

Engineering Yeast for the Production of Biologicals

Veronica Johanna Marie Gast

Department of Biology and Biological Engineering

CHALMERS UNIVERSITY OF TECHNOLOGY

The market for biopharmaceutical proteins, or biologicals, has been expanding rapidly over the last decades and its value was estimated in 2020 to exceed 300 billion US dollars. Efficient cell factories that produce the biologicals fulfill an essential role within this industry. Around 20% of the current biologicals are produced in the yeast species *Saccharomyces cerevisiae*. In this thesis, I focus on the engineering of *S. cerevisiae* as a cell factory for biologicals; Affibody molecules, filgrastim, adalimumab, and insulin precursor. The first strategy focuses on the role of the eIF2α kinase Gcn2 in *S. cerevisiae*. Upon removal of the kinase Gcn2 we showed effectiveness to improve the production of the model protein α-amylase and performed initial experiments on the influence of the removal of the kinase Gcn2 on the production of adalimumab. Our results indicate a novel role of the eIF2α kinase Gcn2 in *S. cerevisiae*. Secondly, I focused on the removal of vacuolar proteases from *S. cerevisiae*. The proteolytic degradation of recombinant proteins by yeast is a known phenomenon that reduces production yield. I identified and removed the specific proteases that degrade the synthetic biologicals, Affibody molecules, which resulted in the production of intact and functional Affibody molecules and I concluded the study with a high production experiment. Additionally, I removed the severe degradation phenotype of a previously engineered *S. cerevisiae* strain and implemented that strain for the production of filgrastim and adalimumab. As a final strategy, I used two proteome constrained genome-scale models of *S. cerevisiae* as engineering guides. One model, ecYeast8, suggested overexpression targets that combined into one strain improved the titers of filgrastim, adalimumab, and insulin precursor. The other model pcSecYeast proved effective to improve insulin precursor and resulted in a 10-fold increase of final insulin precursor concentration. The results presented in this thesis will contribute to the improvement of *S. cerevisiae* as a production host for biologicals and other recombinant proteins.

Keywords: Recombinant protein production, *Saccharomyces cerevisiae,* biopharmaceuticals, biologicals, kinase Gcn2, proteases, genome-scale models.

List of publications.

This thesis is based on the work contained in the following papers.

I. The yeast eIF2 kinase Gcn2 facilitates H_2O_2-mediated feedback inhibition of both protein synthesis and endoplasmic reticulum oxidative folding during recombinant protein production.
Veronica Gast, Kate Campbell, Cecilia Picazo, Martin Engqvist, Verena Siewers, and Mikael Molin. (2021) *Applied and Environmental Microbiology* 87(15) e0030-21 1-16

II. A hypersensitive genetically encoded fluorescent indicator (roGFP2-Prx1) enables continuous measurement of intracellular H_2O_2 during cell micro-cultivation.
Veronica Gast, Verena Siewers and Mikael Molin. (2022) *Bioprotocols* 12 (3) e4317 1-12

III. Engineering *Saccharomyces cerevisiae* for the production and secretion of Affibody molecules.
Veronica Gast, Anna Sandgren, Finn Dunås, Siri Ekblad, Rezan Güler, Staffan Thorén, Marta Tous Mohedano, Mikael Molin, Martin K M Engqvist and Verena Siewers. (2022) *Microbial Cell Factories* 21 (36) 1-15

IV. Improving the production of biologicals in *Saccharomyces cerevisiae* by overexpressing native target genes predicted by two proteome constrained genome-scale models.
Veronica Gast, Feiran Li, Iván Domenzain, Mikael Molin and Verena Siewers. *Manuscript*

Contribution summary.

I. Designed the study, performed most of the experiments, analyzed the data, and wrote the paper.
II. Designed the study, performed all experiments, analyzed the data, and wrote the paper.
III. Co-designed the study, performed most of the experiments, analyzed the data, and wrote the paper.
IV. Designed the study, performed all wet-lab experiments, analyzed the data, and wrote the manuscript.

Preface.

This dissertation is submitted for the partial fulfillment of the degree of Doctor of Philosophy. It is based on the work carried out between January 2018 and April 2022 in the Systems and Synthetic Biology division, Department of Biology and Biological Engineering, Chalmers University of Technology, under the supervision of Research Professor Verena Siewers and Senior Researcher Mikael Molin. Temporary supervisors were Assistant Professor Martin Engqvist and Associate Professor Dina Petranovic Nielsen. This thesis was examined by Professor Ivan Mijakovic. The research was funded by VINNOVA center CellNova (2017-02105).

April 2022.
Veronica Gast.

'When in doubt follow your nose'

Gandalf the Grey, Lord of the Rings

Abbreviations

PCR	Polymerase Chain Reaction
HR	Homologous Recombination
DSB	Double-Stranded Break
PTM	Post Translational Modification
CHO	Chinese Hamster Ovary
SRP	Signal Recognition Particle
ER	Endoplasmic Reticulum
COPII	Coat Protein Complex II
COPI	Coat Protein Complex I
tER	transitional ER
SNARE	Soluble N-ethylmaleimide sensitive factor attachment protein receptor
TRAPPI	Transport protein particle I complex
HDSV	High-Density Sorting Vesicle
LDSV	Low-Density Sorting Vesicle
DCW	Dry Cell Weigth
GEM	Genome-Scale Metabolic model
ABD	Albumin-Binding Domain
ORF	Open Reading Frame
FSEOF	Flux Scanning based on Enforced Objective Flux

Table of Content

1. BACKGROUND 5

1.1 *SACCHAROMYCES CEREVISIAE*. 5
1.2 SYNTHETIC BIOLOGY. 6
1.3 RECOMBINANT PRODUCTION OF BIOLOGICALS. 7
1.4 THE SECRETORY PATHWAY. 9
1.4.1 GENE EXPRESSION AND MRNA TRANSLATION. 9
1.4.2 ENDOPLASMIC RETICULUM. 10
1.4.3 TRANSPORT BETWEEN ER AND GOLGI APPARATUS. 11
1.4.4 GOLGI APPARATUS AND EXOCYTOSIS. 12
1.5 ENGINEERING STRATEGIES TO IMPROVE RECOMBINANT PROTEIN PRODUCTION. 13
1.5.1 SYSTEMS BIOLOGY AS AN ENGINEERING GUIDE. 13

2. RECOMBINANT PROTEIN PRODUCTION AND OXIDATIVE STRESS. 16

2.1 STRESS AND OXIDATIVE STRESS. 16
2.2 BIOSENSORS FOR INTRACELLULAR H_2O_2 MEASUREMENTS. 17
2.3 RECOMBINANT PROTEIN PRODUCTION AND OXIDATIVE STRESS. 19
2.4 THE KINASE GCN2. 20
2.5 THE KINASE GCN2 AND RECOMBINANT PROTEIN PRODUCTION. 21
2.6 IMPACT OF REMOVAL OF GCN2 KINASE ON THE PRODUCTION OF A BIOLOGICAL. 26

3. DEGRADATION OF RECOMBINANT PROTEINS BY PROTEASES. 29

3.1 YEAST PROTEASES. 29
3.2 DEGRADATION OF RECOMBINANT PROTEINS BY PROTEASES. 31
3.3 AFFIBODY MOLECULES. 32
3.4 PRODUCTION OF AFFIBODY MOLECULES BY *S. CEREVISIAE*. 33
3.5 REMOVAL OF PROTEASES FROM *S. CEREVISIAE* TO PRODUCE OTHER BIOLOGICALS. 38

4. APPLICATION OF PROTEOME-CONSTRAINT GENOME-SCALE MODELS (GEMS) FOR CELL FACTORY ENGINEERING. 41

4.1 GENOME SCALE MODELS FOR *S. CEREVISIAE*. 41
4.2 APPLICATION OF TWO YEAST GEMS FOR GUIDED ENGINEERING OF *S. CEREVISIAE* FOR THE OPTIMIZED PRODUCTION OF BIOLOGICALS. 42
4.3 TARGET GENES SELECTED BY THE MODELS. 43
4.4 VERIFICATION OF THE SINGLE OVEREXPRESSION TARGETS. 44
4.5 VERIFICATION OF COMBINED STRAINS. 48

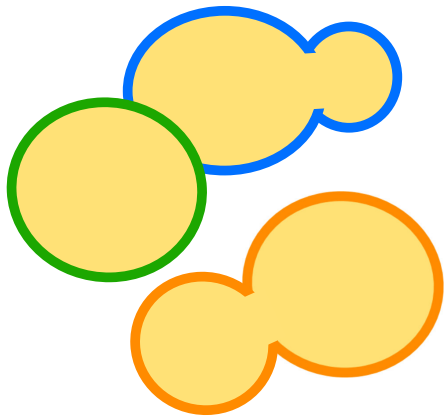
5. CONCLUSION AND PERSPECTIVES 53

5.1 THE KINASE GCN2 AND RECOMBINANT PROTEIN PRODUCTION. 54
5.2 THE REMOVAL OF VACUOLAR PROTEASES FOR YEAST AS A PRODUCTION HOST FOR RECOMBINANT PROTEINS. 56
5.3 THE APPLICATION OF GEMS AS GUIDES FOR THE ENGINEERING OF CELL FACTORIES TO PRODUCE BIOLOGICALS. 58

6. ACKNOWLEGDEMENTS 61

7. REFERENCES. 63

1. Background



1.1 Saccharomyces cerevisiae.

In this thesis, I will describe my research on the production of pharmaceutical recombinant proteins by yeast. Yeasts are unicellular eukaryotic microorganisms, which are part of the fungi kingdom. One of the most popular yeast species and also the production host in this thesis is the yeast species *S. cerevisiae*, also referred to as baker's yeast or budding yeast. *S. cerevisiae* has been a central part of the human application of biotechnology in daily life since 7000 BC (1). Both beer and bread production has been documented by the Egyptians and Babylonians to the extent that different kinds of beers were described. The dominant role of *S. cerevisiae* remained in the bread and beer industry and led to its certification of 'generally regarded as safe' status. Nowadays, this yeast also fulfills a central role within the industrial production of chemicals and pharmaceuticals such as ethanol, artemisinic acid, and insulin precursor (2–4). Alongside its industrial career, *S. cerevisiae* is a model organism for eukaryotic cell biology (5–9). *S. cerevisiae* has 85% percent of its coding genes characterized from which 17% have homologs in humans with roles associated with human diseases (9). Additionally, the genome of *S. cerevisiae* was the first eukaryote genome to be sequenced and also will potentially be the first eukaryotic genome to be completely synthetically constructed (10, 11).

1.2 Synthetic biology.

As mentioned before *S. cerevisiae* is currently employed as a host for the production of artemisinic acid and insulin precursor, which brings us to the use of synthetic biology or more specifically, recombinant DNA technology and engineering of biological systems, *i.e.* bioengineering. The bioengineering field began with the conceptualization of the Central Dogma of molecular Biology, and the direction, in which information can move between DNA, RNA, and proteins (12). The Central Dogma of Molecular Biology is shown in Figure 1. The Central Dogma of Molecular Biology concludes that information can flow from nucleic acid to nucleic acid and protein, but not from protein to nucleic acid. After the conceptualization implementation followed rapidly. The first publication of the application of recombinant DNA technology was in 1973 when antibiotic resistance genes from other bacteria were expressed in *Escherichia coli* (13). Two years later, the first study of the expression of eukaryotic DNA in *E. coli* was published (14).

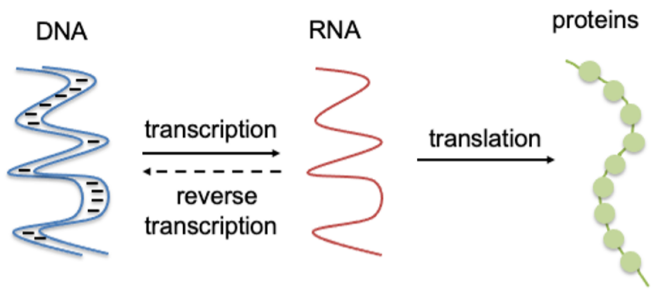


Figure 1. Schematic of the Central Dogma of Molecular Biology.

For yeast bioengineering, the first stable genomic integration was achieved in 1978 (15). After those initial steps within recombinant DNA technology, progress followed rapidly in general with the development of molecular biology techniques, which we still apply broadly today, like the *in vitro* amplification of DNA the polymerase chain reaction (PCR). The PCR technology got major attention since it provided an opportunity for screening human DNA-based diseases and the presence of pathogens in human tissue, like the RT-PCR test for the COVID-19 (16, 17). In the 1990s, large-scale sequencing of DNA emerged and developed in the following 10 years. Developments in technique lowered the costs tremendously and the maximum length of the reads was extended from 35 to 20.000 base pairs. Nowadays whole-genome sequencing is a technique available for the majority of academic laboratories (18). The developments within whole-genome sequencing were feasible due to advances in the data handling (18). Due to the availability of large-scale DNA sequencing, interesting genes within pathways are more easily identified and can be used for the engineering of a host species (19).

Since the first genomic integration in 1978, homologous recombination (HR) played a central role in targeted gene integrations and deletions in *S. cerevisiae*. In the last 15 years, interest increased in endonucleases that can insert double-stranded breaks (DSB) in DNA (19). Especially for species with less efficient HR, these were promising enzymes to improve engineering efficiency. Zinc-finger nucleases and transcription activator-like effector nucleases were initial candidates (19). A more recent discovery was the identification of the bacterial DNA endonuclease known as CRISPR-Cas9. The origin of CRISPR-Cas9 is a defense mechanism of bacteria against viruses that recognizes and removes viral DNA (20). The main advantage of CRISPR-Cas9 compared to the other nucleases was the ability to easily customize the target DNA of the endonuclease. CRISPR-Cas9 improved the effectiveness and elegance of bioengineering rapidly not only in unicellular organisms, but most importantly in human cells, in which homologous recombination had low-efficiency (20–22). Consequently, even for *S. cerevisiae* with its efficient HR machinery CRISPR-Cas9 is currently the go-to genetic engineering technology since it enables the multiplexing of integrations and deletions (19).

1.3 Recombinant production of biologicals.

With the developments within the bioengineering field, new applications arose with it like recombinant production of proteins. In recombinant protein production, an alternative host organism is used to produce a protein of interest. The start of recombinant pharmaceutical protein production was in 1982 when the FDA approved the recombinant production of insulin precursor by *E. coli* (23). Four years later, the first publication on human insulin precursor produced in *S. cerevisiae* appeared which, until this day, remains the dominant industrial host for insulin precursor production. The production of recombinant pharmaceutical proteins, or biologicals, has been an extensively growing market over the last decades. Besides the size, the market is also changing towards the production of more complex proteins like antibodies (24, 25).

In general, the majority of biologicals are produced in bacterial, yeast, insect, or mammalian cells (24–26). All these hosts have advantages and disadvantages. *E. coli* is the dominant bacterium for the production of biopharmaceuticals and around 30% of the biologicals are produced in *E. coli* (27). *E. coli* is a preferred choice since these cells grow the fastest, can reach very high titers, and have low medium requirements (27, 28). A downside of bacterial systems is that they have the tendency to capture the proteins in inclusion bodies (28). Also, bacterial gram-negative species like *E. coli* contain in their cell wall for humans toxic compounds (endotoxins), which are challenging to separate from the proteins (29). Currently. there are regulations in place that require pharmaceuticals to have an endotoxin concentration below a certain threshold to minimize the immune response of the human body (29). Additionally, prokaryotes have fewer post-translational modification (PTM) reactions compared to eukaryotes, which makes some eukaryotic proteins challenging to fold for *E. coli* (27). Nevertheless, over the years, some

of these shortcomings have been compensated by genetic engineering of *E. coli*, like the introduction of glycosylation reactions or the removal of the endotoxins from the cell wall (30, 31). Insect cells are an upcoming alternative stimulated by the developments in the availability of genetic engineering tools. Popular cell lines originate from *Drosphila melanogaster*, *Spodoptera frugiperda* and *Trichoplusia ni*. The cultivation of insect cells is more economic compared to mammalian cells and insect cells have more advanced PTMs compared to yeast and bacteria. The major drawback however remains the time-consuming engineering of the insect cells (32). There has been a clear trend of increasing numbers of approvals of production of biologicals in mammalian cell lines with the increasing number of antibodies entering the market (33). The preference for mammalian cell lines as hosts for antibodies is caused by the glycosylation requirements of antibodies, which impact the efficacy and half-life of the proteins in the human body (34). So far, only human cell lines can reproduce human glycan structures but their productivity remains low. The dominant host for antibody production are Chinese hamster ovary (CHO) cell lines which are engineered to mimic the human-like glycosylation *in vivo* or the produced antibodies will be enzymatically altered *in vitro* before they can be used as pharmaceuticals (25, 34). A downside is that the production costs of mammalian cells are higher compared to all the alternative hosts; therefore, it remains of interest to develop and engineer the lower eukaryotic and prokaryotic organisms further toward the production of biologicals.

Yeasts including *S. cerevisiae* are robust under industrial conditions, have similar post-translational modifications to humans, secrete the proteins, reach relatively high titers and do not require complex medium (26, 35–37). Examples of alternative non-conventional yeasts are the methylotrophic yeasts *Komagataella phaffi* and *Ogataea polymorpha* (38). These yeasts can use methanol as a carbon source and are known to produce high titers of recombinant proteins (39, 40). The downside of these species is that their main strength is also their pitfall since the use of methanol on industrial scale limits the application due to its toxicity and flammable nature (41). An obstacle when using yeast as a production host is the degradation of recombinant proteins by yeast proteases. This topic will be discussed in detail in **Chapter 3**. Another obstacle with yeasts as a production host are their native glycosylation structures, which differ from human glycosylation structures. Particularly *S. cerevisiae* is prone to hypermannosylate proteins, which reduces efficacy and half-life of the proteins in the human body (34). Nevertheless, major progress has been made to humanize N-glycosylation in the yeasts *K. phaffi* and *S. cerevisiae* (24, 34, 42, 43). Currently, *S. cerevisiae* remains the most applied yeast for biopharmaceutical production in the industry (4, 44). An overview of several biologicals produced by *S. cerevisiae* is shown in Table 1 and was based on the overview of Gary Walsh (25).

Table 1. Overview of biologicals produced in industry by *S. cerevisiae* based on the overview of Gary Walsh [25]

Biological	Type	Illness
Insulin	Hormone	Diabetes mellitus
Glucagon	Hormone	Hypoglycemia
Liraglutide	Hormone	Obesity
Albiglutide	Hormone	Diabetes mellitus type 2
Somatropin	Hormone	Growth failure
Liraglutide	Hormone	Diabetes mellitus type 2
HBsAg	Vaccine	Hepatitis B
Combination vaccine	Vaccine	Hepatitis A, Hepatitis B and *H. influenzae*
HPV	Vaccine	Human papillomavirus
VLP	Vaccine	Malaria, Hepatitis B
Hirudin	Blood factors	Venous thrombosis, thrombocytopenia
Factor XIII A-subunit	Blood factors	Congenital factor XIII A-subunit deficiency
GM-CSF	cytokine	Neutropenia
Urate oxidase	Enzyme	Hyperuricemia

1.4 The secretory pathway.

Native and heterologous proteins are synthesized, folded, and secreted by a group of organelles named the secretory pathway. In this subchapter, I will provide an overview of the route proteins pass from their synthesis until secretion, and will mostly focus on the yeast secretory pathway. I will explain the mechanisms and complexity of the secretory pathway and provide background for the studies presented in the upcoming chapters. Additionally, throughout the subchapter previous studies on the improvement of recombinant protein production will be mentioned.

1.4.1 Gene expression and mRNA translation.

The production of proteins starts with the expression of the gene of interest. Often in the case of recombinant protein production, the gene encoding the protein is overexpressed by promoters that are constitutively active or can be induced (45). Several native *S. cerevisiae* promoters are used as promoters in other non-conventional yeasts that often have less defined sets of well-characterized promoters (45). In this thesis, the strong *TPI1* promoter was used to express the majority of the recombinant proteins. In *E. coli*, the overexpression of recombinant protein has been shown to lead to growth retardation partly caused by transcription by removing the ribosomal binding site from the mRNA (46). After transcription, the mRNA will travel to the cytosol where the ribosomes will translate mRNA into a polypeptide chain. Translation is a high resource-consuming process in the cell (47). Translation starts with the assembly of the ribosomal proteins on the mRNA, which is a tightly regulated process that requires at least 24 translation initiation factors (48). Translation can involve co-translocation into the ER which is dependent on a signal sequence emerging on the N-terminus of a polypeptide chain during translation. The signal sequence will bind to the signal recognition particle (SRP) complex blocking translation temporarily while targeting the whole ribosomal complex to the SRP receptor on the cytosolic side of the endoplasmic reticulum (ER) membrane (49). The SRP

complex will be released and translation will continue with the polypeptide chain directly being translocated into the ER by the Sec61 translocon (49). In our studies, we used an N-glycosylated native signal peptide of *S. cerevisiae*, the signal peptide of the α-mating factor for all the proteins (50). This signal peptide mediates post-translational ER-translocalization. Different signal peptides have shown varying effectiveness for different proteins (51). Insulin precursor was for example secreted in higher quantities when using the synthetic *YAP3-TA57* signal peptide instead of the α-factor signal peptide (51). Optimizing the best combination of signal peptide and recombinant protein or the adjustment of the native signal peptide to be effective for a specific protein(s) are both implemented strategies to improve protein production with yeast (52, 53).

1.4.2 Endoplasmic reticulum.

The moment the polypeptide chain enters the ER the folding starts immediately. In the ER, a high amount of proteins and chaperones are present to support the proteins while folding (54). One of the distinct folding mechanisms in the ER is the iterative making and breaking of disulfide bonds within proteins until the correct confirmation is found. This specific folding mechanism is called oxidative folding and will be discussed in more detail in **Chapter 2**. The ER maintains an oxidized environment compared to, for example, the cytosol to stimulate the iterative process of oxidative folding (54). Essential proteins within this mechanism are Pdi1 and Ero1 (55). Another PTM occurring in the ER is glycosylation. The moment the polypeptide chain enters the ER N-glycosylation sites with N-X-T/S sequence can be glycosylated with a tree branch high mannose glycosylation structure (56). The glycan structure will be step-wise trimmed to inform the environment of the folding state of the protein. When the protein is approaching its final state, two competing reactions will either allow the protein to continue folding or mark it for degradation, but it will not enter another round of oxidative folding (56). Besides N-glycosylation, there is also the possibility of O-glycosylation of threonine or serine residues. The O-mannosyl transferases Pmt1, Pmt2, Pmt3, Pmt4 or Pmt6 will attach the initial mannose to T/S (57). The ER is prone to experiencing stress caused by crowding due to high load of (misfolded) proteins. Cells can experience ER and oxidative stress because of ER overload. Oxidative stress and its influence on protein production will be a central topic in **Chapter 2**. There are two mechanisms that can respond to ER stress to regain homeostasis. One of the responses is called the unfolded protein response (UPR) and the other is called ER-associated degradation (ERAD). The UPR will lead to increased expression of ER foldases and chaperones. The central activator of the UPR is the transmembrane protein Ire1. In the ER lumen, Ire1 remains in an inactive form by binding with the folding chaperone Kar2. When an accumulation of unfolded protein occurs Kar2 will dissociate from Ire1 to assist in folding. The dissociation of Kar2 will lead to the activation of Ire1, which will then splice *HAC1* mRNA on the cytosolic ribonuclease domain of the Ire1(58). The spliced mRNA will be translated into the Hac1 transcription factor that will activate the UPR response genes in the nucleus. The UPR is a broad response which involves 381 genes, more than 5% of the ORFs. Among these 381 genes

are several ER folding chaperones including *PDI1*, *ERO1*, *KAR2* but also genes involved in ERAD (49, 59). ERAD is a mechanism that translocates misfolded proteins from the ER to the cytosol to be degraded by the proteasome; additionally, ERAD plays an important role in the inactivation of the UPR (59). The engineering of processes in the ER are popular strategies to improve productivity of cells producing recombinant proteins. Especially the overexpression of foldases like Pdi1. Overxpression of *PDI1* has improved α-amylase production but also has been shown to increase *Schizosaccharomyces pombe* acid phosphatase in *S. cerevisiae* (60, 61). The overexpression of *CWH41*, responsible for the first step in glycan trimming also improved α-amylase production (62), but also the expansion of the ER size by deletion of *OPI1* showed effective for the production of an antibody (63). However, not all intuitive strategies proved successful, for example the overexpression of *KAR2*. In *H. polymorpha* the overexpression of *KAR2* led to a 10-fold reduction of the *Aspergillus niger* glucose oxidase titer. Also in *S. cerevisiae*, overexpression of *KAR2* did not affect the production of *Saccharomycopsis fibuligera* β-glucosidase Bgl1p, *Clostridium thermocellum* endoglucanase CelA and *Aspergillus oryzae* α-amylase whereas in the same study *PDI1* overexpression was effective for increasing production of all three proteins (64, 65).

1.4.3 Transport between ER and Golgi apparatus.

The next step in the secretory pathway is the transport between ER and the Golgi apparatus. This transport operates in both directions. The transport towards the Golgi is mediated by coat protein complex II (COPII) vesicles. The proteins exit the ER through specific ER membrane domains called transitional ER sites (tER). Sec16 and Sec12 fulfill important roles within the organization of the COPII vesicles from the ER membrane. The signal peptide on the (recombinant) proteins leads to the selection of the proteins as cargo. Sec24 and its homologs are responsible for the selection process but Erv29 also plays a role in the case of the α-signal peptide (66, 67). The fusion of the COPII vesicles to the Golgi membrane is organized by the tethering complex TRAPPI and the binding to Sec23 on the COPII vesicle. TRAPPI activates the Rab-family GTPase Ypt1, which is also involved in the retrograde transport towards the ER or the internal transport within the Golgi (66). The final fusion step is mediated by the SNARE set with Sed5, Bos1, Bet1 and Sec22. Moderate overexpression of *SEC16* and overexpression of *ERV29* have shown to be beneficial for improving α-amylase titers in the supernatant (60, 68).
The retrograde transport from Golgi to ER is mediated by the coat protein complex I (COPI) vesicles and should recycle vesicle proteins and potentially missorted ER residents. The ER residents are recognized by their HDEL ER localization tag, which binds to COPI protein Erd2 (66). The formation of the COPI complex is dependent on the Ras-family GTP-ase Arf1 which, is either in the inactive GDP bound form in the cytosol or in its active GTP form bound to a membrane and the coatomer complex (66). Upon arrival at the ER membranes, Arf1 will be inactivated and the vesicle releases the cargo proteins. The disassembly of the COPI vesicles is initiated by two GTPase activating proteins (GAPs), Gcs1 and Glo3, by the exchange of GTP with GDP on Arf1 (69).

Overexpression of either *GCS1* or *GLO3* led to a reduction of the α-amylase titer; however, combined overexpression of *GCS1* or *GLO3* together with one of the proteins involved in ER to Golgi transport (anterograde transport), Sec16, led to improved productivity compared to the sole overexpression of Sec16 (69).

1.4.4 Golgi apparatus and exocytosis.

The Golgi apparatus in *S. cerevisiae,* unlike in higher eukaryotes, has its cisternae scattered through the cell (70, 71). The Golgi has an orientation from *cis*-Golgi to *trans*-Golgi and on the *trans* side proteins will be exported from the Golgi to the trans-Golgi network (TGN) (71). The localization of the Golgi resident proteins involved in these steps is based on a polarized distribution within the cisternae (71). Transport between the stacks is mediated by COPI vesicles. The main processes occurring in the Golgi apparatus are the final mannosylation steps to N- and O-linked glycans and the removal of signal peptides. The mannosylation on the N-glycans can mean the addition of up to 50 mannose residues and provides *S. cerevisiae* with its characteristic hypermannosylation. Enzymes involved in the N-glycosylation are α-1,6-mannosyltransferases Och1, and two complexes of Mnn9 with Van1 and another of Mnn9 with Anp1. The latter complex can add around 40 mannoses to the glycan structure. The final steps of the hypermannosylation are fulfilled by Mnn1, Mnn2 and Mnn5 (70, 72). The O-glycosylation is less severe with a final quantity of around 5 mannoses. In the ER, only the first mannose is attached, and the remaining residues are attached in the Golgi. First, Mnt1 will attach two mannoses and the final two mannoses are attached by Mnn1, which is also involved in N-glycosylation (57). The removal of signal peptides from proteins is performed by three Golgi-located proteases, Kex1, Kex2 and Sec11 (Ste13). Kex2 and Sec11 are of specific interest since those are the proteases involved in processing of the signal peptide used to direct the recombinant proteins through the ER in this thesis (66). Engineering of the Golgi apparatus is mostly directed to improving the transport of the proteins but also to increasing the Golgi retention of Kex2 (60).

After the Golgi, protein transport will continue towards the extracellular environment via exocytosis, which is facilitated by the exocyst (66). Exocytosis starts from the TGN and proceeds via either the light density secretory vesicles (LDSV) or the heavy density secretory vesicles (HDSV) (70). The LDSVs contain cell membrane proteins and the HDSVs contain soluble proteins that are transported for secretion. Both vesicles types move along actin filaments using the V-type myosin motor Myo2 which is recruited by Rab Ypt31/32 and needs to be activated by Sec4 to be able to interact with Myo2 (66). In one study, the translocation from Golgi to the supernatant was optimized by upregulation of several proteins involved in trafficking including Ypt32 and Sec4. The authors observed increased production of endoglucanase from *Clostridium thermocellum* and β-glucosidase from *Saccharomycopsis fibuligera* in soluble form but also when anchored to the membranes. (73). The LDSV will move directly from Golgi to the plasma membrane but the HDSVs will pass through the endosome (70). A sorting mechanism should

recognize which HDSVs contain proteases destined for the vacuole and which vesicles should continue to the plasma membrane for secretion (74). Over 70 proteins involved with vacuolar sorting have been identified. A major sorting protein is the receptor Vps10, which binds vacuolar proteases PrA and CPY (74). PrA and CPY will be of specific interest in **Chapter 3**.

1.5 Engineering strategies to improve recombinant protein production.

Alongside engineering of the secretory pathway, from which examples were given in the previous sub-chapter, there are also other methods available to improve the production of recombinant proteins or biologicals. An example is random engineering by UV-mutagenesis accompanied by high-throughput screening, with proved very effective for the construction of the *S. cerevisiae* strain B184 (75, 76). B184 is employed as the production strain in **Chapters 2** and **3.** Another option is to use library screening. Library screening can be performed by different sorts of libraries, for example, a plasmid-based library of native cDNAs. Yeast can be transformed with this library to see which gene increases recombinant protein abundance simply by an extra copy of that gene being present. This has proven effective to identify several targets in *K. phaffi* including *HAC1* (77). Finally, the engineering of the metabolism rather than the secretory pathway can be an option to improve the productivity of proteins. So far limited studies have been reported on yeast-based metabolic engineering for increased protein production but it is implemented broadly in *E. coli* to reduce acetate production in recombinant protein producing strains (78).

1.5.1 *Systems biology as an engineering guide.*

A new field has been developed over the years in the form of systems biology which can be used as a guiding tool for cell factory engineering (79). Systems biology includes the use of computational and mathematical models to describe biology. Initially, mathematical models were developed that would describe parts of the cellular metabolism (79). The models became more complex and advanced over the years until the construction of the first genome-scale models (GEMs) was achieved. In 2003, the first eukaryotic genome-scale model of *S. cerevisiae* was published (80).

During the last decade, the use of multi-omics to study biological systems has gained a lot of momentum. Omics studies can provide essential information for our understanding of biology by mapping large data sets with information on for example transcriptome and proteome (47, 81). Additionally, the knowledge from omics studies can be applied in the further development of the GEMs. Additions of omic studies have added information on protein abundance and distribution within the cell which can be used to constrain GEMs on the usage of enzymes and energy. The addition of those proteome constraints enabled an *S. cerevisiae* GEM to capture complex biological phenomena like the Crabtree effect (82, 83). GEMs can nowadays also be used as a successful guiding tool to predict engineering targets for cell factory improvement (84, 85). In **Chapter 4**, I studied the

application of two proteome constraint GEMs as a tool for strain engineering for the production of biological proteins. A single previous study was found on the use of a GEM containing the central carbon metabolism to improve the production of a recombinant cytosolic human superoxide dismutase (hSOD) in *K. phaffi* (84).

Aim and outline

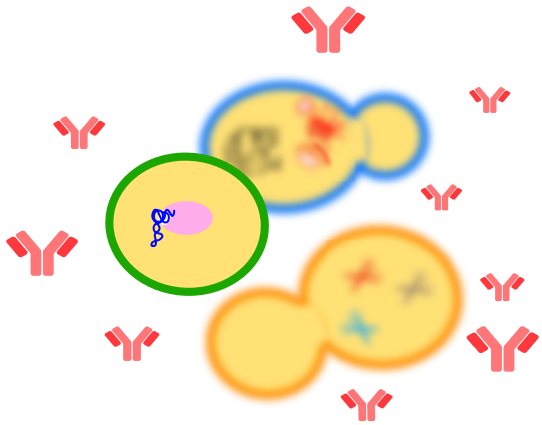
The goal of this thesis was to improve the production of biologicals by *S. cerevisiae*. Throughout the thesis, I will present different engineering strategies to achieve that goal. In total three different strategies were applied and will be presented in separate chapters. The studies include data from **Paper I**, **Paper II**, **Paper III**, and **Paper IV** but also additional experiments.

In **Chapter 2** we examined the protein kinase Gcn2 as a new target to increase recombinant production. Firstly, I demonstrate the use of biosensors to measure differences in cytosolic H_2O_2 *in vivo* as a result of recombinant protein production (**Paper II**). More importantly, we tested our hypothesis that the kinase Gcn2 fulfills a role within a negative feedback loop where an elevated level of cytosolic H_2O_2, as a result of recombinant protein production, activates the protein kinase Gcn2 and reduces general translation and thereby recombinant protein production (**Paper I**).

Chapter 3 is dedicated to the identification and removal of proteases that degrade biologicals and thereby reduce final titers. A part of the chapter will focus on the degradation of synthetic peptides called Affibody molecules (**Paper III**) and a second part will focus on the severe degradation phenotype of a previously engineered *S. cerevisiae* strain.

In **Chapter 4**, I tested two different proteome-constraint GEMs to increase the production of three biologicals. Both GEMs are recent advances to previous *S. cerevisiae* GEMs and in this chapter, we wanted to verify the use of such advanced GEMS as a tool to improve recombinant protein production. One GEM (EcYeast8) includes only metabolic reactions and the other (pcSecYeast) includes metabolic reactions and protein synthesis. Additionally, we hypothesized that either optimizing secretion or metabolism would prove most effective for the high production of recombinant proteins (**Paper IV**).

2. Recombinant protein production and oxidative stress.



In this chapter, I will discuss the results and the impact of the kinase Gcn2 removal on recombinant protein production. The kinase Gcn2 is present in most eukaryotic cell types and based on our studies appears to fulfill a previously unknown role within the oxidative stress response in *S. cerevisiae*. Most of the results that are included in this chapter are based on **Paper I**.

2.1 Stress and oxidative stress.

Many intracellular and environmental changes can induce stress in yeast. Examples of extracellular stress factors are temperature, pressure, and concentrations of specific ions or toxins (86). To cope with stress, biological systems can induce a stress response to regain homeostasis. Stress responses often require a complex network of signaling to adapt to the new condition, which can lead to altered cell growth and adjustments in gene expression (86). In the case of recombinant protein production, many studies are focused on intracellular stress and its impact on the productivity of the cells. As described in the background chapter, ER stress can occur due to recombinant protein production and result in the induction of ER stress responses like the UPR and ERAD. Another example of stress linked to recombinant protein production is oxidative stress (87). Oxidative stress is a condition caused by levels of reactive oxygen species (ROS) exceeding the intracellular buffer capacity. ROS is a group of reactive compounds that are formed by the incomplete reduction of oxygen to water (88). High levels of ROS can be harmful to yeast cells leading to DNA breakage, unwanted oxidation of proteins and lipids, and even apoptosis (8). Most ROS are reactive due to the presence of an unbound electron pair. An example is superoxide (O_2^{*-}) which, like the other oxygen radicals, has a short half-life *in vivo* (89). Such radicals can induce damage and are therefore converted to a more stable species like H_2O_2. Even though H_2O_2 is more stable and less toxic compared to other ROS, H_2O_2 can still inflict damage to the cell and has shown to be lethal to yeast from an extracellular concentration of 2 mM (90).

Yeast contains several antioxidant proteins which can convert H_2O_2 to water and oxygen. H_2O_2 can be broken down to O_2 and H_2O by catalases or be reduced to H_2O by peroxiredoxins or glutathione peroxidases (89, 91). H_2O_2 is produced in several organelles where the oxygen consumption is high. Such organelles are the mitochondria or peroxisomes and, more importantly for this thesis, the ER (89).

The perspective written by Murphy emphasizes the importance of precision in studies regarding ROS, since different ROS species fulfill different roles in cells and the elevated presence of a ROS species is not a direct indication of more oxidative damage (92). However, for many years methods to study and quantify ROS and H_2O_2 within biological systems have been limited. Often, scientists would turn to the use of staining or reagents that are known and documented to be unspecific or even unreliable. Examples are carboxy-H_2-DCFDA, dihydrorhodamine 123, and hydroethidine (93–95). Carboxy-H_2-DCFDA was shown to be unstable with decreasing level of dissolved oxygen in the medium, dihydrorhodamine 123 is converted to the fluorescent compound rhodamine by the oxidation of H_2O_2 by peroxidases but does not measure an H_2O_2 concentration by itself, and hydroethidine, a reagent for O_2^-, was shown to be converted to several products besides the desired fluorescent product (93–95). Fortunately, during the last decades, new technology has entered the field in the form of biosensors, which allows the selective detection of specific ROS species *in vivo* (92).

2.2 Biosensors for intracellular H_2O_2 measurements.

Biosensors are biological sensors that are often based on enzymes and their natural specificity and sensitivity. In the early stages, biosensors would be based on enzymes or microorganisms that would react with, or metabolize a compound of interest leading to a change in for example oxygen tension (96). Nowadays, biosensors are more advanced and can detect extra- and intracellular concentrations of toxins or metabolites (97, 98). Examples of such biosensors are synthetic constructs with an H_2O_2 scavenger connected to a green fluorescent protein (GFP). GFP-linked biosensors are genetically encoded fluorescent indicators (GEFIs). Those GEFIs with an H_2O_2 scavenger can detect intracellular H_2O_2 quantities and translate those quantities into fluorescent signals (99–102). There are also other GEFI variants that can for example detect reduced glutathione (103). Even though the exact proteins within GEFIs can differ, the concept often remains the same. The H_2O_2 scavenger will reduce H_2O_2 to 2 H_2O, which will create an internal disulfide bond that will move within the biosensor towards the GFP protein. The GFP protein is an engineered GFP variant called a roGFP, for which the fluorescence is dependent on the redox state of the protein (104). When the disulfide bond is located within the roGFP the excitation wavelength will shift. A schematic representation of the mechanism is shown in Figure 2. When roGFP is in its reduced state the maximum excitation wavelength will be at 488 nm and upon oxidation, it will shift to 405 nm while the emission peak remains stable at 510 nm in both states. Two of the studies in this thesis

concern the use of a GEFI to measure cytosolic changes in H_2O_2 because of recombinant protein production (**Paper I** and **Paper II**).

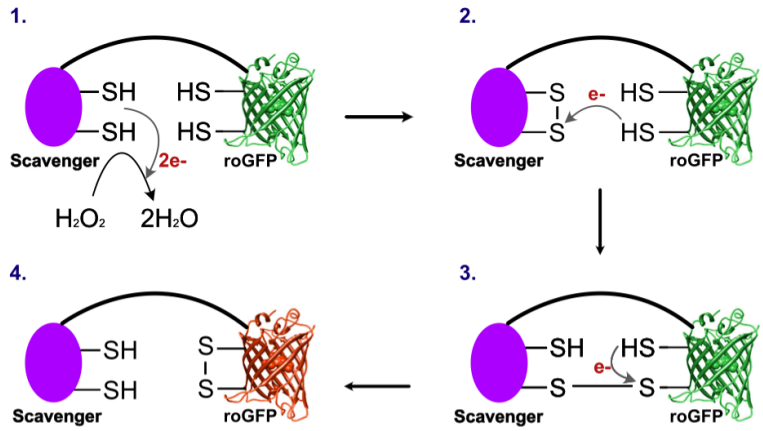


Figure 2. Schematic of the reduction of H_2O_2 by scavenger and the movement of the disulfide bridge through the GEFI until the internal bridge is located within the roGFP and the maximum excitation wavelength changes from 488 to 405 nm.

We were interested to investigate if producing recombinant α-amylase would lead to elevated levels of cytosolic H_2O_2 in *S. cerevisiae*. To study potential differences in intracellular H_2O_2 levels, we used a GEFI based on a roGFP2, with an enhanced GFP as a fluorescent protein (102) and explored several options for H_2O_2 scavengers. We tested three biosensors with different H_2O_2 scavengers, Prx1, PfAOP, and PfAOP^{L409M}, which were all attached to roGFP2 (101, 105). Prx1 is the sole mitochondrial peroxiredoxin in yeast, PfAOP is a peroxiredoxin 5-type protein from *Plasmodium falciparum* and PfAOP^{L409M} is an engineered variant of PfAOP with a mutation of residue Leu^{109}, which improved redox enzyme kinetics (101, 105). PfAOP^{L409M} remains activated at higher concentrations of H_2O_2 but also showed higher sensitivity to lower H_2O_2 concentrations compared to PfAOP (106). The abundance of H_2O_2 is determined based on a [400/488] ratio, also referred to as the [Ox/Red] ratio. This ratio shows the quantity of the sensors in the oxidated state compared to the quantity of the sensors in the reduced state and can be used to compare cytosolic H_2O_2 levels. Due to the ratiometric system of the sensor different quantities of GEFI between strains do not interfere with the comparison. A *S. cerevisiae* strain was transformed with the three GEFIs and the transformants were cultivated in a Biolector and tested for their response to DTT and H_2O_2, respectively. The results shown in Figure 3 show the oxidative index of the different sensors of the [Ox/Red] ratios measured over a 2-h interval after the addition of DTT, H_2O_2, and a control without any addition. RoGFP2-Prx1 displays the expected relation between the oxidative indices where the addition of DTT lowers the index, due to decreased fraction of oxidized biosensor, and the addition of H_2O_2 increases the index, due to an increased fraction of oxidized biosensor, compared to the control. For PfAOP and PfAOP^{L409M,} the control

showed a higher oxidation index compared to the samples where H_2O_2 is added, which indicates that these two sensors did not function as expected in our tested setup. RoGFP2-Prx1 showed a more desired index of the control. RoGFP2-Prx1 was therefore selected as the best performing sensor for our study.

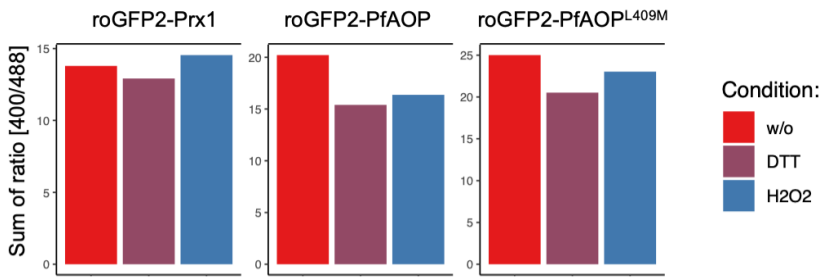


Figure 3. Influence of the addition of DTT and H_2O_2 on the [Ox/red] ratios of the different GEFIs. The bar plots represent of theindex of the [400/488] or [Ox/Red] ratios of the consecutive 2 h after addition of DTT and H_2O_2. DTT and H_2O_2 were added to a final concentration of 1 mM.

2.3 Recombinant protein production and oxidative stress.

As mentioned before, oxidative stress is often observed in relation to recombinant protein production (107, 108). H_2O_2 is produced in the ER with the reduction of oxygen that occurs during the iterative process of the making and breaking of disulfide bonds mediated by protein disulfide isomerase (Pdi1) and its counterpart ER oxidoreductase 1 (Ero1). This process will be referred to as oxidative folding. Pdi1 is a folding chaperone that, in its oxidized state, transfers electrons to cysteines on folding proteins to make internal disulfide bonds. Ero1 reoxidizes Pdi1 afterward and uses oxygen as a final electron donor and reduces O_2 to H_2O_2. If a host cell produces high titers of a recombinant protein, it has been suggested that an overload of the folding machinery in the ER, including the oxidative folding machinery, leads to high production of H_2O_2 and therefore oxidative stress (109). This is supported by a study where *HAC1* was removed from *S. cerevisiae* while producing two recombinant proteins and therefore the ability of *S. cerevisiae* to induce the UPR. Due to the absence of intact UPR *S. cerevisiae* was unable to increase the number of chaperones in the ER. This was hypothesized to result in a reduced general folding rate while iterative oxidative folding would continue without progressing towards the final state of the protein which led to a futile cycle within redox metabolism, resulting in ROS production and an oxidative stress response (109). Another study supports this model where misfolding-inducing conditions, like reduced glycosylation but also increased protein secretion resulted in upregulation of the UPR and an increase in oxidative folding demand in the ER (110). In one of our own experiments, we observed that the addition of riboflavin cofactors, which are known to stimulate oxidative folding (111), improves α-amylase production in AACk. In Figure 4 the results of a western blot experiment against α-amylase in the supernatant are shown. Upon the addition of cofactors riboflavin (FMN), but mainly flavine-adenine-dinucleotide (FAD), the titer of

α-amylase in the supernatant increased, which could be due to a reduction in the fraction of misfolded α-amylase. Another study showed that in *S. cerevisiae* the overexpression of *HAP1,* encoding a transcription factor involved in respiration and the response to oxygen and heme, led to a periodic increase in productivity of α-amylase which was coherent with a lowered degree of oxidative stress during that interval suggesting that oxidative stress can reduce recombinant protein production (112). These studies show that the production of a recombinant protein with internal disulfide bonds can lead to an oxidative stress response which will inhibit the production of the recombinant protein.

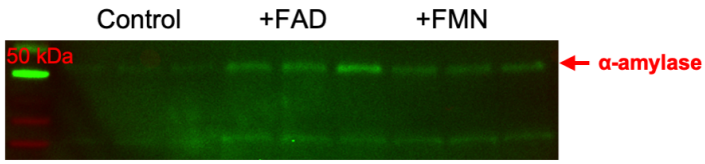


Figure 4. Influence of the addition of favin cofactors on α-amylase production. Three biological replicates of AACk were grown in for 48 h in Delft synthetic media in a volume of 2.5 mL. (Control) cultures without any addition, (+FAD) addition of 10 mM FAD and (+FMN) addition of 10 mM FMN.

2.4 The kinase Gcn2.

Gcn2 is a kinase that is mostly known for its role as an activator of Gcn4, a transcription factor in *S. cerevisiae*, which regulates amino acid biosynthesis (113). The kinase Gcn2 is itself activated by binding of uncharged tRNAs to its HisRS-domain which will lead to a conformational change (114, 115). Upon activation, the kinase Gcn2 will phosphorylate the translation initiation factor eIF2α on Ser-51. EIF2α is part of the complex that delivers the initiator methionyl-tRNA to the ribosome. After initiation of translation, the complex detaches with eIF2α in GDP form, which eIF2B should exchange for GTP before the next translation initiation. EIF2α in phosphorylated form does not allow guanine nucleotide exchange and will remain in GDP form. The GDP form of eIF2α is unable to initiate translation and this mechanism will attenuate general translation (116). The reduction of general translation will result in the translation of the transcription factor Gcn4. Gcn4 will induce the expression of the genes related to the biosynthesis of amino acids (116, 117). In the mRNA leader sequence of *GCN4* four uORFs block the translation of the *GCN4* mRNA under non-stressed conditions. However, when Gcn2 is activated and eIF2α is phosphorylated, those four uORFs will be skipped by the ribosomal complex and *GCN4* mRNA will be translated (118). Attenuation of general translation will reduce protein synthesis and is a sensible response if the cells sense amino acid shortage since it reduces the cellular costs and requirement for metabolites, especially given that protein production is expensive for cells (47). Over the years, several additional inducers of Gcn2 have been identified like exposure to sodium, rapamycin, glucose starvation, and purine starvation, which all activate Gcn2 through the same mechanism of amino acid starvation (118–120). Interestingly, one study showed that the addition of H_2O_2 to the medium also activates the kinase Gcn2, resulting in a decrease in translation efficiency and protein synthesis (119).

Another interesting aspect is that Gcn2 is the sole kinase of eIF2α in *S. cerevisiae* whereas mammalian cells have four eIF2α kinases. Besides GCN2, there are PERK, PRK, and HRI. PERK is transmembrane protein localized in the ER membrane and is activated by the presence of unfolded proteins, indicating that in mammalian cells a mechanism is in place that connects phosphorylation of eIF2α with ER homeostasis (114). In yeast, ER stress and the phosphorylation of eIF2α have thus far not been linked. We hypothesized that if the kinase Gcn2 is induced by the external addition of H_2O_2, it could potentially be activated also by elevated H_2O_2 produced by the overexpression of a recombinant protein. A schematic of the suggested loop is shown in Figure 5.

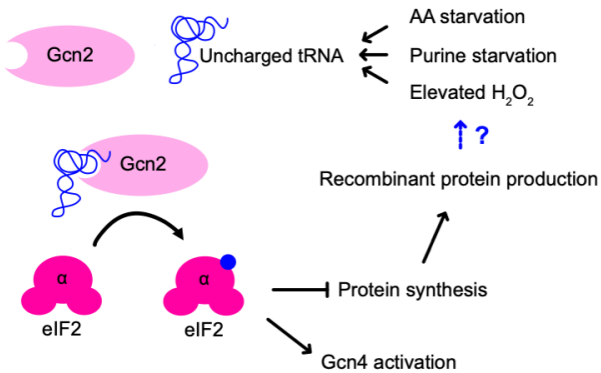


Figure 5. Schematic of the hypothesized negative feedback loop mediated by the kinase Gcn2.

2.5 The kinase Gcn2 and recombinant protein production.

Two different *S. cerevisiae* backgrounds were transformed with the previously described roGFP2-Prx1 GEFI. B184 is an effective producer of α-amylase and glucan-1,4α-glucosidase and was constructed by exposing strain AACk to 3 consecutive rounds of UV-mutagenesis and screening. AACk is a *S. cerevisiae* CEN.PK strain with partial removal and thereby inactivation of Tpi1 (75). The absence of active Tpi1 is complemented by the use of a CPOT plasmid, an expression plasmid often used for recombinant proteins (51). Both strains were compared with and without recombinant α-amylase production effectuated by the presence of a CPOT plasmid carrying the α-amylase gene from *A. oryzae* or a CPOT plasmid with an empty cassette (51). Both B184 and AACk were cultivated in a Biolector with and without producing recombinant α-amylase. The [Ox/Red] ratios were determined and are shown in Figure 6A. B184 showed a stable elevated [Ox/Red] ratio in the strains producing recombinant α-amylase while for AACk no such difference can be observed. These results indicate that the cytosol of strain B184 contains a constantly moderately higher level of H_2O_2 under α-amylase producing conditions, which is potentially also caused by the production of recombinant α-amylase. Based on previous studies performed on B184 and AACk, an increase in H_2O_2 was observed in both B184 and AAC upon production of recombinant α-amylase (76).

However, those measurements were done with another less specific method to detect H_2O_2, namely the dihydrorhodamine 123 dye. This dye shows the level of H_2O_2 oxidizing reactions rather than the actual quantity of H_2O_2 (94).

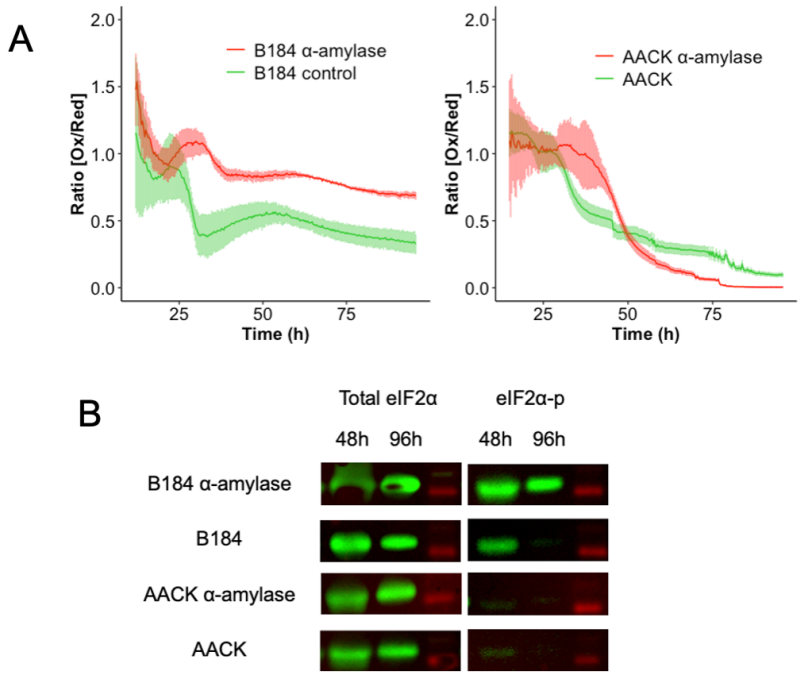


Figure 6. The influence of α-amylase expression on (A) cytosolic H_2O_2 and (B) phosphorylation of eIF2α in AACk and B184.

After confirmation of the elevated levels of cytosolic H_2O_2 in B184, we investigated if the kinase Gcn2 showed activity by checking if eIF2α was phosphorylated. This was done by performing a western blot experiment with an antibody against total eIF2α and another antibody against the eIF2α with phosphorylated Ser-51. The results are shown in Figure 6B. The western blot against the phosphorylated fraction of eIF2α shows that in B184 after 48 h both with and without recombinant protein production eIF2α is phosphorylated. In AACk no phosphorylation of eIF2α is observed at either time point with and without producing recombinant α-amylase. After 96 h, only B184 producing α-amylase still showed a phosphorylated fraction of eIF2α suggesting that the Gcn2 kinase was active and potentially reduced the rate of protein synthesis in that strain.

Since eIF2α was phosphorylated in B184 even after 96 h of cultivation, translation and α-amylase production could have been reduced for the whole duration of the experiment. Therefore, *GCN2* was removed in B184, AACk and K17, an inverse engineered *S. cerevisiae* strain (60), and all three strains and their *gcn2Δ* deficient mutants were

cultivated and sampled for α-amylase titers after 24, 48, and 96 h; results are shown in Figure 7A.

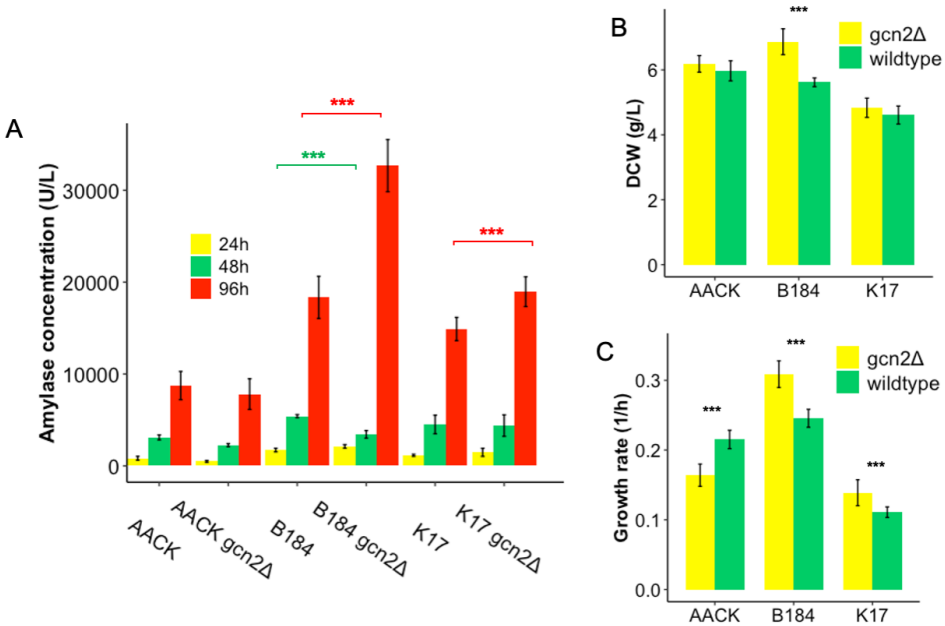


Figure 7. Impact of the removal of kinase Gcn2 from AACk, B184, and K17 on (A) α-amylase production, (B) dry cell weight (DCW), and (C) maximal specific growth rate.

The measurements after 24 h and 48 h show little or even negative impact (B184) of the removal of the kinase Gcn2 but after 96 h both for B184 *gcn2Δ* and K17 *gcn2Δ* the removal of the kinase Gcn2 led to an improvement of the α-amylase titer in the supernatant; for B184 *gcn2Δ* the concentration almost doubled. These results indicate that the presence of kinase Gcn2 reduces α-amylase production in B184 and K17. We showed that B184 suffered from a reduced translation rate in **Paper I** which was elevated by removal of the kinase Gcn2. The absence of impact of the removal of *GCN2* from AACk on α-amylase production resonates with the lack of eIF2α phosphorylation under these conditions (Figure 6B). The removal of the kinase Gcn2 affected other physiological characteristics as well. Measurements of the dry cell weight (DCW) and the maximum specific growth rate are shown in Figure 7B and 7C. In, B184 *gcn2Δ*, both the biomass yield and the maximum specific growth rate were improved. In K17 *gcn2Δ*, only the maximum specific growth rate was improved and in AACk *gcn2Δ* the maximum growth rate was reduced. These data show that even though these strains produce the same recombinant protein, the impact of this single gene deletion leads to a variable impact on recombinant protein production and growth parameters in the different strains.

We showed at this point that the removal of the kinase Gcn2 from B184 resulted in an improved α-amylase titer, increased maximum specific growth rate, and biomass yield. To obtain a further understanding of intracellular mechanisms due to the removal of the kinase Gcn2, qPCR was performed on several genes involved in the UPR and the oxidative stress response. In addition, the ratio of unspliced and spliced *HAC1* was determined. Hac1 is the transcription factor that mediates the UPR response and is spliced by Ire1 when the ER experiences stress. Additionally, several UPR targets were selected that fulfill different roles within the ER. To determine the induction of the oxidative stress response several genes of the antioxidant response were selected. The results are shown in Figures 8A, 8B, and 8C. Figure 8B shows the ratio of spliced/unspliced *HAC1* mRNA. Based on the ratio of *HAC1* splicing, the UPR is induced to a lesser degree in B184 *gcn2Δ* compared to B184. In Figures 8A and 8C, qPCR results are shown and presented as relative expression of genes in B184 *gcn2Δ* compared to B184. The dashed line represents the expression level in B184. Figure 8A shows that the *PDI1* transcript levels were 7-fold increased upon removal of the kinase. *PDI1* overexpression has previously been linked to an improved α-amylase production (60, 75) and the B184 strain has an extra *PDI1* copy in the genome due to a duplication of chromosome III (76). K17 also has an additional copy of *PDI1* that is overexpressed (60). Four out of five of the genes encoding anti-oxidant response genes showed elevated transcripts in B184 *gcn2Δ* as can be seen in Figure 8C. Tsa1 is a cytosolic peroxiredoxin that reduces peroxides. Tsa1 is reduced by thioredoxins like Trx2, and Srx1 reactivates Tsa1 in case it is hyperoxidized (121). All these genes are regulated by the transcription factor Yap1, which is activated under oxidative stress conditions (88, 122). When we examined B184 *gcn2Δ* with roGFP2-Prx1, we found that the cytosolic H_2O_2 was lower compared to B184 (Figure 8D). Because α-amylase production is increased H_2O_2 levels were expected to be higher. However, on the other hand, we do observe increased expression of the Yap1 controlled H_2O_2 scavengers and anti-oxidant genes.

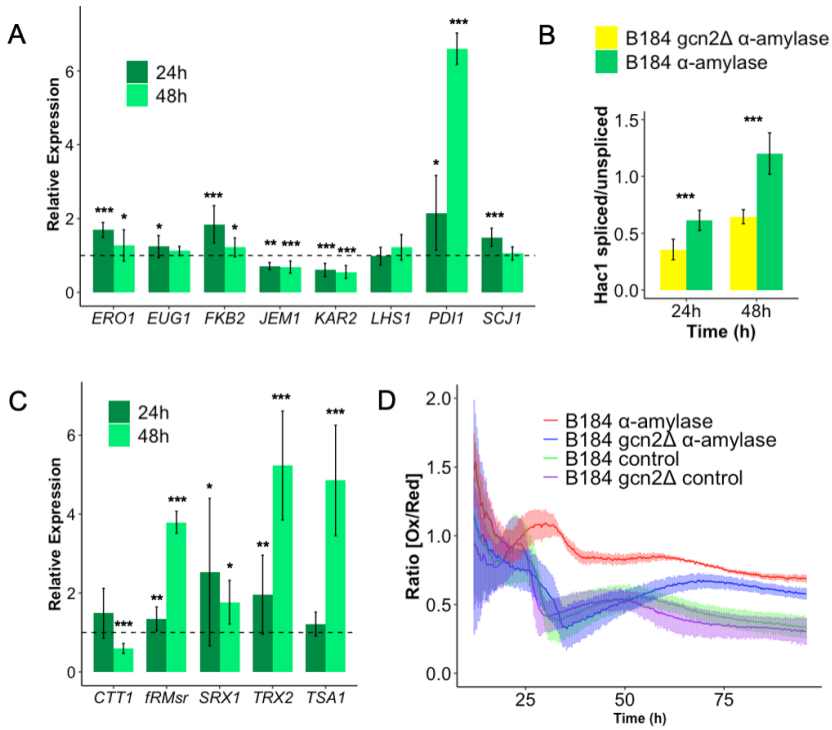


Figure 8. Impact of removal of *GCN2* from B184. (A) qPCR results of UPR target genes in B184 with and without the kinase Gcn2 while producing α-amylase, (B) ratios of qPCR results of *HAC1*spliced and *HAC1*unspliced mRNA (C) qPCR results of oxidative response genes in B184 with and without the kinase Gcn2 while producing α-amylase, and (D) [Ox/Red] ratios of B184 with and without the kinase Gcn2 and α-amylase production, respectively.

Because the H_2O_2 levels do not seem to correlate with the amount of α-amylase produced, the elevated levels of H_2O_2 in B184 with recombinant α-amylase production could reflect H_2O_2 produced in response to some other stimulus induced by recombinant protein production. In a study by Haynes *et al*., they showed that under ER stress conditions the measured ROS was partly generated by the mitochondria rather than oxidative folding or oxidative stress originating from the ER (123). Also, throughout the years more and more studies have shown that H_2O_2 also fulfills the role as a signaling molecule both in directing the oxidative stress response but also mitosis, and even the sensing of light (124–126). The elevated level of H_2O_2 we observed in B184 while producing α-amylase could be a result of the signaling function of H_2O_2 or was partly generated by the mitochondria.

Upon removal of the kinase Gcn2 we observed a reduction of the cytosolic H_2O_2, a reduction of ER stress, increased expression of *PDI1*, increased level of expression of the oxidative stress response genes, and an increase of α-amylase production. The exact role within this mechanism of the kinase Gcn2 remains unclear.

For the connection between the ER stress and activation of the kinase Gcn2 only suggestive literature could be found. As mentioned before, in mammalian cells phosphorylation of eIF2a is connected to misfolding of proteins via the kinase PERK. Yeast does not possess this kinase but it would be possible that Gcn2 fulfills a role in the reduction of translation to cope with ER stress. In the study where the removal of *HAC1* was tested for its impact on α-amylase and insulin precursor production, it was observed that the elimination of the UPR resulted in a downregulation of Gcn4 activity. Thus, the kinase Gcn2 may not have been activated when the UPR could not be induced (109).

In our experiments, we observed a lower H_2O_2 concentration alongside a reduced UPR induction. Several studies connect the UPR with oxidants or oxidative stress. In the study of Haynes *et al*., (2004) the authors observed a positive correlation between the level of UPR induction with the amount of ROS produced, which is in line with our observations. However, a recent study in *S. cerevisiae* showed that the addition of H_2O_2 inhibits induction of the UPR by inactivating Ire1 through an unclear mechanism mediated by the Cys843 residue in Ire1 (127). In this study, H_2O_2 was added extracellularly at a concentration of 5 mM, which is a comparatively high concentration. It could be that different H_2O_2 concentrations influence the correlation. For example in the study of Shenton *et al* (119). The authors showed that the maximal induction of the Gcn2 kinase activity is at 0.5 mM and reduces at higher concentrations. The maximal concentration they tested was 2 mM (119). The exact role of H_2O_2 in coordinating proteins synthesis to oxidative folding and the UPR appears to be complex. However, my data suggest a role of H_2O_2 beyond a stoichiometric by-product of oxidative folding within protein production and indicate a potential link between the kinase Gcn2 and ER stress.

2.6 Impact of removal of Gcn2 kinase on the production of a biological.

Since the removal of the kinase Gcn2 resulted in an improved titer of α-amylase, we tested if the removal of *GCN2* would also increase the productivity of a biological by B184, namely the antibody adalimumab. Adalimumab is a TNF-α neutralizer and creates the most revenue of all biologicals in the market (25, 128). We removed *GCN2* from two B184 strains that express adalimumab in two different constructs. Both adalimumab constructs were expressed from the CPOT plasmid with an additional expression cassette since an antibody is a tetramer consisting of two light and two heavy chains which are expressed as separate ORFs. The gene encoding the light chain was cloned into the original expression cassette and was under the control of the *TPI1* promoter. The additional cassette was used for the expression of the heavy chain and contained either the *TPI1* promoter or the truncated *ADH1* (tr*ADH1*) promoter (129). The folding of the heavy chain in an antibody is dependent on the light chain and literature has shown that a reduced expression of the heavy chain compared to the light chain improved the production of an

antibody in CHO cells (130, 131). Other studies performed on the production of antibodies in *S. cerevisiae* used the same and a strong promoter for light and heavy chain, the *GAL1* promoter (63, 107). We decided to use the *TPI1* promoter as a strong promoter to express the light chain and another less strong promoter, the tr*ADH1* promoter, to achieve a lower expression of the heavy chain (129). The light chain of adalimumab had a His-tag attached to the C-terminus and the heavy chain a Flag-tag to the N-terminus. The B184 strains with and without *GCN2* and expressing the two adalimumab constructs were cultivated for 96 h. The supernatant was analyzed afterward, and the western blots against His- and Flag-tag, respectively, are shown in Figures 9A, 9B, 9C, and 9D. In Figures 9A and 9B the western blots against His-tag are shown. The light chain should have a size around 25 kDa, but we observe a band slightly higher around 30-35 kDa. We excluded ineffective cutting of the signal peptide by Kex2 or the presence of N-glycosylation structures as causes for the increased size of the light chain (data not shown). In Figures 9C and 9D, the western blots against the Flag-tag are shown. The heavy chain has an expected size of 50 kDa which is coherent with the size we observe. Based on the western blot results we observed a difference between the use of the tr*ADH1* and the *TPI1* promoter to produce an antibody. The use of the tr*ADH1* promoter increases the abundance of the heavy chain compared to the light chain, whereas the *TPI1* shows similar presence of both subunits. Upon removal of the kinase Gcn2 for the tr*ADH1* promoter leads to a reduction in the heavy chain and an increase in the light chain and for the *TPI1* promoter construct the blots indicate a similar quantity but in the heavy chain shows degradation as well. The results we obtained in this experiment require further validation to conclude on both the use of different promoter for antibody production and the impact of the kinase Gcn2.

The removal of the kinase Gcn2 led to several observations in B184 producing α-amylase. The improved production we observed for α-amylase was partly caused by the severe increase in *PDI1* expression. As mentioned before, overexpression of *PDI1* has been shown to improve α-amylase production. α-Amylase is a moderate complex protein one N-glycosylation site, and four internal disulfide bonds for nine cysteines. Due to the uneven number of cysteines, the oxidative folding of α-amylase could become a bottleneck in folding when overexpressing α-amylase. Adalimumab is a large tetramer, which contains intra- and inter disulfide bonds and has 2-N-glycosylation sites and is a protein that potentially has other folding demands than α-amylase. Another study showed that the increase of the ER size and the overexpression of the peptidyl-propyl isomerase (PPIase) *CPR5* improved the production of an antibody in *S. cerevisiae* and the overexpression of *PDI1* did not (63). In the folding process of an antibody, the folding mechanism of the heavy chain requires an essential isomerization reaction which is catalyzed by a PPIase (130). Since the removal of the Gcn2 kinase increases the expression of *PDI1* it might remove a bottleneck in α-amylase but not for the production and folding of adalimumab.

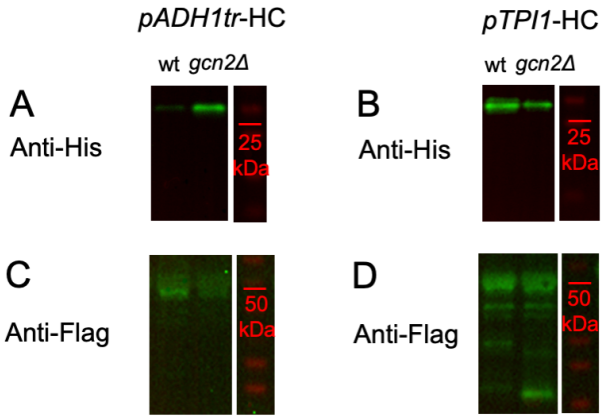
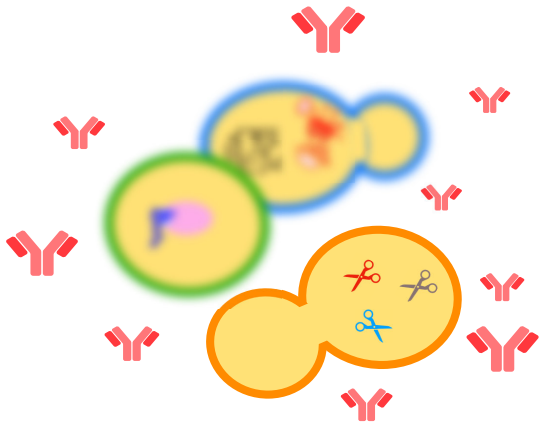


Figure 9. Influence of removal of kinase Gcn2 on production of adalimumab. Western blot against the His-tag (light chain) (A) and Flag-tag (heavy chain) (B) of adalimumab. The lanes marked with wt contain supernatant of a cultivation with B184 expressing adalimumab and lanes marked with *gcn2Δ* contain supernatant of a cultivation with B184 *gcn2Δ* expressing adalimumab.

Overall, we showed that the kinase Gcn2 can reduce the production of α-amylase. For now, we did not detect a positive impact of the removal of the kinase on adalimumab production but more experiments need to be done to draw final conclusions.

3. Degradation of recombinant proteins by proteases.



In this chapter, the removal of proteases to increase intact titers of biologicals is discussed. A part of the chapter is dedicated to the degradation of a specific type of recombinant proteins called Affibody molecules and is based on the results presented in **Paper III**, and a second part of the chapter focuses on the degradation of biologicals by the engineered strain K17 (60).

3.1 Yeast proteases.

S. cerevisiae has proteases localized in several organelles; the cytosol, the Golgi apparatus, the vacuole, and attached to several membranes. In the cytosol, there are several large proteolytic complexes called proteasomes (132). Proteasomes in *S. cerevisiae* degrade ubiquitinated proteins and fulfill a central role in protein degradation in the cell. They degrade all sorts of proteins including misfolded proteins from the ER directed by ERAD but also many short-lived proteins including the transcription factor Rpn4, regulating the expression of many proteasomal genes (132). Within the secretory pathway, proteases fulfill a PTM role. Proteases like Kex2 and Sec11 remove the signal peptides from native proteins that travel through the secretory pathway to be secreted. Examples of native proteins from which the signal peptide is cleaved by Sec11 are invertase and acid phosphatase (133). Sec11 is the only signal peptidase that is essential in *S. cerevisiae* (134). *S. cerevisiae* also contains proteases that are anchored to the plasma membrane or cell wall. These anchored proteases include the yapsin family. Instead of traveling from the Golgi to the vacuole, membrane-anchored proteins will be shuttled by the LDSVs to their destination, the plasma membrane. Yapsins fulfill different roles within cell wall integrity during external stress conditions like heat or elevated concentrations of salts (135).

As a final group, there are the vacuolar proteases. The vacuole is a central organelle in *S. cerevisiae*. Functions of the vacuole include storage of nutrients like amino acids, pH regulation, and protein degradation (136, 137). Molecules and proteins can reach the vacuole via several transport routes including the late Golgi transport and endocytosis from the plasma membrane, or from the cytosol (138). Proteins that are shuttled to the vacuole are degraded and their amino acids are reused. The rate of vacuolar degradation is dependent on nutrient availability and increases under starvation conditions, for example when yeast goes into the stationary phase (139). The vacuolar proteases will pass through the secretory pathway before reaching the vacuole. The vacuolar proteases contain a signal peptide with a pre- and a pro-peptide. The pre-peptide directs the proteases through the secretory pathway and the pro-peptide directs the proteases from the late Golgi to their final destination, the vacuole (140). Additionally, the pro-peptide keeps the protease in an inactive precursor form and ensures correct folding and maturation of the protease in the ER and Golgi (137). The absence of the pro-peptide results in slow maturation and transport of the proteases or even degradation (141, 142). The vacuolar proteases reach their active form by removal of their pro-peptide(s) upon arrival to the vacuole or in transport towards the vacuole.

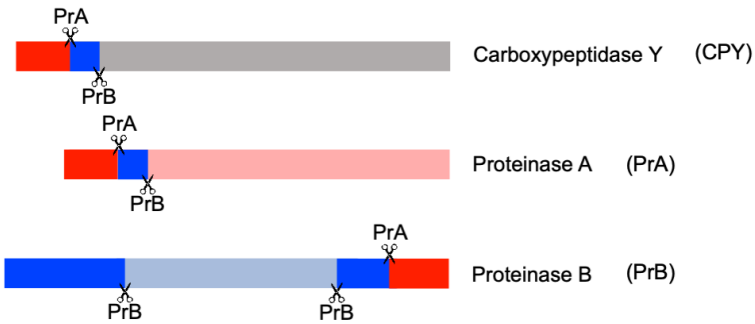


Figure 10. Schematic on the organisation and processing of pro-peptides in carboxypeptidase Y, proteinase A and proteinase B.

For this part of the thesis, three vacuolar proteases are of interest, proteinase A (PrA), proteinase B (PrB), and carboxypeptidase Y (CPY). PrA and PrB are endo-peptidases and CPY is an exopeptidase (139). Endopeptidases catalyze cleavage within an amino sequence whereas exopeptidases catalyze the cleavage of the terminal peptide, and in the case of CPY, this is at the C-terminus of a protein (143). The activation of the vacuolar proteases is regulated by a cascade that starts with the activation of PrA. Several vacuolar proteases are activated by PrA and/of PrB. The exact confirmation of the pro-peptides differs from protease to protease. In Figure 10 a schematic is shown of PrA, PrB, and CPY with the pro-peptides, and their activators. Initially, PrA was thought to autoactivate upon arrival to the vacuole, which would be triggered by the local low pH; however, a more recent study shows that the activation might also happen during the vesicle transport and is not dependent on the low pH (144, 145). PrA, PrB, and CPY all require cleavage of a pro-peptide by PrA and PrB. The activation cascade begins with autoactivation of PrA

which will remove a part of the pro-peptide from its N-terminus followed by complete removal of the pro-peptide by PrB. CPY also has a pro-peptide on the N-terminus which is partially removed by PrA and completely by PrB. PrB has pro peptides both at the C- and N-terminus. The pro-peptide at the N-terminus is removed by PrB in the ER and the pro-peptide at the C-terminus is partly removed by PrA and completely by PrB in the vacuole (137).

3.2 Degradation of recombinant proteins by proteases.

Theoretically, the vacuolar proteases remain in an inactive form when they travel through the secretory pathway until they reach their destination, the vacuole. This should prevent the degradation of other proteins in the secretory pathway (139). However, it has been shown that yeast can secrete active vacuolar proteases into the supernatant. This secretion is induced by high glucose concentrations during the early stages of cultivation but will increase regardless after a certain duration of a cultivation (146, 147).

Due to the secretion of vacuolar proteases but also the presence of cell wall anchored proteases yeasts can degrade recombinant proteins in the supernatant. Therefore, the removal of yeast proteases is a widely applied method to reduce the degradation and increase the titer of recombinant proteins. The proteases that are linked to the degradation include vacuolar proteases PrA, PrB, and CPY, but also membrane-anchored proteases like yapsins (148, 149). The combined removal of PrA and PrB to improve recombinant protein production has been realized in *S. cerevisiae*, *K. phaffi,* and *Candida boidinii* (150–152). Since PrA and PrB are responsible for the activation of several vacuolar proteases, including CPY, removing them should keep other vacuolar proteases in inactive form (152). Also, the removal of other protease combinations has been applied successfully to improve the stability of recombinant proteins in the supernatant of yeast. A fusion construct of human serum albumin and human parathyroid hormone (66 kDa) was produced by *K. phaffi* but was partly degraded in the supernatant. The removal of several proteases including vacuolar proteases PrA, PrB, and six yapsins, was tested. The combined removal of *PEP4* (PrA) and *YPS1* resulted in the highest titer of the fusion protein (148). In another study, several yapsins from *S. cerevisiae* were removed to improve the production of the human parathyroid hormone. *YPS1*, *YPS2*, *YPS3*, *YPS6,* and *YPS7* were removed both individually and combined and it was reported that combined removal of *YPS1*, *YPS2*, *YPS3*, *YPS6* increased the stability of human parathyroid hormone the most (149).

An alternative to the removal of proteases to reduce degradation can be altering the medium composition and cultivation conditions. In *K. phaffi* maintaining a pH of 4 instead of 5 in combination with lowering the temperature from 30°C to 22°C during a fed-batch fermentation was found to increase the titer of a fusion protein of cellulose-binding molecule of *Neocallimastix patriciarum* cellulase 6A and lipase B from *Candida antarctica* (CBM-CALB) 3.3-fold. Additionally, all the secreted CBM-CALB remained

intact (153). In *S. cerevisiae* the degradation of human serum albumin was reduced through the addition of different nitrogen sources like arginine or ammonium salts. It was suspected that the additional feeding of carbon source, galactose to induce the *GAL10* promoter, without adding a nitrogen source led to nitrogen starvation and induced severe degradation (146).

3.3 Affibody molecules.

This chapter will focus on description of the production and degradation of several biologicals. Specific interest was in the realization of the stable production of Affibody molecules. Affibody molecules are synthetic peptides that can be altered to bind to human target proteins. The binding capacity of Affibody molecules originates from a synthetic peptide called the Z domain. This domain is based on the B domain of the immunoglobulin-binding staphylococcal protein A (154). Randomization of 13 amino acids within the Z domain alters its specificity and affinity (155, 156). The small size of Affibody molecules, 6.7 kDa for a single Z domain, allows the molecules to penetrate tissue more easily compared to, for example, antibodies. Affibody molecules based on only Z domain(s) are degraded rapidly in the human body which compromises their functionality. Affibody molecules, with their small size, are below the glomerular filtration barrier of 60 kDa. By adding another peptide called the albumin-binding domain (ABD) that binds to human serum albumin, a protein above the kidney barrier, the half-life of the Affibody molecules increases, and restores their functionality (157, 158). Currently, applications of Affibodies are within diagnostics and therapeutics. An Affibody molecule that functions as an IL-17-specific ligand trap for the treatment of plaque psoriasis is currently in clinical trials and has completed phase I (158). Another example of a relevant target protein of Affibody molecules is HER3. Elevated expression of HER3 is connected to several malignant cancer types (159). For the moment, Affibody molecules are produced in *E. coli*. However, the outer membrane of *E. coli* contains toxic lipopolysaccharides, also called endotoxins, which are challenging and costly to remove from the final product and can induce an immune response in the human body. Therefore, it was of interest to explore other potential production hosts. In this part of the study, three different Affibody molecule conformations of a Z_{HER3_1}, a Z domain with an affinity for the cancer marker HER3, in combination with an ABD were produced in *S. cerevisiae* (159). The different configurations of the Affibody molecules are shown in Figure 11.

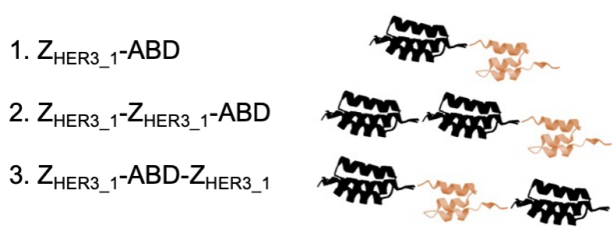


Figure 11. Schematic of Affibody molecule structures, the black molecule is the Z_{HER3_1} and the orange molecule is the albumin binding domain (ABD).

3.4 Production of Affibody molecules by S. cerevisiae.

The three Affibody molecules were expressed in *S. cerevisiae* strain B184, and after a 48 h cultivation, the supernatant of the different strains was analyzed. Two western blots were performed, one against the Z_{HER3_1} domain and another against the ABD. The western blot against the Z_{HER3_1} showed Z_{HER3_1}-ABD and Z_{HER3_1}-Z_{HER3_1}-ABD at their expected band sizes of 12 kDa and 18.9 kDa but also some additional smaller bands. Z_{HER3_1}-ABD-Z_{HER3_1} was expected to have shown a band around 18.9 kDa but several bands with smaller sizes, around 12 kDa, can be seen on the western blot against Z_{HER3_1} (Figure 12A). The western blot against the ABD, shown in Figure 12B, shows none or very little signal for any of the Affibody molecules. We suspected this to be caused by the degradation of the ABD.

To identify the proteases responsible for the degradation we performed an experiment with specific protease inhibitors. We inoculated spent supernatant from a previous cultivation of B184 producing α-amylase and added separate components from a protease inhibitor cocktail together with a standard of Z_{HER3_1}-ABD-Z_{HER3_1}. After incubation for 24 h, all the mixtures were separated using a reducing SDS-PAGE. The results, shown in Figure 12C, showed that the addition of pepstatin A, which targets aspartyl proteases, inhibits the degradation of Z_{HER3_1}-ABD-Z_{HER3_1}.

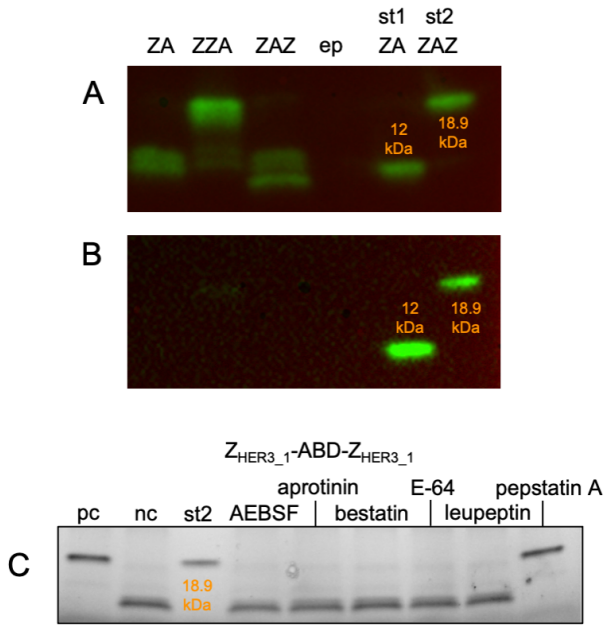


Figure 12. Degradation of Affibody molecules by an aspartyl protease. (A) Western blot against the Z_{HER3_1} domain of the supernatant of B184 producing the Affibody molecules, (B) Western blot against the ABD of the supernatant of B184 producing the Affibody molecules and (C) SDS-PAGE after incubation in spent supernatant of the Affibody molecule standard and the separate components of a protease inhibitor cocktail. St1 indicates the lane with Z_{HER3_1}-ABD standard dissolved in water, St2 indicates the lane with Z_{HER3_1}-ABD-Z_{HER3_1} standard dissolved in water, pc indicates a lane the lane with Z_{HER3_1}-ABD-Z_{HER3_1} standard and the complete protease inhibitor cocktail in spent supernatant and nc indicates indicates the lane with Z_{HER3_1}-ABD-Z_{HER3_1} standard in the spent supernatant. The other indicate lanes with addition of components of the protease inhibitor cocktail.

PrA is an aspartyl protease and due to excessive literature on the undesired degradation of recombinant proteins by PrA, we assumed that PrA would be the responsible aspartyl protease degrading the Affibody molecules (148, 150, 160). However, upon removal of *PEP4* encoding PrA from B184 we only observed intact production of Z_{HER3_1}-ABD-Z_{HER3_1} based on a western blot against the ABD, shown in Figure 13. Z_{HER3_1}-ABD-Z_{HER3_1} was the only Affibody molecule of the three with the ABD enclosed by two Z_{HER3_1} domains. Therefore, we suspected that additional exopeptidase could be degrading the Affibody molecules, for example, CPY.

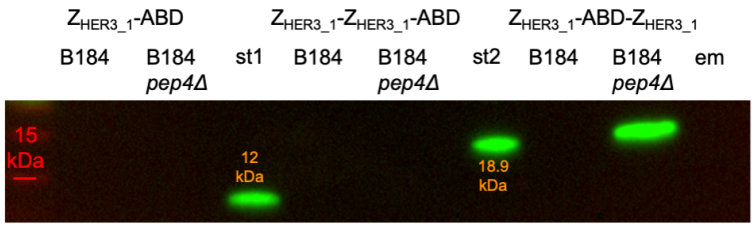


Figure 13. Production of Affibody molecules by B184 *pep4Δ*. Western blot against the ABD of supernatant of B184 and B184 *pep4Δ* producing the Affibody molecules. St1 indicates the lane with Z_{HER3_1}-ABD standard dissolved in water, St2 indicates the lane with Z_{HER3_1}-ABD-Z_{HER3_1} standard dissolved in water and em indicates the lane with medium.

Therefore, the *PRC1* gene encoding CPY was removed but also *PRB1* encoding PrB, since PrB works alongside PrA as an activator of many other vacuolar proteases and was shown as a successful target to improve recombinant protein titers (137, 150). Also, a triple deletion strain was constructed with all the three proteases removed. The double deletion of *PEP4* with either *PRB1* or *PRC1* led to similar quantities of intact Affibody molecules based on the western blots shown in Figures 14A and 14B. In addition, the triple deletion strain showed a similar quantity of Z_{HER3_1}-ABD and Z_{HER3_1}-Z_{HER3_1}-ABD. Based on these results, the removal of *PEP4* with either *PRB1* or *PRC1* is sufficient to produce intact Z_{HER3_1}-ABD and Z_{HER3_1}-Z_{HER3_1}-ABD. The location of the ABD within the molecules indicated activity from an exopeptidase rather than an endopeptidase. Since the removal of PrB or CPY shows the same impact on the degradation it seems highly probable that it is CPY that was the responsible protease alongside PrA for the degradation of Affibody molecules.

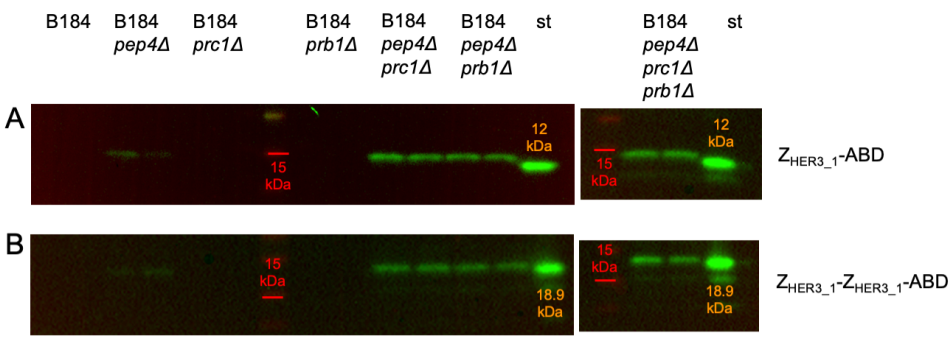


Figure 14. Production of Affibody molecules by B184 mutants (A) Western blot against ABD of supernatant of B184 mutants producing Z_{HER3_1}-ABD and (B) Western blot against ABD of supernatant of B184 mutants producing Z_{HER3_1}-Z_{HER3_1}-ABD. St indicates the lane with an Affibody standard dissolved in water.

Both PrA and PrB need to be intact for complete activation of CPY but also that they both need to be removed for complete inactivation (161). In a study, the activity of CPY and PrB in a *pep4-3 S. cerevisiae* strain was determined to be at 3 (or less) and 7% of their activity with intact *PEP4* gene (162). Interestingly, in a follow-up study it was discovered that there is a delayed effect of the *pep4-3* mutation on CPY inactivation. In fact, the activity of CPY was maintained for up to 20 generations in meiotic spores with a *pep4-3* genotype (163). In that same study, a link between PrB and CPY activity was proposed since the combined *pep4-3* mutation and *prb1-1122* mutation did not show this delayed phenotype (163). Based on these studies and our results it can be concluded that to remove extracellular protease activity by CPY targeting recombinant proteins the deletion of *PEP4* should be combined with either *PRB1* or *PRC1* deletion.

For continuation of the study, B184 *pep4Δ* expressing Z_{HER3_1}-ABD-Z_{HER3_1} was used as a proof of concept to show that *S. cerevisiae* can be a host for Affibody production. A binding assay was performed on the Z_{HER3_1}-ABD-Z_{HER3_1} molecule produced by B184 *pep4Δ* and the current production host *E. coli*, respectively, to ensure that the Affibody molecule efficacy was not affected using *S. cerevisiae* as the production host instead of *E. coli*.

Table 2. Binding kinetics of Z_{HER3_1}-ABD-Z_{HER3_1} produced by *E. coli* and B184 *pep4Δ*.

Production host	k_a1 (1/Ms)	k_a2 (1/RUs)	k_d1 (1/s)	k_d2 (1/s)	K_D1 (M)	K_D2 (M)
E. coli	$5.21x10^5$	$1.23x10^{-3}$	$1.43x10^{-2}$	$2.29x10^{-5}$	$2.75x10^{-8}$	$1.85x10^{-2}$
S. cerevisiae	$3.83x10^5$	$1.71x10^{-3}$	$1.79x10^{-2}$	$2.76x10^{-5}$	$4.68x10^{-8}$	$1.62x10^{-2}$

Based on the results shown in Table 2, both hosts produce Z_{HER3_1}-ABD-Z_{HER3_1} with similar binding kinetics, indicating that the molecule produced by B184 *pep4Δ* performs similarly to the molecule produced by *E. coli*. Secondly, we performed a fed-batch bioreactor experiment with the goal to produce a high titer of Z_{HER3_1}-ABD-Z_{HER3_1}. An aerated, glucose-limited fed-batch was performed with over 150 h of concentrated feed addition. Measurements during the cultivation are visible in Figures 15A and 15B. A final biomass concentration of 100 g/L and a Z_{HER3_1}-ABD-Z_{HER3_1} titer just above 500 mg/L, based on Bio-Layer Interferometry (BLI) protein quantification, was measured at the end of the cultivation (Figure 15C). This is a high titer compared to an overview of titers of biopharmaceuticals produced by *S. cerevisiae* as presented by Nielsen (2013). The titers of two of six biopharmaceutical proteins produced in *S. cerevisiae* are 3 g/L and 1.8 g/L for human serum albumin and human transferrin, respectively, whereas the titers of hirudin, insulin precursor, glucagon, and hepatitis surface antigen all were below the titer we reached in this study (164). For *E. coli* higher production titers of biopharmaceutical proteins can be found. Some examples are 8.5 g/L for insulin-like growth factor and 4.2 g/L of human granulocyte colony-stimulating factor (165, 166). Compared to those, the titer of Affibody molecules reached by B184 *pep4Δ* is ten-fold lower.

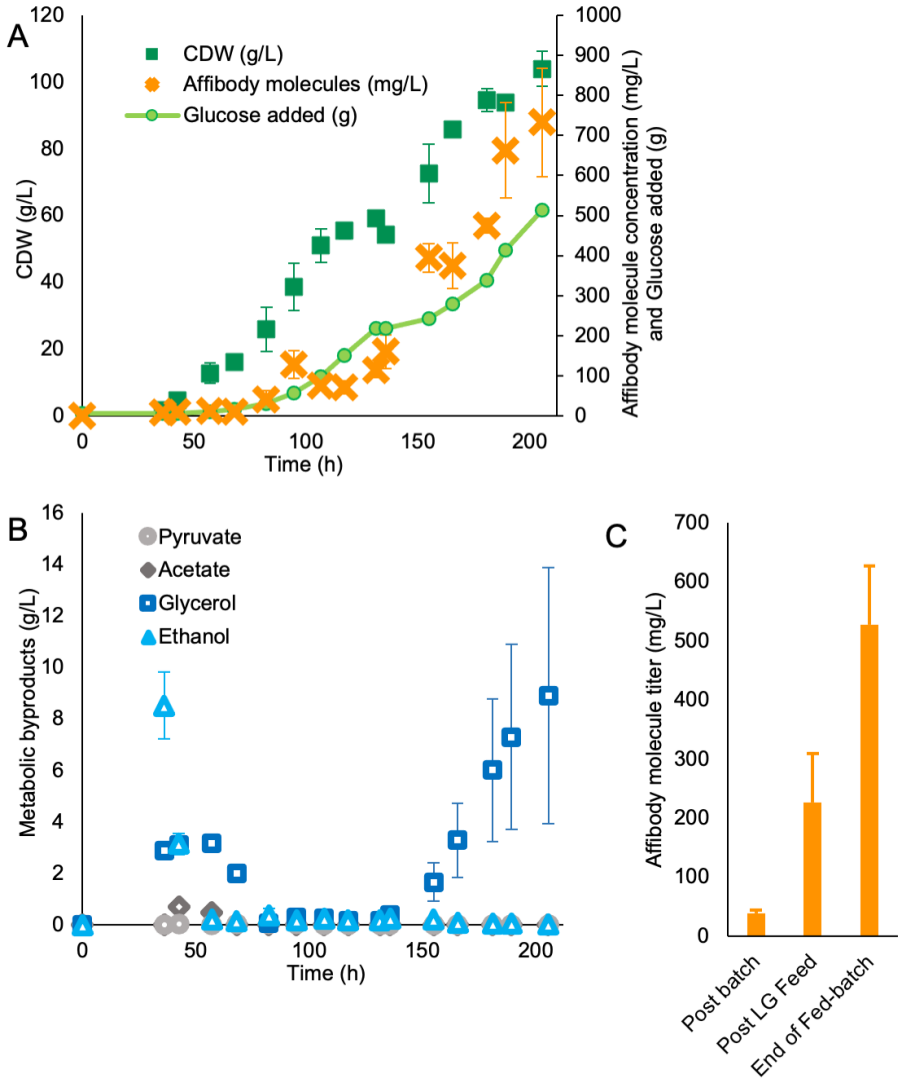


Figure 15. Fed-batch cultivation with B184 *pep4Δ* producing Z_{HER3_1}-ABD-Z_{HER3_1}. (A&B) Measurements during fed-batch of Z_{HER3_1}-ABD-Z_{HER3_1}. (C) Quantification of Z_{HER3_1}-ABD-Z_{HER3_1} concentration during feb-batch based on Bio-Layer Interferometry (BLI).

For the remaining part of this chapter, the removal of proteases from the previously engineered *S. cerevisiae* strain K17 will be discussed.

3.5 Removal of proteases from S. cerevisiae to produce other biologicals.

K17 is an *S. cerevisiae* strain that was reverse-engineered based on the studies in which B184 was constructed and analyzed (60, 75, 76). The genotype of K17 is CEN.PK 530.1CK [*MATa URA3 HIS3 LAU2 TRP1 SUC2 MAL2-8^{C} tpi1(41-707) Δhda2 Δvps5 Δtda3 PGK1p-COG5 Dgos1::amdSYM-TEF1p-PDI1*). K17 and B184 are both improved producers of α-amylase and glucan 1,4-α-glucosidase compared to their background strain AAC (60, 75). Therefore, we decided to use these strains as production hosts for the biological filgrastim. Both strains were transformed with a CPOT plasmid expressing filgrastim. Filgrastim has a C-terminal His-tag which was used to verify the presence and concentration of filgrastim in the supernatant. K17 and B184 expressing filgrastim were grown for 96 h and the supernatant was analyzed with western blot against the His-tag. The results are shown in the blot in Figure 16A.

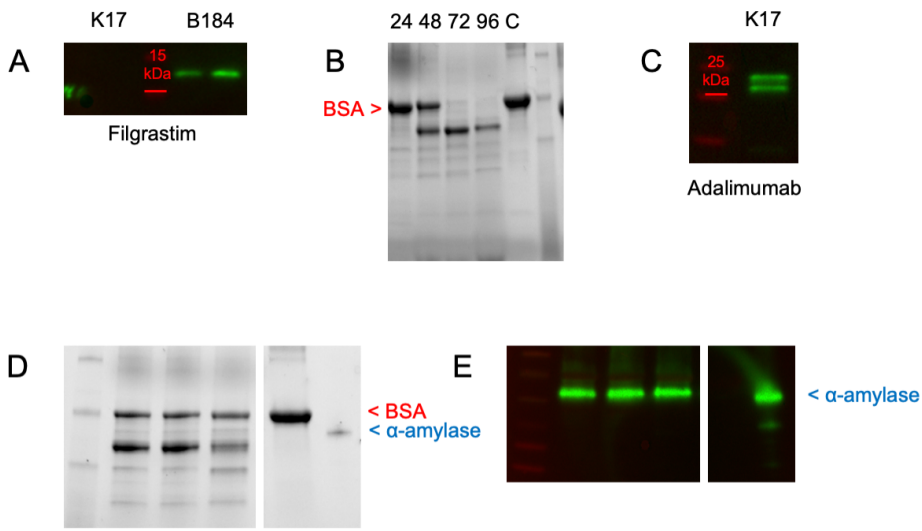


Figure 16. Degradation of filgrastim by K17. (A) Western blot against the His-tag with supernatant of K17 and B184 expressing filgrastim. (B) SDS-PAGE of supernatant of K17 producing filgrastim. (C) Western blot against the His-tag of the light chain of adalimumab with supernatant of K17 expressing adalimumab. SDS-PAGE (D) and western blot (E) of supernatant of three replicate cultures of K17 expressing α-amylase

For B184 filgrastim appeared in the correct size of 18 kDa but no bands were visible in the supernatant of K17. The SDS-PAGE gels for K17 showed that one of the media components, BSA, was severely degraded during the cultivation, shown in Figure 16B. Besides we had also noticed some degradation of adalimumab light chain produced by K17 in another experiment shown in the western blot in 16C. We repeated experiment of filgrastim with K17 that produces α-amylase and analyzed the supernatant. The results are shown in Figures 16D and E. The SDS-PAGE shows degradation of BSA but α-amylase remains intact. We hypothesize that the same protease degrading BSA might be

responsible also for the absence of filgrastim and the degradation of adalimumab in the supernatant. To realize K17 as a production host for biologicals, the responsible protease(s) needed to be removed or their activity reduced.

We cultivated several intermediate strains from the original paper of K17 to potentially identify the responsible engineering step that caused this degradation phenotype. We cultivated the strains for 96 h and analyzed the supernatant by SDS-PAGE shown in Figure 17 (60). The SDS-PAGE shows that the lane with K03 BSA is degraded but the lanes with K01 and K02 show BSA intact. The lane marked with K03 shows the supernatant of AAC *vps5Δ*. Vps5 is a nexin-1 homolog and is required for trafficking from the endosome back to the Golgi (167, 168). Removal of *VPS5* leads to the missorting of Kex2 and Vps10 to the vacuole and a distinct vacuolar morphology with many small vacuoles instead of one, which was also observed in K17 (data not shown) and more importantly results in the secretion of CPY in precursor form (167).

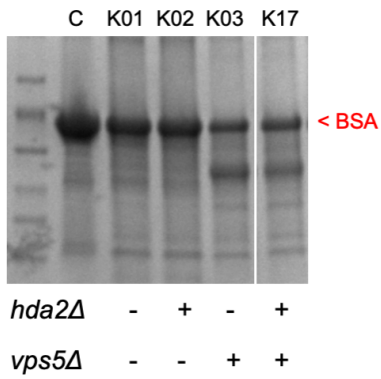


Figure 17. Degradation of BSA by intermediate strains before construction of K17. SDS-PAGE of supernatant of different intermediate strains before construction of K17. C indicates the control lane with BSA dissolved in water

In the study on the production of Affibody molecules, we also observed degradation by CPY. To reduce the degradation, we wanted to reintegrate the *VPS5* gene into its native genomic location, but this proved challenging. As an alternative, we removed both the *PEP4* and *PRC1* genes from K17. Both genes were removed separately and combined in K17. K17, K17 *prc1Δ*, K17 *pep4Δ,* and K17 *prc1Δ pep4Δ* were constructed. All four strains were transformed with the CPOT plasmid expressing adalimumab with the heavy chain under the tr*ADH1* promoter, the same construct that was used for Figure 16C. The four strains were cultivated and the supernatant was analyzed for the presence of degradation. The western blot was against the His-tag attached to the light chain of adalimumab. The results are shown in Figure 18A. The SDS-PAGE shows that the deletion of *PRC1* reduces the degradation to some extent but more importantly the deletion of *PEP4* reduced the degradation of BSA. The western blot against the His-tag shows that both proteases were degrading the light chain of the antibody and the combined

removal results in the highest titer of intact light chain of adalimumab. K17 and K17 *prc1Δ pep4Δ* were also tested to produce filgrastim and the results are shown in Figure 18B. Even though the BSA seems to remain intact filgrastim is degraded by K17 but in K17 *prc1Δ pep4Δ* two bands are visible around the expected size. We suspect one of the bands to be filgrastim of the correct size and the other filgrastim partly degraded by another protease(s). In the previous study on the removal of the *VPS5* gene, the authors hypothesize that the degradation of the mislocalized Kex2 in a *vps5Δ* mutant is caused by PrA in the vacuole (167). Based on our results, PrA seems to be secreted and degrades proteins in the supernatant which can be connected to the use of 2% glucose media (147).

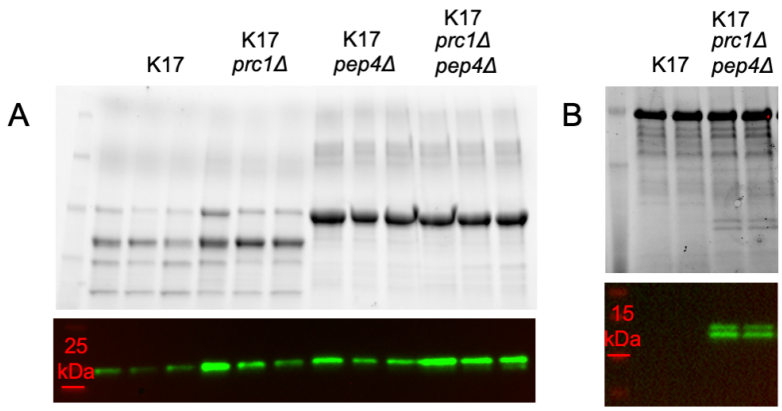
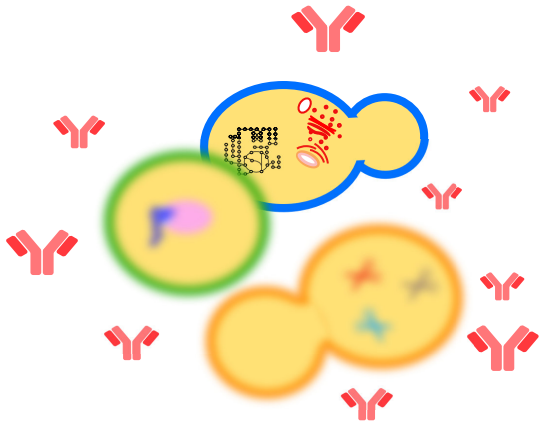


Figure 18. The removal of proteases from K17. Reducing SDS-PAGE and western blot against the His-tag (light chain) of adalimumab (A) and filgrastim (B).

Based on the results shown in this chapter yeast proteases PrA and CPY appear to degrade several biologicals. However, the removal of the genes encoding these proteases from *S. cerevisiae* turned out to be an effective method to increase titers of intact biologicals in the supernatant.

4. Application of proteome-constraint genome-scale models (GEMs) for cell factory engineering.



In this chapter of this thesis, GEMs were used as an engineering guide for optimizing biological production by *S. cerevisiae*. I present results on the validation of the application of GEMs as engineering guides for recombinant protein production. The data presented in this chapter are based on **Paper IV**.

4.1 Genome scale models for S. cerevisiae.

As mentioned in the background chapter, the first GEM for *S. cerevisiae* was published in 2003 (80). Since then, the complexity and fine-tuning of the yeast GEMs have made major progress. Over the years, large numbers of metabolic reactions were added alongside information on enzyme kinetics leading to the creation of proteome- and enzyme-constrained models. With these additions, the prediction performance of the models increased tremendously and enabled the capturing of complex biological phenomena like the Crabtree effect (82, 83).

In this chapter, we focus on the use of advanced GEMs to select targets to improve the production of biologicals. After years of reverse engineering, random mutagenesis, and the screening of libraries, now also GEM-guided engineering can be implemented as an effective strategy for improving yeast cell factories. Most GEMs are based solely on metabolic reactions and therefore can only optimize metabolism to produce a product. An example of a successful application of a GEM for the engineering of a cell factory for a heterologous metabolic product was the production of sesquiterpenes. In this specific example, the authors used *in silico* testing of deleting metabolic genes and their influence on metabolic fluxes. The promising targets were implemented *in vivo*. The model suggested the deletion of glutamate dehydrogenase encoded by *GDH1*. After removal of *GDH1*, the growth was severely reduced due to cofactor imbalance, so the authors decided to overexpress *GDH2* to compensate. This strain reached a 3-fold increase in

sesquiterpene titer when a 10-fold improvement was predicted. Only a single study on the use of stoichiometric GEMs for the enhancement of production of a recombinant protein could be found. In the study of Nocon *et al*., they improved the intracellular production of human copper/zinc superoxide dismutase (hSOD) in *K. phaffi* by engineering the central carbon metabolism (84). The fluxes through the central carbon metabolism were determined experimentally and compared to the model, where the authors found an increased flux through the TCA cycle in the strain producing hSOD and linked this observation to increase in energy demand. Seven genes were suggested for upregulation and six genes for downregulation. From the upregulation targets, *SOL3*, *GDH3,* and *MDH1* and the deletion of targets *ADH2* and *GUT2* showed to improve the intracellular hSOD concentration (84).

4.2 Application of two yeast GEMs for guided engineering of S. cerevisiae for the optimized production of biologicals.

In this chapter, two proteome-constraint models were used to predict targets for optimizing the production of three biologicals. One of the models is ecYeast8, a coarse-grained proteome constrained genome-scale model (169, 170). Coarse-grained models include maximum capacities of reaction fluxes. All the metabolic reactions have constraints based on turnover rates, which include the catalyzing enzyme abundance and the corresponding k_{cat} value. The protein demand associated with the turnover rates in the cell are constrained by total proteome abundance. The addition of proteome constraints improved the predictions of the model significantly (169). Yeast8 represents the latest update of the metabolic model for *S. cerevisiae* and ecYeast8 is based on Yeast8. After the protein of interest is added to the model targets were selected by Flux scanning based on Enforced Objective Flux (FSEOF). FSEOF enforces the flux towards a specific product within the model. All the reaction fluxes that increase to facilitate the increased flux towards the product represent overexpression targets (171). All targets will be ranked for several parameters including the presence of isoenzymes and the influence of the target abundance on product increase. Additionally, EcYeast8 can simulate cumulative engineering steps and validate the combination of targets. EcYeast8 in combination with FSEOF and simulation of target combination was implemented successfully to increase the quantity of intracellular heme (85). The second GEM is pcSecYeast and is a so-called fine-grained model (170, 172). Fine-grained models contain metabolic reactions and constraints but have additional reactions for protein synthesis. Within pcSecYeast, metabolism is linked to protein synthesis and will include energetic demands and process of ribosome and enzyme synthesis. As a result, the model does not have a constant but a dynamic biomass equation depending on enzyme composition. pcSecYeast includes reactions for complex secretory mechanisms like protein translocation from cytosol to the ER, glycosylation, oxidative folding, protein degradation, and vesicle sorting. The FSEOF approach was also used with pcSecYeast to select engineering targets and includes a ranking of the targets by including if the target is part of a complex, has homologs, and whether the protein occupies a large fraction of the proteome (171, 172). These

considerations should enable the model to select the most effective targets. pcSecYeast can suggest both metabolic targets and enzymes involved in protein secretion. pcSecYeast was verified for the selection of targets to improve the production of α-amylase and selected three metabolic enzymes and fourteen enzymes from the secretory pathway (172). From the metabolic genes, only overexpression of *CYS4* showed effectiveness and for the secretory pathway, fourteen overexpression targets were selected. *SEC16*, *CWH41*, *PDI1,* and *ERV29* were previously verified for improving α-amylase production whereas *MNS1*, *SEC65*, *ERV2*, *ERO1,* and *SWA2* showed effective during the experimental validation (60, 62, 68, 172).

The input for the models includes medium composition, the amino acid sequences of the recombinant proteins, and for pcSecYeast also post-translational modifications. The three biologicals included in this study were insulin precursor, filgrastim, and adalimumab (173, 174). In the construction of adalimumab the light chain was expressed by the *TPI1* promoter and the heavy chain was expressed by the tr*ADH1* promoter (129). After obtaining the predictions from both models we found that for ecYeast8 ten targets were mutual for all three proteins and eleven targets for pcSecYeast. This led to the decision to include only common targets between the three biologicals and try to realize a chassis strain to produce these three biologicals. Additionally, ecYeast8 will predict targets to optimize metabolism whereas pcSecYeast would optimize both metabolism and secretion. Therefore, a comparison could potentially be made between the impact of metabolism versus the secretory capacity for increasing recombinant protein production.

4.3 Target genes selected by the models.

In Table 3, an overview of the selected genes per model is shown. EcYeast8 selected ten targets of which six are involved in amino acid metabolism and four encode aminoacyl-tRNA synthetases. All the genes involved in amino acid biosynthesis were involved in the biosynthesis of either arginine, isoleucine/valine, or aromatic amino acids. The aminoacyl-tRNA synthetases were surprising and interesting targets since limited literature could be found on the overexpression of these proteins in yeast. The targets selected by pcSecYeast were ranked at the highest priority targets were all involved in protein secretion. A schematic of the localization of the proteins within the secretory pathway is shown in Figure 19. Vps1 and Swa2 are involved in vesicle transport from Golgi to the endosome (175, 176). Ire1 is the transmembrane kinase/nuclease that activates the UPR via the activation of Hac1 translation and is activated due to the dissociation of Kar2 in the ER lumen (58, 177). Lhs1 and Sil1 are chaperones involved in protein transport and folding into the ER and are nucleotide exchange factors for Kar2 (177, 178). Erv2 is an ER-based flavoprotein that reoxidizes Pdi1 alongside Ero1 (179). Erv29 is a transmembrane protein involved in the transport from ER to Golgi and plays an essential role in ERAD. *SEC17*, *USO1, YPT1,* and *SEC16* all encode proteins involved in the transport between ER and Golgi. Sec17 facilitates the loading of SNARE complexes (180), Ypt1 is a GTPase from the Rab-family that mediates the transport between the ER

and Golgi (181). Besides, Ypt1 is responsible for the decay of *HAC1* mRNA and the recovery from ER stress (182). Uso1 interacts with Ypt1 and fulfills a role within directing the transport (181). Sec16 is involved in COPII vesicle formation (68). Interestingly, both models solely selected overexpression targets as priorities. This also included non-mutual targets between the three biologicals. The models can also suggest deletion or downregulation of genes.

Table 3. Overview of the selected overexpression targets suggested by the ecYeast8 and pcSecYeast models.

ecYeast8		pcSecYeast	
Target	**Fulfills a role within**	**Target**	**Fulfills a role within**
ARO1	Aromatic amino acid biosynthesis	*VPS1*	Vesicle formation Golgi
ARO2	Aromatic amino acid biosynthesis	*SWA2*	Vesicle uncoating endosome
ILV2	Isoleucine and Valine biosynthesis	*IRE1*	UPR induction ER
ILV5	Isoleucine and Valine biosynthesis	*ERV2*	Oxidative folding ER
ARG56	Arginine biosynthesis	*ERV29*	ER-Golgi transport
ARG8	Arginine biosynthesis	*LHS1*	Transport into ER
GUS1	Glutamyl-tRNA synthesis	*SIL1*	Transport into ER
MES1	Methionyl-tRNA synthesis	*SEC17*	ER-Golgi transport
THS1	Threonyl-tRNA synthesis	*USO1*	ER-Golgi transport
GLN4	Glutaminyl-tRNA synthesis	*YPT1*	ER-Golgi transport
		SEC16	ER-Golgi transport

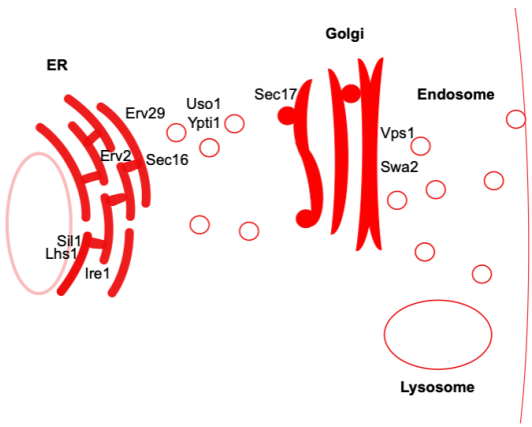


Figure 19. Schematic of the targets selected by psSecYeast and their localization in the secretory pathway.

4.4 Verification of the single overexpression targets.

The first experiments were dedicated to verifying the impact of the single overexpression of the targets. All the genes were cloned into centromeric overexpression plasmids and the yeast strains expressing the recombinant proteins were transformed with the plasmids. An obstacle for this study was the absence of a screening method that could be used at relatively high throughput. The insulin precursor concentrations were analyzed with a previously developed reversed-phase HPLC method (51). The quantification of the other

two proteins in the supernatant was based on a developed SEC-HPLC method, which is described in more detail in **Paper IV**. In short, the method measures the quantity of total secreted proteins which we assumed to be our protein of interest alongside native secreted proteins. By using a negative control, supernatant of the same background strain without recombinant protein expression, we normalized the measurements and assume that the surplus of proteins in the supernatant of recombinant protein-expressing strain is mostly our protein of interest. We compared quantities measured with the SEC-HPLC method with western blot and concluded the method to be sufficiently reliable for a semi-quantitative assessment and proceeded with the analysis of the biologicals in the supernatant.

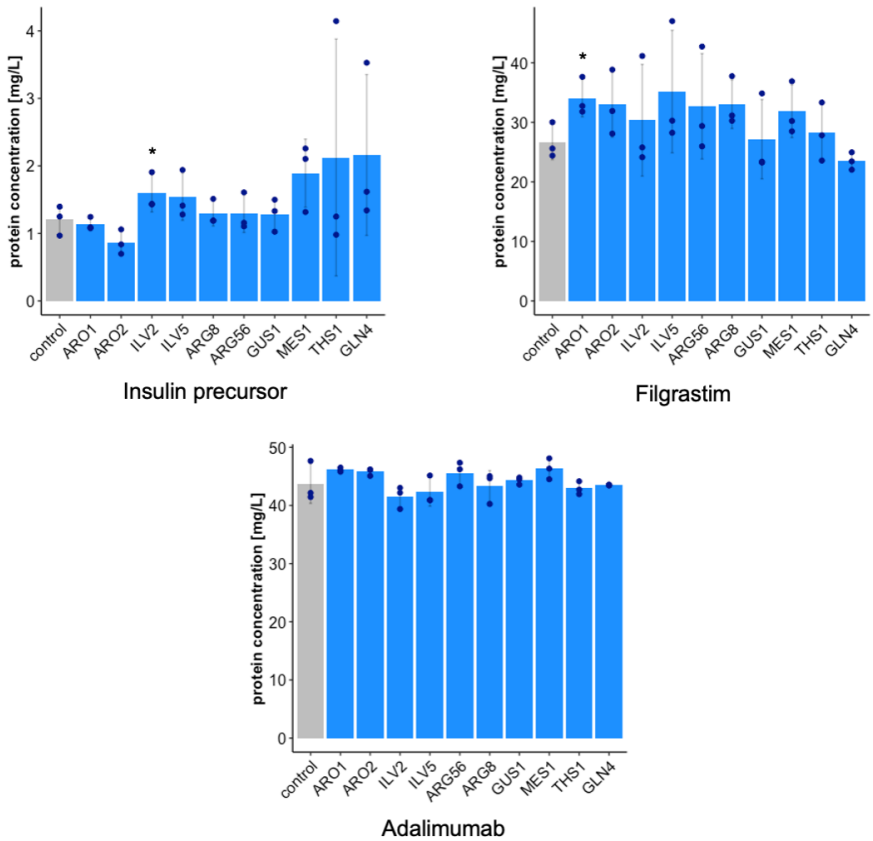


Figure 20. Concentrations of the biologicals measured in supernatant with reverse phase-HPLC or SEC-HPLC after overexpression of the targets selected by ecYeast8. Significance was determined using t-tests and * refers to P< 0.05, ** refers to P<0.01 and *** refers to P<0.005.

In Figure 20, the results of the overexpression targets selected by ecYeast8 are presented. A small number of overexpression targets led to a significant difference compared to the

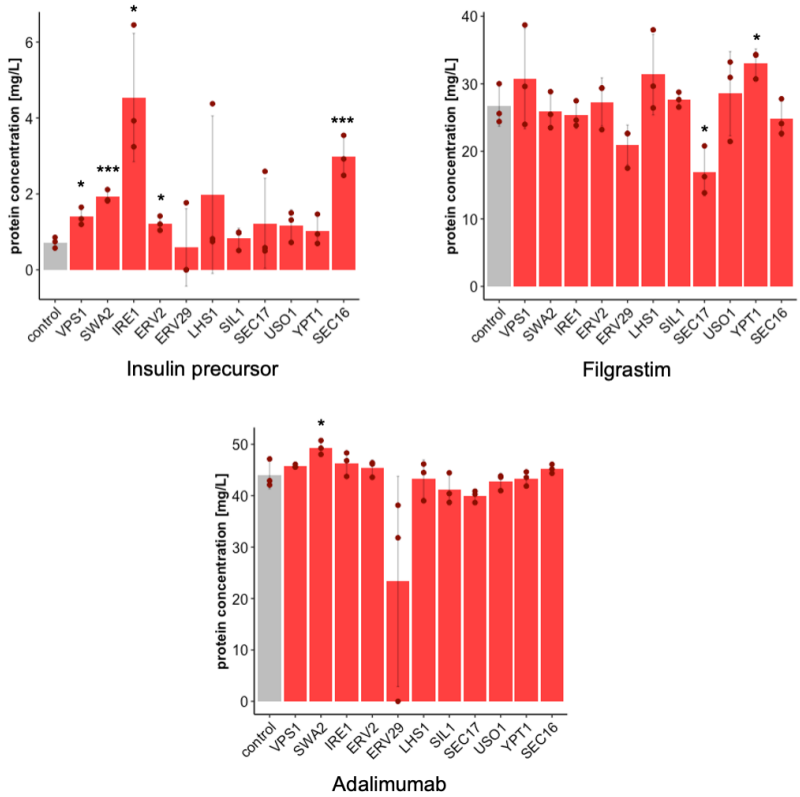


Figure 21. Concentrations of the biologicals measured in supernatant with reverse phase HPLC or SEC-HPLC after overexpression of the targets selected by pcSecYeast. Significance was determined using t-tests and * refers to P< 0.05, ** refers to P<0.01 and *** refers to P<0.005.

control. Overexpression of *ILV2* for insulin precursor and *ARO1* for filgrastim resulted in modest improvements in recombinant protein titers.

The results of the overexpression of the targets from pcSecYeast are shown in Figure 21. Overexpression of *VPS1*, *SWA2*, *IRE1*, *ERV2*, and *SEC16* had a positive influence on the titer of insulin precursor, and overexpression of *SWA2* had a positive influence on the adalimumab titer. The filgrastim titer was increased by overexpression of *YPT1* and reduced by overexpression of *SEC17*. For insulin production, the overexpression of *IRE1* and *ERV2* showed effective which indicates that increased folding capacity in the ER stimulates insulin precursor production. *VPS1* and *SWA2* are involved in the vesicle formation from Golgi to endosome indicating another bottleneck in insulin precursor production. In the original publication of pcSecYeast, α-amylase was used as a model protein. Interestingly, *SEC16*, *ERV29*, *USO1*, *IRE1*, *ERV2*, *SWA2*, and *VPS1* were also suggested as targets to increase α-amylase production. Overexpression of *SEC16*, *ERV29*, *ERV2*, and *SWA2* had been shown effective to increase α-amylase production (60, 68, 172) . Overexpression on *USO1*, *IRE1*, and *VPS1* did not have an impact on the α-amylase

titer in the original study on pcSecYeast (172). However, *VPS1* and *IRE1* improved the production of insulin precursor in our study.

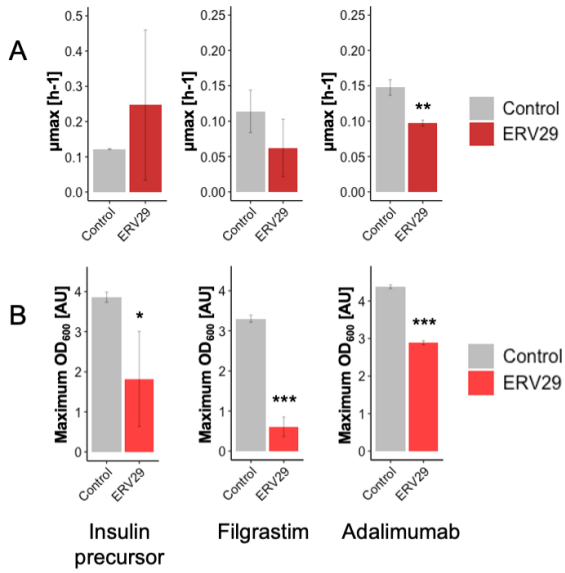


Figure 22. Specific maximum growthrates (A) and OD_{600} (B) of the controls and the strains overexpressing *ERV29*. Significance was determined using t-tests and * refers to P< 0.05, ** refers to P<0.01 and *** refers to P<0.005.

Overexpression of *ERV29* led to a growth defect in all three strains biological producing, shown in Figure 22A and 22B. Previously, overexpression of *ERV29* was proven successful in improving α-amylase production in *S. cerevisiae* (183, 184). In those two studies, no growth defect was observed. In our experiment, we used a low-copy plasmid combined with expression from the *TEF1* promoter. In one of the other studies, it was observed that the single overexpression of *ERV29* resulted in improved α-amylase production, but if the overexpression of *ERV29* was combined with overexpression of *GOS1* the α-amylase titer would reduce. This was observed using both a high and low-copy plasmid. The authors used another expression system for α-amylase, namely p426GPD amylase with amylase under the control of the p*GPD* promoter (60). Due to the negative impact on growth physiology, *ERV29* was excluded for the remainder of the study.

4.5 Verification of combined strains.

After the verification of the single overexpression targets, the next step was to combine the overexpression targets. ecYeast8 simulated the combination of the engineering targets. The overexpression targets per model were combined in two sets which were later combined in one final strain. The intermediates were called M1, M2, S1, and S2, and the final strains M12 and S12. The construction of the combined strains proved challenging. Overexpression of *ARO1* was in the end excluded from the combined strains M1 and M12, and integration of the expression cassette of *USO1* and *SIL1* could not be realized for filgrastim and insulin precursor in the S12 strain. Also, the p*PGK1* promoter integration upstream of *SEC16* was not achieved in the S12 strain expressing filgrastim. Details on the strains are presented in Table 4.

Table 4. overview of strain names and integrations cassettes or promoter integration.

		Cassette integration		
		p*TEF1*-t*CYC1*	p*PGK1*-t*ADH1*	p*PGK1* integration
M12	M1		*ARO2*	
	M1	*ILV2*	*ILV5*	
	M1	*ARG8*	*ARG56*	
	M2	*GUS1*	*MES1*	
	M2	*THS1*	*GLN4*	
S12	S1	*VPS1*	*SWA2*	
	S1	*IRE1*	*YPT1*	
	S1	*ERV2*	*LHS1*	
	S2	*SIL1*		
	S2	*SEC17**	*USO1**	
	S2			*SEC16***

* Was not integrated in the insulin precursor and filgrastim producing strain S12.
** Was not integrated in the filgrastim producing strain S12.

In Figure 23 the results are shown of the M1, M2 and M12 strains. Insulin precursor titer was determined with reversed-phase HPLC and was improved in the M12 strain compared to the control (Figure 23A). Filgrastim and adalimumab titers were determined with western blot. The filgrastim titer was determined by western blot against the His-tag attached to filgrastim in the supernatant (18.8 kDa). The titer was improved in the M12 strain but further validation is required. The control for filgrastim in that experiment grew slower compared to the M1, M2 and M12 mutants which could have contributed to the low titer of filgrastim for the control. The M1 and M2 mutants show variable increases in titer (Figure 23B). For adalimumab, western blots were performed against the His-tag attached to the light chain (25 kDa) and the Flag-tag attached to the heavy chain (50 kDa) (Figure 23C and 23D).

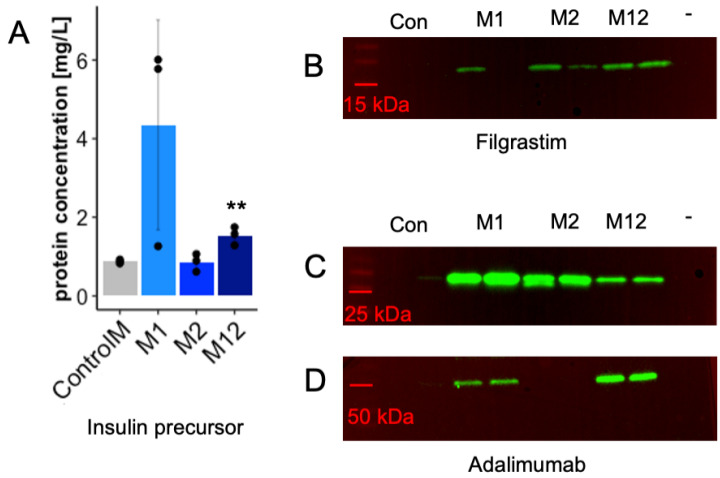


Figure 23. Insulin precursor, filgrastim and adalimumab production after combined integration of the targets selected by ecYeast8. The strains after integration of the target were analysed after 48-h or 72-h cultivation in aerated 96-well plates. (A) Concentration of Insulin precursor determined by reversed phase-HPLC. These concentrations were determined after 72-h of cultivation. (B) Western blot against the His-tag attached to filgrastim. (C&D) Western blot against the His-tag attached to the light chain (C) or against the Flag-tag attached to the heavy chain of adalimumab (D). Con contain supernatant from cultivation with background strains. M1 lanes contain supernatant from cultivation with M1 strains. M2 lanes contain supernatant from cultivation with the M2 strains. M12 lanes contain supernatant produced by the M12 strains. – lane contains supernatant from cultivation with background strains without expression a biological. These concentration were determined after 48-h of cultivation. Significance was determined using t-tests and * refers to P< 0.05, ** refers to P<0.01 and *** refers to P<0.005.

The results show that the overexpression of the genes involved in amino acid biosynthesis improved the titer of both the light and the heavy chain. The M2 strain with the tRNA synthetases overexpressed showed only improvement of the light chain production. The M12 strain shows mostly increased production of the heavy chain and a lower titer of the light chain. The folding of antibodies is a complex mechanism where the folding of the heavy chain depends on the light chain (130). The results indicate that the M12 strain led to the highest production of the heavy chain. However, also these data need further validation as the control showed low growth in this experiment.

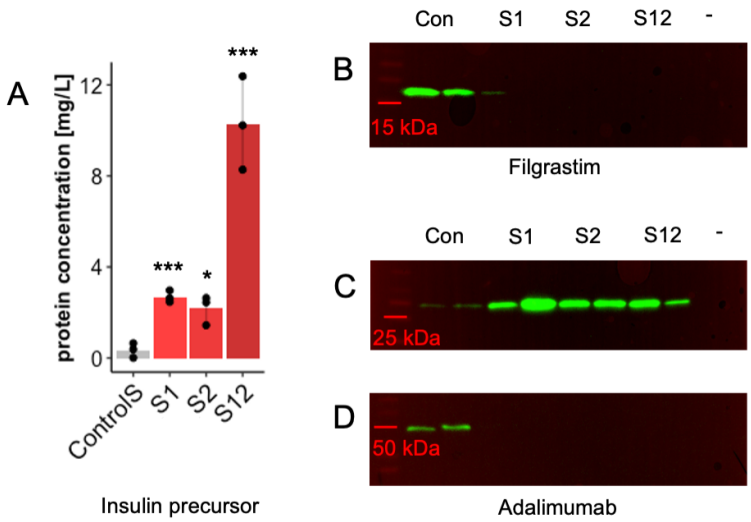


Figure 24. Insulin precursor, filgrastim and adalimumab production after combined integration of the targets selected by pcSecYeast in the genome. The strains after integration of the target were analysed after 48-h cultivation in aerated 96-well plates. (A) Concentration of Insulin precursor determined by reversed. (B) Western blot against the His-tag attached to filgrastim. (C&D) Western blot against the His-tag attached to the light chain (C) or against the Flag-tag attached to the heavy chain of adalimumab (D). Con contain supernatant from cultivation with background strains. S1 lanes contain supernatant from cultivation with S1 strains. S2 lanes contain supernatant from cultivation with the S2 strains. S12 lanes contain supernatant produced by the S12 strains. – lane contains supernatant from cultivation with background strains without expression a biological. Significance was determined using t-tests and * refers to P< 0.05, ** refers to P<0.01 and *** refers to P<0.005.

In Figure 24, the results for the combined integration of the targets selected by pcSecYeast are shown. In Figure 24A, the titers of insulin precursor are shown. The combined overexpression of the targets shows a positive influence on the insulin titer in all three combinations. However, the highest titer is reached by the S12 strain with a more than 10-fold increase of insulin precursor compared to the control. In Figure 24B, the western blot shows that the combined overexpression of the targets did not improve filgrastim titers. In Figures 24C and 24D, the western blots of adalimumab show that the heavy chain titers were reduced in the strain with the combined overexpression of the targets, but the light chain titers do show an increase. These results suggest that the combination of the pcSecYeast targets improves the secretion of smaller or easy-to-fold proteins.

Overall, both GEMs suggested valuable targets to improve the production of one or all biologicals. The targets selected by ecYeast8 did not improve titer when the targets were overexpressed as single genes, but the combined overexpression indicates an improvement for all three proteins based on these initial results.
This study was performed on Delft minimal media which means that all the amino acids need to be synthesized by *S. cerevisiae* rather than taken up from the medium. Most other studies regarding recombinant protein production are performed with rich media, either

YPD or amino acid supplementation since this was shown to improve productivity due to reduced metabolic costs (185–187). In this study, our results indicate that overexpression of several genes in the biosynthesis of amino acids also improves production. Additionally, the overexpression of the amino acid biosynthesis genes in combination with the overexpression of aminoacyl-tRNA synthetases seems even more effective. This is interesting since the aminoacyl-tRNA synthetases and the genes selected from the biosynthetic pathways target different amino acids. The aminoacyl-tRNA synthetases include targeting the synthesis of tRNAs with methionine, glutamate, threonine, and glutamine, whereas the genes from the biosynthetic pathway are involved with the synthesis of arginine, valine, and aromatic amino acids.

For pcSecYeast different results were obtained. Since the model did not simulate the combined overexpression of the targets, we expected the single overexpression targets to have the most impact, but a very significant improvement was observed for combined integration. The highest increase in production was obtained for the insulin precursor both during the overexpression of the single target and for the combined integration of the target genes. For filgrastim and adalimumab, moderate results were obtained both for the overexpression of the single targets and the combined integration of the genes. An interesting result however was the increase of titer only for the light chain of adalimumab. This observation, together with the improvement of the insulin precursor in the combined strain, indicates that the secretion of smaller easy-to-fold proteins benefited from the optimization of secretion achieved in the combined strain but the secretion of the more complex proteins did not.

Based on these initial results ecYeast8 indicates to be a GEM with the capacity to select targes that improve recombinant protein production. Also, the feature to simulate the combination of several targets can lead to the improvement of titers of several proteins and therefore the construction of an improved cell factory for biologicals. For pcSecYeast excellent results were obtained for the improved production of insulin precursor with a 10-fold increase. At the moment, the results indicate both metabolism and secretion fulfill important roles in the production of recombinant proteins. However, to draw any further conclusions more experiments need to be done to confirm our initial observations.

5. Conclusion and perspectives.

The overall goal of this thesis was to implement different strategies to improve the production of biologicals in *S. cerevisiae*. In the different chapters of this thesis, several different approaches to increase protein production have been presented. In **Chapter 2,** I discussed the removal of the kinase Gcn2 and the role of this kinase in recombinant protein production. In **Chapter 3,** the degradation of biologicals by vacuolar proteases by *S. cerevisiae is* addressed and how this can reduce titers of biologicals, and in **Chapter 4,** I demonstrated the implementation of proteome constrained models as engineering guides to increase the production of biologicals.

Conclusions.

When engineering yeast to improve the production of recombinant proteins most studies focus on secretion. Often applied strategies are the optimization of protein folding and translocation through the secretory pathway. There has been very limited interest in the role of translation rate and control. In our study, we showed the improvement of α-amylase production in two yeast strains by removal of the kinase Gcn2. We hypothesized that elevated levels of cytosolic H_2O_2, induced by recombinant α-amylase production led to the activation of the kinase Gcn2. We showed that the strain B184 had increased levels of cytosolic H_2O_2 under α-amylase production and upon removal of the kinase the α-amylase titer increased. Also, we observed increased antioxidant protein transcripts, a reduction in the level of cytosolic H_2O_2 and increased transcripts of several foldases including *PDI1*. We were unable to fully clarify the underlying mechanism of our observations. Nevertheless, our study did show that the kinase Gcn2 indeed reduces protein synthesis upon recombinant protein production but, in addition, that it is connected to the UPR and H_2O_2 concentration which, to the best of our knowledge, are novel findings in *S. cerevisiae*. Finally, we tried if the removal of the kinase Gcn2 would also improve the production of the antibody adalimumab and did not obtain similar improvement results as for α-amylase.

Perspectives.

Regarding the future and the impact of the kinase Gcn2 on recombinant protein production, the removal of the kinase Gcn2 in the yeast strain B184 in combination with the production of α-amylase was a proof of concept. However, the broader applicability of the deletion as a target for recombinant protein production is to be seen. The increase in the α-amylase production upon removal of the kinase in K17 was less profound and we did not reach similar increased production with adalimumab based on the experiments presented. However, would I recommend further validation of those results. An additional experiment here that would provide more insight is to use the Prx1-roGFP2 GEFI and monitor the cytosolic levels of H_2O_2 in B184 under adalimumab producing conditions to see if increases in cytosolic H_2O_2 can be observed. If no such increase is observed there is more support for the link between Gcn2 kinase activity and cytosolic level of H_2O_2 and would explain why we do not observe an increase of adalimumab during the initial experiments. Something we did not investigate at the time is that in addition to activation of Gcn2 leading to the phosphorylation of eIF2α also the activity of the phosphatase would dephosphorylate eIF2α could play a role. In a previous study, B184 was analyzed by transcriptome analysis and it was found that *ADR1* was downregulated in B184 compared to AAC (76). *ADR1* encodes one of the regulators of Glc7, the responsible phosphatase that should dephosphorylate eIF2α (188). Additional experiments on the activity of Glc7 would be an interesting follow-up on the mechanism regulating eIF2α phosphorylation in B184 under α-amylase-producing conditions. Since both B184 strains

showed phosphorylation of eIF2α at the 48 h timepoint but only B184 producing α-amylase at 96 h, it can be that both the activities of Gcn2 or the phosphatase are modulated under α-amylase-producing conditions. The reduction of translation in B184 producing α-amylase complicates the design of an experiment around the expression of Glc7. Additionally, the regulation of Glc7 is complex and dependent on regulators like Glc8 [189]. However, one could mutate the eIF2γ subunit of eIF2 as described in the study of Rojas *et al*. [190]. The eIF2γ subunit is responsible for recruiting Glc7 for the eIF2α dephosphorylation [190]. By mutating the eIF2γ subunit the recruitment of Glc7 will be reduced. One could compare the phosphorylation of eIF2α in B184 with a mutated eIF2γ. If the phosphorylation of eIF2α remains in B184 without α-amylase production during later time points as well this would indicate that the activity of Glc7 is also part of the prolonged phosphorylation state of eIF2α in B184 while producing α-amylase.

If the kinase Gcn2 will have a role as a general engineering target for *S. cerevisiae,* further investigations regarding recombinant protein production, cytosolic H_2O_2, and sole yeast kinase of eIF2a would be desired. Especially the link between the UPR and the Gcn2 would be interesting to explore further. Obvious experiments like the addition of ER stress-inducing compounds like DTT or tunicamycin did not reduce translation initiation in *S. cerevisiae*, which indicates that the UPR may not activate Gcn2 directly [191]. Also, in the study where the impact of H_2O_2 on Gcn2 activation is demonstrated, the authors show that Gcn2 activity is only partly responsible for the reduced rate in translation. They also observed inhibition of post-initiation translation which could also be an interesting aspect to study in more detail in relation to recombinant protein production.

Nevertheless, our findings showed a connection between cytosolic H_2O_2, the kinase Gcn2, and the UPR. Further unraveling the mechanism underlying these observations would be a contribution to the yeast cellular biology field with the potential for the engineering of cell factories.

Since the publication of **Paper I** several other studies have been published that focus on translational control in yeast. Recently, two studies were published addressing this topic in *K. phaffi*. One was on a method to quantify translation rate and in this study, the authors showed that the removal of the kinase Gcn2 from *K. phaffi* results in an increased translation rate under glucose-limited conditions (192). In a second study, they examined the role of a large number of translational factors and implemented overexpression of single or combined closed-loop translation initiation factors to improve the productivity of a nanobody protein and human serum albumin (193). These recent publications and **Paper I** show the increasing interest in the role of translation rate on recombinant production in yeast.

5.2 The removal of vacuolar proteases for yeast as a production host for recombinant proteins.

Conclusions.

The results of the study of the production of Affibody molecules by *S. cerevisiae* showed that Affibody molecules and more specifically the ABD are subjected to degradation by vacuolar proteases. We showed that B184 produced intact filgrastim, even though filgrastim was completely degraded by K17, whereas B184 did degrade the ABD. The removal of *PEP4* and *PRB1* or *PEP4* and *PRC1* led to the intact production of Affibody molecules and other biologicals in *S. cerevisiae*. After realization of the production of intact Affibody molecules, we verified the binding kinetics of the produced Affibody molecules by yeast and performed a high production experiment in which we reached a final Affibody titer above 0.5 g Z_{HER3_1}-ABD-Z_{HER3_1}/L. The subchapter on the removal of proteases from strain K17 has shown how the deletion of *VPS5* resulted in the degradation of BSA. After the removal of the genes of PrA and CPY, the degradation of BSA was reduced and higher titers of secreted biologicals were observed in the supernatant. Based on these results we conclude that *S. cerevisiae* secretes vacuolar proteases that reduce the titers of several recombinant proteins. Removal of PrA combined with PrB or CPY can reduce the degradation and improve the final recombinant protein titers obtained.

Perspectives.

Regarding the production of Affibody molecules, several additional experiments can be performed. For now, we only tested Z_{HER3_1}-ABD-Z_{HER3_1} in the fed-batch experiment and binding kinetics assay. Additional experiments regarding the production of Z_{HER3_1}-ABD and Z_{HER3_1}-Z_{HER3_1}-ABD by B184 *pep4Δprc1Δ* or B184 *pep4Δprb1Δ* would be an addition to verify that *S. cerevisiae* can produce all the Affibody molecules in this study. Additionally, I would suggest optimizing the fed-batch production experiment to increase the final titer. A final titer of 0.5 g/L was reached without any optimization of the setup. Looking back at the data from the fed-batch experiment the concentration of Affibody molecules was increasing rapidly in the final stages. If the experiment would have continued longer a higher titer could potentially have been achieved.

The deletion of *VPS5* from K17 resulted in the severe degradation of BSA. After removal of the genes of PrA and CPY, the degradation of BSA reduced and higher titers of secreted biologicals were observed in the supernatant. K17 was presented in the original study as an efficient producer of α-amylase and glucan 1,4-α-glucosidase (60). In that publication SDS-PAGE or western blot results were absent. We showed that α-amylase remained stable in the supernatant but did not test for the α-amylase production in K17 *pep4Δprc1Δ* and therefore we cannot exclude that the α-amylase is at least partially degraded as well. The degradation by K17 might have been noticed by the authors if they had included an SDS-PAGE and/or western blot in the original study. The secretion of CPY due to missorting of an *S. cerevisiae vps5Δ* mutant was previously known and is a common

phenotype for several *VPS* deletions (167, 194). Therefore, a take-home message from this chapter is that SDS-PAGE and western blots analysis should be included when working with yeast as a recombinant protein production host.

The secretion of proteases by yeast has been known for decades. Most reviews on the use of yeast as a host for recombinant protein production include a chapter on the degradation of recombinant protein by yeast. However, the application of this knowledge is absent in many studies regarding recombinant protein production or at least not mentioned. In our studies, we observed degradation by three different *S. cerevisiae* strains, K17, B184 and degradation by AACk was shown in **Paper III**. B184 and K17 both have AACk as background strain so the coherence in their degradation phenotypes was to be expected. AACk is derived from the CEN.PK *S. cerevisiae* strain background. CEN.PK was characterized 20 years ago as having several beneficial traits for cell factory research (195). It is therefore likely that many laboratory studies on recombinant protein production with *S. cerevisiae* have been performed in CEN.PK strains without the awareness of the degradation phenotype. For the future, I would suggest that the removal of vacuolar proteases should become standard practice when working with yeast cell factories to produce recombinant proteins.

Fortunately, the removal of proteases is applied in the field but seems to be more centered around the actual application of yeasts as recombinant protein production hosts rather than research dedicated to the topic. In *K. phaffi* the removal of proteases is implemented in several commercial strains like, pps-9016, SMD1163, PichiaPinkTM and SuperMan5, in which *PEP4* is removed and in some also *PRB1* (196). Based on our observations the combination of removal of *PEP4* and *PRB1* reduces the degradation significantly and in **Paper III** we showed that there is limited impact of removing these proteases on the growth. In a study where *S. cerevisiae* was engineered specifically for the production of human proteins, the authors did remove *PEP4* and *PRB1* and increased the titer of human interferon 10-fold and did not observe a severe growth penalty in the absence of the proteases (197).

5.3 The application of GEMs as guides for the engineering of cell factories to produce biologicals.

Conclusions.

Two proteome constrained models, ecYeast8 and pcSecYeast, were applied as cell factory engineering guides to produce biologicals. After the initial simulations, several targets common for the three biologicals of interest, insulin precursor, filgrastim, and adalimumab, were selected. Those, in total twenty-one, targets were first overexpressed at single targets and later combined by strain engineering. Based on our results both GEMs appear to predict accurate targets to improve the production of biologicals. The targets selected by ecYeast8 had a more positive impact in the combined engineered strain compared to the single overexpression of the targets based on our current experiments. The combination of all the selected targets by ecYeast8 improved the production of insulin precursor, filgrastim, and adalimumab, all our proteins of interest. The targets selected by pcSecYeast showed effectiveness for the increased production of insulin precursor. The combination of the targets selected by pcSecYeast resulted in a 10-fold improved production in insulin precursor production. Based on our current results GEMs show useful tools for guided engineering to improve recombinant protein production.

Perspectives.

The study has several interesting aspects however it should be noted that additional experimental validation is required. The results of the combined integration of the strain were based on single experiments. However, the current results indicate several things. First, they indicate that optimization of amino acid metabolism improves recombinant protein production in synthetic media. The results in this study provide new insights into the use of the native amino acid metabolism of yeast rather than to use of medium supplements. A second interesting aspect was the suggested overexpression of aminoacyl-tRNA synthetases, which, to the best of our knowledge, are new targets for the optimization of cell factories.

The ecYeast8 model did prove very effective for target selection to increase intracellular heme where a 70-fold increase was achieved by eleven target deletions/overexpressions (85). Our study currently supports that this model performs well as a guide for cell engineering, even for recombinant protein production. To continue this study more experimental validation of the filgrastim and adalimumab concentration is required. For now, we used an SEC-HPLC method but preferably another more sensitive method should be applied to quantify the protein concentrations.

PcSecYeast also showed promising results, but mostly for insulin precursor production. I suspect that the results obtained by this model, especially for the overexpression of the single targets suffered under the use of the SEC-HPLC method (**Paper IV**). In hindsight, this method was potentially not a sensitive enough setup to validate those overexpression

titers. Unfortunately, pcSecYeast does not have the capacity, momentarily, to verify the combination of targets. For the future, it would make an interesting addition to the model. The combination of the targets for insulin increased the production 10-fold in synthetic media, which shows the potential influence of optimizing secretion for the increase in recombinant protein titers. For the continuation of this study, it would have been interesting to combine the targets selected by both models into one strain and verify the titers could be reached when all twenty-one targets are combined.

Some final suggestions for the continuation of the study would be to finetune the overexpression of the targets. In this study, we did not compare the actual expression levels or enzyme activity after overexpression of the proteins. Especially since we used non-native promoters to the genes, p*TEF1,* and p*PGK1* to overexpression the proteins. A recent study was published on the use of a deep neural network to analyze the influence of DNA sequences of the non-coding DNA flanking a gene, the promoter, terminator, and gene itself on gene expression. The authors found that the use of 'strong' or 'weak promoters can differ in actual expression strength based on the combination of gene and terminator (198). Potentially one could use their model to simulate the combination of the selected target genes with these two promoters and terminators and predict the actual expression of the targets. The level of overexpression of *SEC16* showed to have a large impact on the α-amylase titer (68). On the same note, the overexpression of *ERV29* led to growth retardation in this study which does not align with the literature. In the future, I would first repeat the cloning of *ERV29* and/or try to adjust the expression levels to see if that improves the growth retardation and potentially product titers.

Based on our current dataset both coarse- and fine-grained GEMs show useful tools to predict targets to improve the production of recombinant proteins. Also, other advanced GEMs are now being published like the recent pcYeast8 which contains compartmentalization of organelles *S. cerevisiae* and models transcription and translation (199). The modeling of translation would be interesting to simulate in relation to recombinant protein production based on our findings in **Chapter 1**. One of the ongoing challenges to this day in recombinant protein production research is that engineering approaches for one protein show less significance or are ineffective to increase the production of another protein. Potentially the use of these models could guide research to detect bottlenecks and improve protein-independent productivity and increase our understanding of why certain strategies work for one protein and do not for another.

6. Acknowlegdements.

First of all, I want to thank my main supervisor Verena, who was the only supervisor who was there through my whole PhD. You were present during my first interview and while other supervisors came and left, you remained. I have learned so much from you during the last four years, how to perform research, write papers and be a scientist. You did not let me cut any corners and even when reviewers did not have comments on manuscripts, you did. This was a moment I will remember you by since I think it really shows the supervisor you are (in a good way). Also, during the writing of my thesis, you made me step up a notch which I am very grateful for. Secondly Mikael, you have also fulfilled a big role within my supervision. First as collaborator and then as a second supervisor. I admire your passion for your work, you really are unsatiable. Your enthusiasm led you to push me always to do more experiments, but also translated into the endless surprised and excited reactions to new data. Now that you have your office at the ground floor I try waving at you sometimes in the morning but you cannot be disturbed. I always think you must be reading about peroxiredoxins and what they are up to. I would also like to thank Dina and Martin. Even though the supervision was temporary I also learned a lot from you and your supervision. Additionally, I would like to thank Ivan and Jens for being my examiners.

Secondly, I would like to thank all my collaborators. All the collaborators from Affibody AB and more specifically, Anna, Finn, and Karin, I really enjoyed working with you on the Affibody project. All of you were always very interested and invested in the project. Additionally, when I would ask for help regarding experiments or antibodies you all always provided everything without any hesitation. Marta, thank you for the help with the fed-batch experiment. I realize it could not have been easy to work with someone as chaotic as me. Thank you Kate for the setup of the biosensors and Cecilia for the help with the Gcn4 expression experiment. And finally, Iván and Feiran for help with the modelling project, I can imagine that explaining me how genome models work and what proteome constraints are can be a tedious experience.

Next are all the other people from Sysbio and Indbio I met at work during the last 4 years. To work in a place as diverse and big as Sysbio/Indbio was a truly unique experience. I have met so many great people in these divisions and have many good memories of the after works, lunches, fikas, BBQs, parties, game night and movies. Especially during a time as strange as the last two years, I felt very lucky to be able to come to work and spend time with you all. A special shoutout to ‘the office’. It really is quite funny since this office did not change for 3 years, and therefore it felt a bit like time stood still in there. But John, Dany, Max, Oliver and Xiaowei I will miss all the chats and joking around. A special thanks to Ela and Feiran for proofreading my thesis. Another special thanks to the research engineers for running the lab and the people working in administration for enabling us to do our jobs.

Offcourse also a big thanks to my other friends who I met here in Göteborg; Doris, Monika, Emmy, Sina, Sonali, Boyana, Franzi, Laura, Eliana, Lisa, and Anandi. The last four years are filled with fond memories of you all. Especially when living abroad your friends are very important and you all marked the last four years in a special way. Also, all the people I played squash with during the last years it was always something I would look forward to during the week and I apologize if I hit you with the squashbal.

Additionally my friends in the Netherlands cannot be forgotten. All my friends who spend hours on the phone with me but also all of you who came and visited or will visit me here in 'Geitenberg'. Several of you are coming now for the second time for the defense and it really means a lot to me.

And finally, my family which really is a base of unconditional support. Mam, Pap, Daav en A, I am very aware and grateful for all of you. Even though I really enjoyed living abroad the last 4.5 years I really missed spending time with you. And offcourse Antonio, you have been such a support for me here in Göteborg and I feel very lucky to have you in my life.

7. References.

1. McGovern PE, Zhang J, Tang J, Zhang Z, Hall GR, Moreau RA, Nuñez A, Butrym ED, Richards MP, Wang CS, Cheng G, Zhao Z, Wang C. 2004. Fermented beverages of pre- and proto-historic China. Proc Natl Acad Sci U S A 101:17593–17598.
2. Parapouli M, Vasileiadis A, Afendra AS, Hatziloukas E. 2020. *Saccharomyces cerevisiae* and its industrial applications. AIMS Microbiol 6:1–31.
3. Kung SH, Lund S, Murarka A, McPhee D, Paddon CJ. 2018. Approaches and recent developments for the commercial production of semi-synthetic artemisinin. Front Plant Sci 9:1–7.
4. Wang G, Huang M, Nielsen J. 2017. Exploring the potential of *Saccharomyces cerevisiae* for biopharmaceutical protein production. Curr Opin Biotechnol 48:77–84.
5. Bro C, Regenberg B, Förster J, Nielsen J. 2006. *In silico* aided metabolic engineering of *Saccharomyces cerevisiae* for improved bioethanol production. Metab Eng 8:102–111.
6. Zeng BX, Yao MD, Wang Y, Xiao WH, Yuan YJ. 2020. Metabolic Engineering of *Saccharomyces cerevisiae* for Enhanced Dihydroartemisinic Acid Production. Front Bioeng Biotechnol 8:1–11.
7. Chen X, Ji B, Hao X, Li X, Eisele F, Nyström T, Petranovic D. 2020. FMN reduces Amyloid-β toxicity in yeast by regulating redox status and cellular metabolism. Nat Commun 11:1–16.
8. Farrugia G, Balzan R. 2012. Oxidative stress and programmed cell death in yeast. Front Oncol 2:1–21.
9. Botstein D, Fink GR. 2011. Yeast: An experimental organism for 21st century biology. Genetics 189:695–704.
10. Belda I, Ruiz J, Santos A, Van Wyk N, Pretorius IS. 2019. *Saccharomyces cerevisiae*. Trends Genet 35:956–957.
11. Goffeau A, Barrell BG, Bussey H, Davis RW, Dujon B, Feldmann H, Hoheisel JD, Jacq C, Johnston M, Louis EJ, Mewes HW, Murakami Y, Tettelin H, Oliver SG. 1996. Life with 6000 Genes. Science 274:563–567.
12. Crick F. 1970. Central Dogma of Molecular Biology. Nature 227:561–563.
13. Cohen SN, Chang AC, Hsu L. 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc Natl Acad Sci U S A 69:2110–2114.
14. Morrow JF, Cohen SN, Chang ACY, Boyer HW, Goodman HM, Helling RB. 1974. Replication and transcription of eukaryotic DNA in *Escherichia coli*. Proc Natl Acad Sci U S A 71:1743–1747.
15. Scherer S, Davis RW. 1979. Replacement of chromosome segments with altered DNA sequences constructed *in vitro*. Proc Natl Acad Sci U S A 76:4951–4955.
16. Sue C, Miller H, Witherow DS. 1993. Molecular Biology Techniques. Anal Chem 65:12.
17. Waller J V., Kaur P, Tucker A, Lin KK, Diaz MJ, Henry TS, Hope M. 2020. Diagnostic Tools for Coronavirus Disease (COVID-19): Comparing CT and RT-PCR Viral Nucleic Acid Testing. Am J Roentgenol 215:834–838.
18. Thermes C. 2014. Ten years of next-generation sequencing technology. Trends Genet 30:418–426.
19. David F, Siewers V. 2015. Advances in yeast genome engineering. FEMS Yeast

Res 15:1.
20. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. 2012. A Programmable Dual-RNA – Guided DNA Endonuclease in Adaptive Bacterial Immunity 337:816–822.
21. Heidari R, Shaw DM, Elger BS. 2017. CRISPR and the Rebirth of Synthetic Biology. Sci Eng Ethics 23:351–363.
22. Meissner TB, Mandal PK, Ferreira LMR, Rossi DJ, Cowan CA. 2014. Genome editing for human gene therapy. Methods in Enzymology, 1st ed. Elsevier Inc.
23. White Junod S. 2019. Celebrating a milestone: FDA's approval of first genetically-engineered product. Am Counc Sci Heal 6 (4).
24. Walsh G. 2014. Biopharmaceutical benchmarks 2014. Nat Biotechnol 32:992–1000.
25. Walsh G. 2018. Biopharmaceutical benchmarks 2018. Nat Biotechnol 36:1136–1145.
26. Sanchez-Garcia L, Martín L, Mangues R, Ferrer-Miralles N, Vázquez E, Villaverde A. 2016. Recombinant pharmaceuticals from microbial cells: A 2015 update. Microb Cell Fact 15 (33).
27. Overton TW. 2014. Recombinant protein production in bacterial hosts. Drug Discov Today 19:590–601.
28. Bhatwa A, Wang W, Hassan YI, Abraham N, Li XZ, Zhou T. 2021. Challenges Associated With the Formation of Recombinant Protein Inclusion Bodies in *Escherichia coli* and Strategies to Address Them for Industrial Applications. Front Bioeng Biotechnol 9:1–18.
29. Schneier M, Razdan S, Miller AM, Briceno ME, Barua S. 2020. Current technologies to endotoxin detection and removal for biopharmaceutical purification. Biotechnol Bioeng 117:2588–2609.
30. Mamat U, Wilke K, Bramhill D, Schromm AB, Lindner B, Kohl TA, Corchero JL, Villaverde A, Schaffer L, Head SR, Souvignier C, Meredith TC, Woodard RW. 2015. Detoxifying *Escherichia coli* for endotoxin-free production of recombinant proteins. Microb Cell Fact 14 (1).
31. Wacker M, Linton D, Hitchen PG, Nita-Lazar M, Haslam SM, North SJ, Panico M, Morris HR, Dell A, Wren BW, Aebi M. 2002. N-linked glycosylation in *Campylobacter jejuni* and its functional transfer into *E. coli*. Science 298:1790–1793.
32. Yee CM, Zak AJ, Hill BD, Wen F. 2019. The Coming Age of Insect Cells for Manufacturing and Development of Protein Therapeutics. Burger's Med Chem Drug Discov 57:289–344.
33. Rettenbacher LA, Arauzo-Aguilera K, Buscajoni L, Castillo-Corujo A, Ferrero-Bordera B, Kostopoulou A, Moran-Torres R, Núñez-Nepomuceno D, Öktem A, Palma A, Pisent B, Puricelli M, Schilling T, Tungekar AA, Walgraeve J, Humphreys D, von der Haar T, Gasser B, Mattanovich D, Ruddock L, van Dijl JM. 2021. Microbial protein cell factories fight back? Trends Biotechnol 40:576–590.
34. Van Landuyt L, Lonigro C, Meuris L, Callewaert N. 2019. Customized protein glycosylation to improve biopharmaceutical function and targeting. Curr Opin Biotechnol 60:17–28.
35. Schmidt FR. 2004. Recombinant expression systems in the pharmaceutical industry. Appl Microbiol Biotechnol 65:363–372.
36. Nielsen J. 2013. Production of biopharmaceutical proteins by yeast.

Bioengineered 4:207–211.
37. Thompson DM, Lu C, Green PJ, Parker R. 2008. tRNA cleavage is a conserved response to oxidative stress in eukaryotes. RNA 14:2095–2103.
38. Kulagina N, Besseau S, Godon C, Goldman GH, Papon N, Courdavault V. 2021. Yeasts as Biopharmaceutical Production Platforms. Front Fungal Biol 2:1–8.
39. Cregg JM, Cereghino JL, Shi J, Higgins DR. 2000. Recombinant protein expression in *Pichia pastoris*. Mol Biotechnol 16:23–52.
40. Manfrão-netto JHC, Milton A, Gomes V. 2019. Advances in Using *Hansenula polymorpha* as Chassis for Recombinant Protein Production 7:1–13.
41. Shen W, Xue Y, Liu Y, Kong C, Wang X, Huang M, Cai M, Zhou X, Zhang Y, Zhou M. 2016. A novel methanol-free *Pichia pastoris* system for recombinant protein expression. Microb Cell Fact 15:1–11.
42. Jacobs PP, Geysens S, Vervecken W, Contreras R, Callewaert N. 2009. Engineering complex-type N-glycosylation in *Pichia pastoris* using GlycoSwitch technology. Nat Protoc 4:58–70.
43. Piirainen MA, Salminen H, Frey AD. 2022. Production of galactosylated complex-type N-glycans in glycoengineered *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 106:301–315.
44. Wagner JM, Alper HS. 2016. Synthetic biology and molecular genetics in non-conventional yeasts: Current tools and future advances. Fungal Genet Biol 89:126–136.
45. Çelik E, Çalik P. 2012. Production of recombinant proteins by yeast cells. Biotechnol Adv 30:1108–1118.
46. Li Z, Rinas U. 2020. Recombinant protein production associated growth inhibition results mainly from transcription and not from translation. Microb Cell Fact 19:1–11.
47. Björkeroth J, Campbell K, Malina C, Yu R, Bartolomeo F Di, Nielsen J. 2020. Proteome reallocation from amino acid biosynthesis to ribosomes enables yeast to grow faster in rich media. Proc Natl Acad Sci U S A 117:21804–21812.
48. Von der Haar T, McCarthy JEG. 2002. Intracellular translation initiation factor levels in *Saccharomyces cerevisiae* and their role in cap-complex function. Mol Microbiol 46:531–544.
49. Hermesh O, Jansen RP. 2013. Take the (RN)A-train: Localization of mRNA to the endoplasmic reticulum. Biochim Biophys Acta - Mol Cell Res 1833:2519–2525.
50. Brake AJ, Merryweather JP, Coit DG, Heberlein UA, Masiarz FR, Mullenbach GT, Urdea MS, Valenzuela P, Barr PJ. 1984. α-Factor-directed synthesis and secretion of mature foreign proteins in *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A 81:4642–4646.
51. Liu Z, Tyo KEJ, Martínez JL, Petranovic D, Nielsen J. 2012. Different expression systems for production of recombinant proteins in *Saccharomyces cerevisiae*. Biotechnol Bioeng 109:1259–1268.
52. Aza P, Molpeceres G, de Salas F, Camarero S. 2021. Design of an improved universal signal peptide based on the α-factor mating secretion signal for enzyme production in yeast. Cell Mol Life Sci 78:3691–3707.
53. Kjærulff S, Jensen MR. 2005. Comparison of different signal peptides for secretion of heterologous proteins in fission yeast. Biochem Biophys Res Commun 336:974–982.

54. Walczak CP, Bernardi KM, Tsai B. 2012. Endoplasmic reticulum-dependent redox reactions control endoplasmic reticulum-associated degradation and pathogen entry. Antioxidants Redox Signal 16:809–818.
55. Frand AR, Kaiser CA. 1998. The *ERO1* gene of yeast is required for oxidation of protein dithiols in the endoplasmic reticulum. Mol Cell 1:161–170.
56. Smith MH, Ploegh HL, Weissman JS. 2011. Road to ruin: Targeting proteins for degradation in the endoplasmic reticulum. Science 334:1086–1090.
57. Strahl-Bolsinger S, Gentzsch M, Tanner W. 1999. Protein O-mannosylation. Biochim Biophys Acta - Gen Subj 1426:297–307.
58. Kimata Y, Kimata YI, Shimizu Y, Abe H, Farcasanu IC, Takeuchi M, Rose MD, Kohno K. 2003. Genetic evidence for a role of BiP/Kar2 that regulates Ire1 in response to accumulation of unfolded proteins. Mol Biol Cell 14:2559 –2569.
59. Lockhart DJ, Wodicka L, Travers KJ, Patil CK, Walter P, Weissman JS. 2004. Functional and Genomic Analyses Reveal an Essential Coordination between the Unfolded Protein Response and ER-Associated Degradation. Cell 101:249–258.
60. Huang M, Wang G, Qin J, Petranovic D, Nielsen J. 2018. Engineering the protein secretory pathway of *Saccharomyces cerevisiae* enables improved protein production. Proc Natl Acad Sci U S A 115:E11025–E11032.
61. Robinson AS, Hines V, Wittrup KD. 1994. Protein disulfide isomerase overexpression increases secretion of foreign proteins in *Saccharomyces cerevisiae*. Bio/Technology 12:381–384.
62. Qi Q, Li F, Yu R, Engqvist MKM, Siewers V, Fuchs J, Nielsen J. 2020. Different routes of protein folding contribute to improved protein production in *Saccharomyces cerevisiae*. MBio 11:6.
63. de Ruijter JC, Koskela E V., Frey AD. 2016. Enhancing antibody folding and secretion by tailoring the *Saccharomyces cerevisiae* endoplasmic reticulum. Microb Cell Fact 15:87.
64. Van Der Heide M, Hollenberg C, Van Der Klei I, Veenhuis M. 2002. Overproduction of BiP negatively affects the secretion of Aspergillus niger glucose oxidase by the yeast *Hansenula polymorpha*. Appl Microbiol Biotechnol 58:487–494.
65. Tang H, Bao X, Shen Y, Song M, Wang S, Wang C, Hou J. 2015. Engineering protein folding and translocation improves heterologous protein secretion in *Saccharomyces cerevisiae*. Biotechnol Bioeng 112:1872–1882.
66. Delic M, Valli M, Graf AB, Pfeffer M, Mattanovich D, Gasser B. 2013. The secretory pathway: Exploring yeast diversity. FEMS Microbiol Rev 37:872–914.
67. Belden WJ, Barlowe C. 2001. Role of Erv29p in collecting soluble secretory proteins into ER-derived transport vesicles. Science 294:1528–1531.
68. Bao J, Huang M, Petranovic D, Nielsen J. 2017. Moderate expression of *SEC16* increases protein secretion by *Saccharomyces cerevisiae*. Appl Environ Microbiol 83:E03400–E03416.
69. Bao J, Huang M, Petranovic D, Nielsen J. 2018. Balanced trafficking between the ER and the Golgi apparatus increases protein secretion in yeast. AMB Express 8:1–10.
70. Hou J, Tyo KEJ, Liu Z, Petranovic D, Nielsen J. 2012. Metabolic engineering of recombinant protein secretion by *Saccharomyces cerevisiae*. FEMS Yeast Res 12:491–510.
71. Papanikou E, Glick BS. 2009. The yeast Golgi apparatus: Insights and

mysteries. FEBS Lett 583:3746–3751.
72. Jungmann J, Munro S. 1998. Multi-protein complexes in the cis Golgi of *Saccharomyces cerevisiae* with α-1,6-mannosyltransferase activity. EMBO J 17:423–434.
73. Tang H, Song M, He Y, Wang J, Wang S, Shen Y, Hou J, Bao X. 2017. Engineering vesicle trafficking improves the extracellular activity and surface display efficiency of cellulases in *Saccharomyces cerevisiae*. Biotechnol Biofuels 10:1–13.
74. Bowers K, Stevens TH. 2005. Protein transport from the late Golgi to the vacuole in the yeast *Saccharomyces cerevisiae*. Biochim Biophys Acta - Mol Cell Res 1744:438–454.
75. Huang M, Bai Y, Sjostrom SL, Hallström BM, Liu Z, Petranovic D, Uhlén M, Joensson HN, Andersson-Svahn H, Nielsen J. 2015. Microfluidic screening and whole-genome sequencing identifies mutations associated with improved protein secretion by yeast. Proc Natl Acad Sci U S A 112:E4689–E4696.
76. Huang M, Bao J, Hallström BM, Petranovic D, Nielsen J. 2017. Efficient protein production by yeast requires global tuning of metabolism. Nat Commun 8:1131.
77. Stadlmayr G, Benakovitsch K, Gasser B, Mattanovich D, Sauer M. 2010. Genome-scale analysis of library sorting (GALibSo): Isolation of secretion enhancing factors for recombinant protein production in *Pichia pastoris*. Biotechnol Bioeng 105:543–555.
78. Waegeman H, Soetaert W. 2011. Increasing recombinant protein production in *Escherichia coli* through metabolic and genetic engineering. J Ind Microbiol Biotechnol 38:1891–1910.
79. Yu R, Nielsen J. 2019. Big data in yeast systems biology. FEMS Yeast Res 19:1–10.
80. Förster J, Famili I, Fu P, Palsson B, Nielsen J. 2003. Genome-scale reconstruction of the *Saccharomyces cerevisiae* metabolic network. Genome Res 13:244–253.
81. Campbell K, Westholm J, Kasvandik S, Di Bartolomeo F, Mormino M, Nielsen J. 2020. Building blocks are synthesized on demand during the yeast cell cycle. Proc Natl Acad Sci U S A 117:7575–7583.
82. Nilsson A, Nielsen J. 2016. Metabolic Trade-offs in Yeast are Caused by F1F0-ATP synthase. Sci Rep 6:1–11.
83. Sánchez BJ, Zhang C, Nilsson A, Lahtvee P, Kerkhoven EJ, Nielsen J. 2017. Improving the phenotype predictions of a yeast genome-scale metabolic model by incorporating enzymatic constraints. Mol Syst Biol 13:935.
84. Nocon J, Steiger MG, Pfeffer M, Sohn SB, Kim TY, Maurer M, Rußmayer H, Pflügl S, Ask M, Haberhauer-Troyer C, Ortmayr K, Hann S, Koellensperger G, Gasser B, Lee SY, Mattanovich D. 2014. Model based engineering of *Pichia pastoris* central metabolism enhances recombinant protein production. Metab Eng 24:129–138.
85. Ishchuk OP, Domenzain I, Sánchez BJ, Muñiz-Paredes F, Martínez JL, Nielsen J, Petranovic D. 2022. Genome-scale modeling drives 70-fold improvement of heme production by *Saccharomyces cerevisiae*. *Accepted for publication in* Proc Natl Acad Sci
86. Hohmann S, Mager WH. 2003. Introduction, p. 1–387. *In* Yeast Stress responses.
87. Mattanovich D, Gasser B, Hohenblum H, Sauer M. 2004. Stress in recombinant

protein producing yeasts. J Biotechnol 113:121–135.
88. Jamieson DJ. 1998. Oxidative stress responses of the yeast *Saccharomyces cerevisiae*. Yeast 14:1511–1527.
89. Phaniendra A, Jestadi DB, Periyasamy L. 2015. Free Radicals: Properties, Sources, Targets, and Their Implication in Various Diseases. Indian J Clin Biochem 30:11–26.
90. Poljak A, Dawes IW, Ingelse BA, Duncan MW, Smythe GA, Grant CM. 2003. Oxidative damage to proteins in yeast cells exposed to adaptive levels of H_2O_2. Redox Rep 8:371–377.
91. Grant CM, Perrone G, Dawes JiW. 1998. Glutathione and catalase provide overlapping defenses for protection against respiration-generated hydrogen peroxide in *Haemophilus influenzae*. Biochem Biophys Res Commun 253:893–898.
92. Murphy MP, Holmgren A, Larsson NG, Halliwell B, Chang CJ, Kalyanaraman B, Rhee SG, Thornalley PJ, Partridge L, Gems D, Nyström T, Belousov V, Schumacker PT, Winterbourn CC. 2011. Unraveling the biological roles of reactive oxygen species. Cell Metab 13:361–366.
93. Dirmeier R, O'Brien KM, Engle M, Dodd A, Spears E, Poyton RO. 2002. Exposure of yeast cells to anoxia induces transient oxidative stress: Implications for the induction of hypoxic genes. J Biol Chem 277:34773–34784.
94. Henderson LM, Chappell JB. 1993. Dihydrorhodamine 123: a fluorescent probe for superoxide generation? Eur J Biochem 217:973–980.
95. Benov L, Sztejnberg L, Fridovich I. 1998. Critical evaluation of the use of hydroethidine as a measure of superoxide anion radical. Free Radic Biol Med 25:826–831.
96. Turner APF, Karube I, Wilson GS. 1987. The Biological component, p. 1–154. *In* Biosensors: Fundamentals and Applications. Oxford Science Publications.
97. Martin-Yken H. 2020. Yeast-Based Biosensors : Current Applications and new developments. Biosensors 10:1–19.
98. Adeniran A, Sherer M, Tyo KEJ. 2015. Yeast-based biosensors: Design and applications. FEMS Yeast Res 15:1–15.
99. Lukyanov KA, Belousov V V. 2014. Genetically encoded fluorescent redox sensors. Biochim Biophys Acta - Gen Subj 1840:745–756.
100. Roma LP, Deponte M, Riemer J, Morgan B. 2018. Mechanisms and applications of redox-sensitive green fluorescent protein-based hydrogen peroxide probes. Antioxidants Redox Signal 29:6.
101. Morgan B, Van Laer K, Owusu TNE, Ezeriņa D, Pastor-Flores D, Amponsah PS, Tursch A, Dick TP. 2016. Real-time monitoring of basal H_2O_2 levels with peroxiredoxin-based probes. Nat Chem Biol 12:437–443.
102. Schwarzländer M, Dick TP, Meyer AJ, Morgan B. 2016. Dissecting redox biology using fluorescent protein sensors. Antioxidants Redox Signal 24:680–712.
103. Gutscher M, Pauleau AL, Marty L, Brach T, Wabnitz GH, Samstag Y, Meyer AJ, Dick TP. 2008. Real-time imaging of the intracellular glutathione redox potential. Nat Methods 5:553–559.
104. Hanson GT, Aggeler R, Oglesbee D, Cannon M, Capaldi RA, Tsien RY, Remington SJ. 2004. Investigating Mitochondrial Redox Potential with Redox-sensitive Green Fluorescent Protein Indicators. J Biol Chem 279:13044–13053.
105. Staudacher V, Trujillo M, Diederichs T, Dick TP, Radi R, Morgan B, Deponte

M. 2018. Redox-sensitive GFP fusions for monitoring the catalytic mechanism and inactivation of peroxiredoxins in living cells. Redox Biol 14:549–556.
106. Staudacher V, Djuika CF, Koduka J, Schlossarek S, Kopp J, Büchler M, Lanzer M, Deponte M. 2015. Plasmodium falciparum antioxidant protein reveals a novel mechanism for balancing turnover and inactivation of peroxiredoxins. Free Radic Biol Med 85:228–236.
107. de Ruijter JC, Koskela E V., Nandania J, Frey AD, Velagapudi V. 2018. Understanding the metabolic burden of recombinant antibody production in *Saccharomyces cerevisiae* using a quantitative metabolomics approach. Yeast 35:331–341.
108. Malhotra JD, Miao H, Zhang K, Wolfson A, Pennathur S, Pipe SW, Kaufman RJ. 2008. Antioxidants reduce endoplasmic reticulum stress and improve protein secretion. Proc Natl Acad Sci U S A 105:18525–18530.
109. Tyo KEJ, Liu Z, Petranovic D, Nielsen J. 2012. Imbalance of heterologous protein folding and disulfide bond formation rates yields runaway oxidative stress. BMC Biol 10:16.
110. Merksamer PI, Trusina A, Papa FR. 2008. Real-Time Redox Measurements during Endoplasmic Reticulum Stress Reveal Interlinked Protein Folding Functions. Cell 135:933–947.
111. Tu BP, Weissman JS, Francisco S. 2002. The FAD- and O 2 -Dependent Reaction Cycle of Ero1-Mediated Oxidative Protein Folding in the Endoplasmic Reticulum 10:983–994.
112. Martínez JL, Meza E, Petranovic D, Nielsen J. 2016. The impact of respiration and oxidative stress response on recombinant α-amylase production by *Saccharomyces cerevisiae*. Metab Eng Commun 3:205–210.
113. Hinnebusch AG. 1984. Evidence for translational regulation of the activator of general amino acid control in yeast. Proc Natl Acad Sci U S A 81:6442–6446.
114. Mascarenhas C, Edwards-Ingram LC, Zeef L, Shenton D, Ashe MP, Grant CM. 2008. Gcn4 is required for the response to peroxide stress in the yeast *Saccharomyces cerevisiae*. Mol Biol Cell 19:2995–3007.
115. Qiu H, Dong J, Hu C, Francklyn CS, Hinnebusch AG. 2001. The tRNA-binding moiety in *GCN2* contains a dimerization domain that interacts with the kinase domain and is required for tRNA binding and kinase activation. EMBO J 20:1425–1438.
116. Castilho BA, Shanmugam R, Silva RC, Ramesh R, Himme BM, Sattlegger E. 2014. Keeping the eIF2α kinase Gcn2 in check. Biochim Biophys Acta - Mol Cell Res 1843:1948–1968.
117. Hinnebusch AG. 2005. Translational regulation of *GCN4* and the general amino acid control of yeast. Annu Rev Microbiol 59:407–450.
118. Rolfes RJ, Hinnebusch AG. 1993. Translation of the yeast transcriptional activator *GCN4* is stimulated by purine limitation: implications for activation of the protein kinase *GCN2*. Mol Cell Biol 12:5099–5111.
119. Shenton D, Smirnova JB, Selley JN, Carroll K, Hubbard SJ, Pavitt GD, Ashe MP, Grant CM. 2006. Global translational responses to oxidative stress impact upon multiple levels of protein synthesis. J Biol Chem 281:29011–29021.
120. Yang R, Wek SA, Wek RC. 2000. Glucose Limitation Induces *GCN4* Translation by Activation of Gcn2 Protein Kinase. Mol Cell Biol 20:2706–2717.
121. Molin M, Yang J, Hanzén S, Toledano MB, Labarre J, Nyström T. 2011. Life Span Extension and H_2O_2 Resistance Elicited by Caloric Restriction Require the

Peroxiredoxin Tsa1 in *Saccharomyces cerevisiae*. Mol Cell 43:823–833.
122. Stephen DWS, Rivers SL, Jamieson DJ. 1995. The role of the *YAP1* and *YAP2* genes in the regulation of the adaptive oxidative stress responses of *Saccharomyces cerevisiae*. Mol Microbiol 16:415–423.
123. Haynes CM, Titus EA, Cooper AA. 2004. Degradation of misfolded proteins prevents ER-derived oxidative stress and cell death. Mol Cell 15:767–776.
124. Veal EA, Day AM, Morgan BA. 2007. Hydrogen Peroxide Sensing and Signaling. Mol Cell 13:1–14.
125. Bodvard K, Peeters K, Roger F, Romanov N, Igbaria A, Welkenhuysen N, Palais G, Reiter W, Toledano MB, Käll M, Molin M. 2017. Light-sensing via hydrogen peroxide and a peroxiredoxin. Nat Commun 145:757–767.
126. Giorgio M, Trinei M, Migliaccio E, Pelicci PG. 2007. Hydrogen peroxide: A metabolic by-product or a common mediator of ageing signals? Nat Rev Mol Cell Biol 8:722–728.
127. Guerra-Moreno A, Ang J, Welsch H, Jochem M, Hanna J. 2019. Regulation of the unfolded protein response in yeast by oxidative stress. FEBS Lett 593:1080–1088.
128. Bang LM, Keating GM. 2004. Adalimumab: A review of its use in rheumatoid arthritis. BioDrugs 18:121–139.
129. Ruohonen L, Aalto MK, Keränen S. 1995. Modifications to the *ADH1* promoter of *Saccharomyces cerevisiae* for efficient production of heterologous proteins. J Biotechnol 39:193–203.
130. Feige MJ, Hendershot LM, Buchner J. 2010. How antibodies fold. Trends Biochem Sci 35:189–198.
131. Ho SCL, Koh EYC, van Beers M, Mueller M, Wan C, Teo G, Song Z, Tong YW, Bardor M, Yang Y. 2013. Control of IgG LC: HC ratio in stably transfected CHO cells and study of the impact on expression, aggregation, glycosylation and conformational stability. J Biotechnol 165:157–166.
132. Finley D, Ulrich HD, Sommer T, Kaiser P. 2012. The ubiquitin-proteasome system of *Saccharomyces cerevisiae*. Genetics 192:319–360.
133. Bohni PC, Deshaies RJ, Schekman RW. 1988. *SEC11* is required for signal peptide processing and yeast cell growth. J Cell Biol 106:1035–1042.
134. Jones EW. 1991. Three proteolytic systems in the yeast *Saccharomyces cerevisiae*. J Biol Chem 266:7963–7966.
135. Gagnon-Arsenault I, Tremblay J, Bourbonnais Y. 2006. Fungal yapsins and cell wall: A unique family of aspartic peptidases for a distinctive cellular function. FEMS Yeast Res 6:966–978.
136. Thumm M. 2000. Structure and function of the yeast vacuole and its role in autophagy. Microsc Res Tech 51:563–572.
137. Van Den Hazel HB, Kielland-Brandt MC, Winther JR. 1996. Review: Biosynthesis and function of yeast vacuolar proteases. Yeast 12:1–16.
138. Bryant NJ, Stevens TH. 1998. Vacuole Biogenesis in *Saccharomyces cerevisiae* : Protein Transport Pathways to the Yeast Vacuole . Microbiol Mol Biol Rev 62:230–247.
139. Hecht KA, O'Donnell AF, Brodsky JL. 2014. The proteolytic landscape of the yeast vacuole. Cell Logist 4:e28023.
140. Klionsky DJ, Banta LM, Emr SD. 1988. Intracellular sorting and processing of a yeast vacuolar hydrolase: proteinase A propeptide contains vacuolar targeting information. Mol Cell Biol 8:2105–2116.

141. Ramos C, Winther JR, Kielland-Brandt MC. 1994. Requirement of the propeptide for *in vivo* formation of active yeast carboxypeptidase Y. J Biol Chem 269:7006–7012.
142. Van den Hazel HB, Kielland-Brandt MC, Winther JR. 1993. The propeptide is required for *in vivo* formation of stable active yeast proteinase A and can function even when not covalently linked to the mature region. J Biol Chem 268:18002–18007.
143. Jung G, Ueno H, Hayaski R. 1999. Carboxypeptidase Y: Structural Basis for Protein Sorting and Catalytic Triad. JB Rev J Biochem 126:1–6.
144. Woolford CA, Daniels LB, Park FJ, Jones EW, Van Arsdell JN, Innis MA. 1986. The *PEP4* gene encodes an aspartyl protease implicated in the posttranslational regulation of *Saccharomyces cerevisiae* vacuolar hydrolases. Mol Cell Biol 6:2500–2510.
145. Rupp S, Hirsch HH, Wolf DH. 1991. Biogenesis of the yeast vacuole (lysosome) Active site mutation in the vacuolar aspartate proteinase yscA blocks maturation of vacuolar proteinases. FEBS Lett 293:62–66.
146. Kang HA, Choi ES, Hong WK, Kim JY, Ko SM, Sohn JH, Rhee SK. 2000. Proteolytic stability of recombinant human serum albumin secreted in the yeast *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 53:575–582.
147. Dong L, Li F, Piao Y, Sun D, Zhao R, Li C, Cong L, Zhao C. 2015. Characterization of proteinase A excretion from *Saccharomyces cerevisiae* in high sugar stress conditions. J Korean Soc Appl Biol Chem 58:203–208.
148. Wu M, Shen Q, Yang Y, Zhang S, Qu W, Chen J, Sun H, Chen S. 2013. Disruption of *YPS1* and *PEP4* genes reduces proteolytic degradation of secreted HSA/PTH in *Pichia pastoris* GS115. J Ind Microbiol Biotechnol 40:589–599.
149. Cho EY, Cheon SA, Kim H, Choo J, Lee DJ, Ryu HM, Rhee SK, Chung BH, Kim JY, Kang HA. 2010. Multiple-yapsin-deficient mutant strains for high-level production of intact recombinant proteins in *Saccharomyces cerevisiae*. J Biotechnol 20:1–7.
150. Komeda T, Sakai Y, Kato N, Kondo K. 2002. Construction of protease-deficient *Candida boidinii* strains useful for recombinant protein production: Cloning and disruption of proteinase A gene (*PEP4*) and proteinase B gene (*PRB1*). Biosci Biotechnol Biochem 66:628–631.
151. Idiris A, Tohda H, Kumagai H, Takegawa K. 2010. Engineering of protein secretion in yeast: Strategies and impact on protein production. Appl Microbiol Biotechnol 86:403–417.
152. Gleeson MA, White CE, Meininger DP, Komives EA. 1998. Generation of protease-deficient strains and their use in heterologous protein expression. Methods Mol Biol 103:81–94.
153. Jahic M, Gustavsson M, Jansen AK, Martinelle M, Enfors SO. 2003. Analysis and control of proteolysis of a fusion protein in *Pichia pastoris* fed-batch processes. J Biotechnol 102:45–53.
154. Nilsson B, Moks T, Jansson B, Abrahmsén L, Elmblad A, Holmgren E, Henrichson C, Jones TA, Uhlén M. 1987. A synthetic IgG-binding domain based on staphylococcal protein a. Protein Eng Des Sel 1:107–113.
155. Nord K, Gunneriusson E, Ringdahl J, Stahl S, Uhlen M, Nygren A. 1997. Binding proteins selected fromm combinatorial libraries of an α-helical bacterial receptor domain. Nat Biotechnol 15:722–777.
156. Rönnmark J, Grönlund H, Uhlén M, Nygren PÅ. 2002. Human immunoglobulin

A (IgA)-specific ligands from combinatorial engineering of protein A. Eur J Biochem 269:2647–2655.
157. Tove Boström JN and SH. 2012. Purification Systems Based on Bacterial Surface Proteins, p. 89–136. *In* Protein Purification. Stockholm.
158. Frejd FY, Kim KT. 2017. Affibody molecules as engineered protein drugs. Exp Mol Med 96: e306.
159. Mishra R, Patel H, Alanazi S, Yuan L, Garrett JT. 2018. HER3 signaling and targeted therapy in cancer. Oncol Rev 12:45–62.
160. Wang ZY, He XP, Zhang BR. 2007. Over-expression of *GSH1* gene and disruption of *PEP4* gene in self-cloning industrial brewer's yeast. Int J Food Microbiol 119:192–199.
161. Mechler B, Müller H, Wolf DH. 1987. Maturation of vacuolar (lysosomal) enzymes in yeast: proteinase yscA and proteinase yscB are catalysts of the processing and activation event of carboxypeptidase yscY. EMBO J 6:2157–2163.
162. Hemmings BA, Zubenko GS, Hasilik A, Jones EW. 1981. Mutant defective in processing of an enzyme located in the lysosome-like vacuole of *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A 78:435–439.
163. Zubenko GS, Park FJ, Jones EW. 1982. Genetic properties of mutations at the *PEP4* locus in *Saccharomyces cerevisiae*. Genetics 102:679–690.
164. Nielsen J. 2013. Production of biopharmaceutical proteins by yeast: Advances through metabolic engineering. Bioengineered 4:207–211.
165. Joly JC, Leung WS, Swartz JR. 1998. Overexpression of *Escherichia coli* oxidoreductases increases recombinant insulin-like growth factor-I accumulation. Proc Natl Acad Sci U S A 95:2773–2777.
166. Yim S, Jeong K, Chang H, Lee S. 2001. High-level secretory production of human granulocyte-colony stimulating factor by fed-batch culture of recombinant *Escherichia coli*. Bioprocess Biosyst Eng 24:249–254.
167. Nothwehr SF, Hindes AE. 1997. The yeast *VPS5/GRD2* gene encodes a sorting nexin-1-like protein required for localizing membrane proteins to the late Golgi. J Cell Sci 110:1063–1072.
168. Horazdovsky BF, Davies BA, Seaman MNJ, McLaughlin SA, Yoon SH, Emr SD. 1997. A sorting nexin-1 homologue, Vps5p, forms a complex with Vps17p and is required for recycling the vacuolar protein-sorting receptor. Mol Biol Cell 8:1529–41.
169. Lu H, Li F, Sánchez BJ, Zhu Z, Li G, Domenzain I, Marci S, Anton PM, Lappa D, Lieven C, Beber ME, Sonnenschein N, Kerkhoven EJ, Nielsen J. 2019. A consensus *S. cerevisiae* metabolic model Yeast8 and its ecosystem for comprehensively probing cellular metabolism. Nat Commun 10:3586.
170. Yang L, Yurkovich JT, King ZA, Palsson BO. 2018. Modeling the multi-scale mechanisms of macromolecular resource allocation. Curr Opin Microbiol 45:8–15.
171. Choi HS, Lee SY, Kim TY, Woo HM. 2010. *In silico* identification of gene amplification targets for improvement of lycopene production. Appl Environ Microbiol 76:3097–3105.
172. Li F, Chen Y, Qi Q, Wang Y, Yuan L, Huang M, Elsemman IE, Feizi1 A, Kerkhoven EJ, Nielsen J. 2021. Genome scale modeling of the protein secretory pathway reveals novel targets for improved recombinant protein production in yeast. BioRxiv 0:0–0.

173. Thim L, Hansen MT, Norris K, Hoegh I, Boel E, Forstrom J, Ammerer G, Fiil NP. 1986. Secretion and processing of insulin precursors in yeast. Proc Natl Acad Sci U S A 83:6766–6770.
174. Vanz ALS, Renard G, Palma MS, Chies JM, Dalmora SL, Basso LA, Santos DS. 2008. Human granulocyte colony stimulating factor (hG-CSF): cloning, overexpression, purification and characterization 12:1–12.
175. Smaczynska-de Rooij II, Marklew CJ, Allwood EG, Palmer SE, Booth WI, Mishra R, Goldberg MW, Ayscough KR. 2016. Phosphorylation Regulates the Endocytic Function of the Yeast Dynamin-Related Protein Vps1. Mol Cell Biol 36:742–755.
176. Gall WE, Higginbotham MA, Chen CY, Ingram MF, Cyr DM, Graham TR. 2000. The auxilin-like phosphoprotein Swa2p, is required for clathrin function in yeast. Curr Biol 10:1349–1358.
177. Thibault G, Ismail N, Ng DTW. 2011. The unfolded protein response supports cellular robustness as a broad-spectrum compensatory pathway. Proc Natl Acad Sci U S A 108:20597–20602.
178. Tyson JR, Stirling CJ. 2000. *LHS1* and *SIL1* provide a lumenal function that is essential for protein translocation into the endoplasmic reticulum. EMBO J 19:6440–6452.
179. Sevier CS, Cuozzo JW, Vala A, Åslund F, Kaiser CA. 2001. A flavoprotein oxidase defines a new endoplasmic reticulum pathway for biosynthetic disulphide bond formation. Nat Cell Biol 3:874–882.
180. Griff IC, Schekman R, Rothman JE, Kaiser CA. 1992. The yeast *SEC17* gene product is functionally equivalent to mammalian α- SNAP protein. J Biol Chem 267:12106–12115.
181. Heo Y, Yoon HJ, Ko H, Jang S, Lee HH. 2020. Crystal structures of Uso1 membrane tether reveal an alternative conformation in the globular head domain. Sci Rep 10:1–9.
182. Tsvetanova NG, Riordan DP, Brown PO. 2012. The yeast rab GTPase Ypt1 modulates unfolded protein response dynamics by regulating the stability of *HAC1* RNA. PLoS Genet 8:1–11.
183. Liu Y, Huang H. 2018. Expression of single-domain antibody in different systems. Appl Microbiol Biotechnol 102:539-551.
184. Besada-Lombana PB, Da Silva NA. 2019. Engineering the early secretory pathway for increased protein secretion in *Saccharomyces cerevisiae*. Metab Eng 55:142–151.
185. Görgens JF, Van Zyl WH, Knoetze JH, Hahn-Hägerdal B. 2005. Amino acid supplementation improves heterologous protein production by *Saccharomyces cerevisiae* in defined medium. Appl Microbiol Biotechnol 67:684–91.
186. Heyland J, Fu J, Blank LM, Schmid A. 2011. Carbon metabolism limits recombinant protein production in *Pichia pastoris*. Biotechnol Bioeng 108:1942–1953.
187. Wittrup KD, Benig V. 1994. Optimization of amino acid supplements for heterologous protein secretion in *Saccharomyces cerevisiae*. Biotechnol Tech 8:161–166.
188. Wek RC, Cannon JF, Dever TE, Hinnebusch AG. 1992. Truncated protein phosphatase *GLC7* restores translational activation of *GCN4* expression in yeast mutants defective for the eIF-2α kinase *GCN2*. Mol Cell Biol 12:5700–5710.
189. Tan YSH, Morcos PA, Cannon JF. 2003. Pho85 phosphorylates the Glc7 protein

phosphatase regulator Glc8 *in vivo*. J Biol Chem 278:147–153.
190. Rojas M, Gingras AC, Dever TE. 2014. Protein phosphatase *PP1/GLC7* interaction domain in yeast eIF2γ bypasses targeting subunit requirement for eIF2α dephosphorylation. Proc Natl Acad Sci U S A 111:E1344–E1353.
191. Deloche O, De La Cruz J, Kressler D, Doère M, Linder P. 2004. A Membrane Transport Defect Leads to a Rapid Attenuation of Translation Initiation in *Saccharomyces cerevisiae*. Mol Cell 13:357–366.
192. Staudacher J, Rebnegger C, Gasser B. 2021. Treatment with surfactants enables quantification of translational activity by O-propargyl-puromycin labelling in yeast. BMC Microbiol 21:1–10.
193. Staudacher J, Rebnegger C, Dohnal T, Landes N, Mattanovich D, Gasser B. 2022. Going beyond the limit: Increasing global translation activity leads to increased productivity of recombinant secreted proteins in *Pichia pastoris*. Metab Eng 70:181–195.
194. Raymond CK, Howald-Stevenson I, Vater CA, Stevens TH. 1992. Morphological classification of the yeast vacuolar protein sorting mutants: Evidence for a prevacuolar compartment in class E vps mutants. Mol Biol Cell 3:1389–1402.
195. van Dijken JP, Bauer J, Brambilla L, Dupoc P, Francois JM, Gancedo C, Giuseppin MLF, Heijnen JJ, Hoare M, Lange HC, Madden HC, Niederberger P, Nielsen J, Parrou JL, Petit T, Porro D, Reuss M, van Riel N, Rizzi M, Steensma HY, Verrips CT, Vindelov J, Pronk JT. 2000. An interlaboratory comparison of physiological and genetic properties of four *Saccharomyces cerevisiae* strains. Enzyme Microb Technol 14:706–714.
196. Safder I, Khan S, Islam I, Ali MK, Bibi Z, Waqas M. 2018. Pichia pastoris expression system: An impending candidate to express protein in industrial and biopharmaceutical domains. Biomed Lett 4:1–14.
197. Tomimoto K, Fujita Y, Iwaki T, Chiba Y, Jigami Y, Nakayama KI, Nakajima Y, Abe H. 2013. Protease-deficient *Saccharomyces cerevisiae* strains for the synthesis of human-compatible glycoproteins. Biosci Biotechnol Biochem 77:2461–2466.
198. Zrimec J, Börlin CS, Buric F, Muhammad AS, Chen R, Siewers V, Verendel V, Nielsen J, Töpel M, Zelezniak A. 2020. Deep learning suggests that gene expression is encoded in all parts of a co-evolving interacting gene regulatory structure. Nat Commun 11:6141.
199. Elsemman IE, Rodriguez Prado A, Grigaitis P, Garcia Albornoz M, Harman V, Holman SW, van Heerden J, Bruggeman FJ, Bisschops MMM, Sonnenschein N, Hubbard S, Beynon R, Daran-Lapujade P, Nielsen J, Teusink B. 2022. Whole-cell modeling in yeast predicts compartment-specific proteome constraints that drive metabolic strategies. Nat Commun 13:1–12.