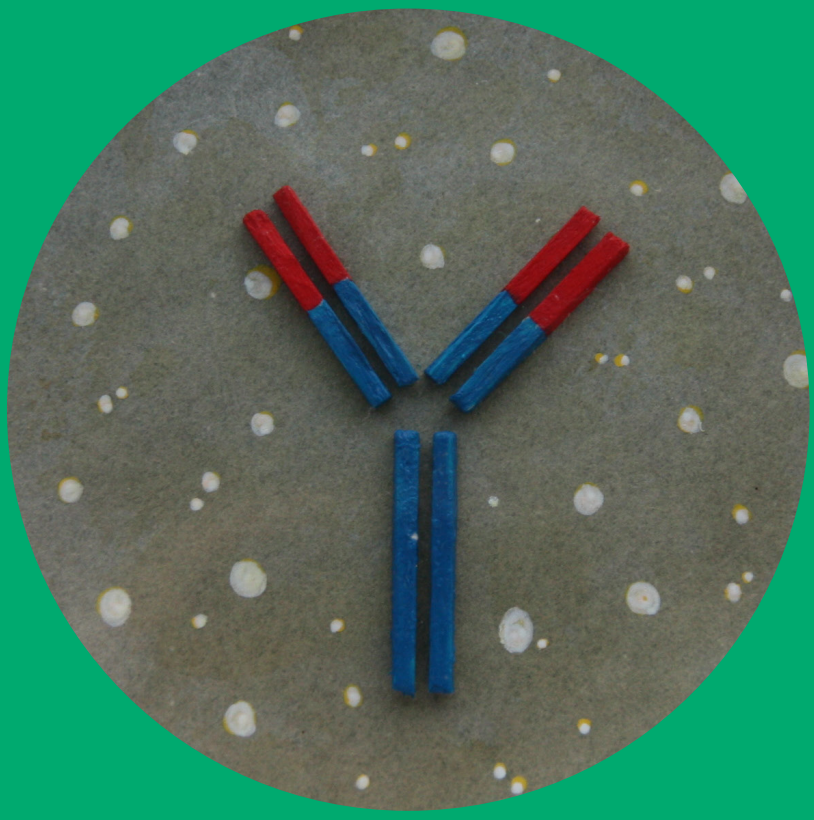
Department of Biotechnology and Chemical Technology

Development of *Saccharomyces cerevisiae* as a recombinant antibody factory

Jorg C. de Ruijter



Aalto University

DOCTORAL DISSERTATIONS

Aalto University publication series
DOCTORAL DISSERTATIONS 277/2016

Development of *Saccharomyces cerevisiae* as a recombinant antibody factory

Jorg C. de Ruijter

A doctoral dissertation for the degree of Doctor of Science in Technology to be presented, with due permission of the Aalto University School of Chemical Technology, for public examination and debate at lecture hall KE1 of the Aalto University School of Chemical Technology (Kemistintie 1, Espoo, Finland) on the 27th of January 2017, at 12 noon.

Aalto University
School of Chemical Technology
Department of Biotechnology and Chemical Technology
Molecular Biotechnology

Supervising professor
Professor Alexander D. Frey, Aalto University, Finland

Preliminary examiners
Dr. Christopher P. Landowski, VTT, Finland
Dr. David Pincus, Whitehead Institute for Biomedical Research, USA

Opponent
Professor Lloyd W. Ruddock, Biocenter Oulu and University of Oulu, Faculty of Biochemistry and Molecular Medicine, Finland

Aalto University publication series
DOCTORAL DISSERTATIONS 277/2016

ISBN 978-952-60-7223-4 (printed)
ISBN 978-952-60-7222-7 (pdf)
ISSN-L 1799-4934
ISSN 1799-4934 (printed)
ISSN 1799-4942 (pdf)
http://urn.fi/URN:ISBN:978-952-60-7222-7

Images: Picture and Antibody petridish by Siiri Viljanen

Unigrafia Oy
Helsinki 2016

Finland

441 697
Printed matter

Author
Jorg C. de Ruijter

Name of the doctoral dissertation
Development of *Saccharomyces cerevisiae* as a recombinant antibody factory

Publisher School of Chemical Technology

Unit Department of Biotechnology and Chemical Technology

Series Aalto University publication series DOCTORAL DISSERTATIONS 277/2016

Field of research Molecular Biotechnology

Manuscript submitted 18 August 2016 **Date of the defence** 27 January 2017

Permission to publish granted (date) 2 November 2016 **Language** English

☐ **Monograph** ☒ **Article dissertation** ☐ **Essay dissertation**

Abstract

The yeast *Saccharomyces cerevisiae* has been widely used as an expression host for the manufacturing of products like biofuels, small molecules, and of recombinant proteins. To increase the yields of economically interesting proteins, the secretory pathway has been engineered extensively, however, secretion titers have often remained low. One example for these shortcomings are full-length IgG antibodies, which are currently mostly produced in CHO cells, although microbial and plant based production platforms are emerging. We believe that *S. cerevisiae* has the potential to become an industrially relevant antibody factory. In this thesis, using targeted and random screening approaches, we aimed to identify genetic factors that can be used to create strains with an increased IgG secretion efficiency.

First, we focused on genes that are regulated by the unfolded protein response and encode proteins affecting the ER folding environment. We enlarged the functional folding space in the ER through deletion of the *OPI1* gene, a modification that increased IgG titers by up to 4.8-fold. Out of a screen of folding catalysts and molecular chaperones, the overexpression of the peptidyl-prolyl isomerase Cpr5p provided the most beneficial effect, increasing IgG titers by up to 3.26-fold. Finally, by combining the *OPI1* deletion with *CPR5* overexpression IgG secretion was increased over ten-fold when compared with the wild type background. In contrast, in a set of strains with deletions of genes encoding proteins of the ER associated degradation pathway only deletion of *HTM1* increased titers by 1.15-fold. Development of a clearance assay allowed us to distinguish differences in cellular IgG clearance among the ERAD deletion strains.

As targets for rational strain engineering are limited, we developed a high throughput method for screening a transposon mediated yeast deletion library and identified genes that influence IgG secretion. With this approach, we were able to identify the genes *VPS30* and *TAR1* that after deletion improved IgG secretion by up to 2.5-fold and up 1.13-fold, respectively, thus validating the applicability of the method. Finally, we aimed to gain insight into the changes that recombinant antibody production inflicts on selected intracellular metabolites. Metabolic footprints of strains expressing a scFv, a scFv-Fc fusion, and a full-length IgG were found to be significantly different based on a semi-quantitative metabolomics method. The most apparent changes were found in metabolites involved in amino acid and redox metabolism.

In conclusion, we identified genes at several places along the secretory pathway that can be used to improve IgG secretion in *S. cerevisiae*.

Keywords *Saccharomyces cerevisiae*, antibody, UPR, ERAD, protein folding, cell factory

ISBN (printed) 978-952-60-7223-4 **ISBN (pdf)** 978-952-60-7222-7

ISSN-L 1799-4934 **ISSN (printed)** 1799-4934 **ISSN (pdf)** 1799-4942

Location of publisher Helsinki **Location of printing** Helsinki **Year** 2016

Pages 189 **urn** http://urn.fi/URN:ISBN:978-952-60-7222-7

Preface

The work for this thesis was carried out in the research group of Molecular Biotechnology, in the Department of Biotechnology and Chemical Technology, at the School of Chemical Technology of Aalto University, during the years 2012-2016.

First, I would like to thank my supervising professor Dr. Alexander Frey. Alex, I will forever be grateful for getting the opportunity to do my doctoral research in your group, these four years have been a pleasure and went by incredibly quick. Thank you for all your help and advice during this time, especially in the moments when it was difficult to push through frustrations when things didn't work or when I was stuck in the writing process. In particular, I really appreciate the effort you put in to help me finish writing my thesis during this summer, before my move to Northern Ireland.

I would also like to thank my preliminary examiners Dr. Christopher Landowski and Dr. David Pincus for evaluating my thesis. Your feedback and suggestions for improvements were much appreciated. Also thanks to Dr. András Pásztor and Dr. Edita Jurak for valuable feedback on my writing before it was finished.

Without my co-authors of the publications in this thesis, I would have never been able to finish this work, so I would like to thank Essi Koskela, Dr. German Jurgens, Jatin Nandania, Dr. Vidya Velagapudi, and Dr. Alexander Frey. It was a pleasure working together with all of you, and your scientific expertise and insights helped me bring this research to a next level.

An important part of doing research is the group of people you have around you every day in the labs, offices, and obviously during the well-deserved break times. Sharing the office with Salem Shamekh and Filip Mollerup was always a delight, thank you for making it such an enjoyable time!

I have naturally spent a lot of time with the people in our Molecular Biotechnology research group; it was great to work with you, to share and discuss our ideas and results, and to enjoy the occasional summer BBQ. Also thanks to all the other people in the research groups of our department for all the great coffee breaks, lunch breaks, and of course valuable research related discussions, I feel that many of you have become friends during these four years.

So, all of you, thank you! Essi Koskela, Mari Piirainen, András Pásztor, Galina Mai-Gisondi, Maciej Spuś, Anne Usvalampi, German Jurgens, Maija Haapakangas, Mariana Anastácio, Sami Havukainen, Edita Jurak, Ville Aumala, Emma Master, Janne Wallenius, Markus Räsänen, Ulla Moilanen, Sasikala Anbarasan, He Li, Ossi Turunen, Tero Eerikäinen, Georg Schmidt, Heli Viskari, Pezhman Mohammadi, Bart Rooijakkers, Christopher Jonkergouw, Markus Linder, Suvi Arola, Katja Osmekhina, Estefania Boix, Salla Virtanen, Sesilja Aranko, Laura Lemetti, Martina Ikonen, Pihla Savola, Esa Uosukainen, Seppo Jääskeläinen, Auli Murrola, Johanna Aura. It was a pleasure working with all of you!

Finally, I want to thank my friends and family for their support. Especially I want to thank Eric and Alice de Ruijter, my parents, as ever since I was young, they have always supported me in making the most of myself. Without the values you taught me during my upbringing, I would have never gotten where I am now.

And last, but certainly not least, I want to thank Siiri for keeping me sane during the seemingly never ending writing process this summer, for always believing in me, making me stop work every once in a while, and for always showing me the wonder and beauty that life brings us and that we can see all around us if we just take the time to take a good look.

Portadown, October 2016

Jorg de Ruijter

CONTENTS

List of publications

This thesis consists of an overview of the following four publications, which from here on are referred to as Roman numerals in the text:

I De Ruijter J.C., Frey A.D.:
Analysis of antibody production in *Saccharomyces cerevisiae*: effects of ER protein quality control disruption,
Appl Microbiol Biotechnol 2015, 99:9061–9071

II De Ruijter J.C.*, Koskela E.V.*, Frey A.D.:
Enhancing antibody folding and secretion by tailoring the *Saccharomyces cerevisiae* endoplasmic reticulum,
Microb Cell Fact 2016, 15:87

III De Ruijter J.C.*, Jurgens G.*, Frey A.D.:
Screening for novel genes of *Saccharomyces cerevisiae* involved in recombinant antibody production,
FEMS Yeast Research, DOI: http://dx.doi.org/10.1093/femsyr/fow104

IV De Ruijter J.C., Koskela E.V., Nandania J., Frey A.D., Velagapudi V.:
Understanding the metabolic burden of recombinant antibody production in *Saccharomyces cerevisiae* using a quantitative metabolomics approach,
Manuscript submitted to *Metabolic Engineering communications*

* = equal contributions

Author's contributions

Publication I: Analysis of antibody production in *Saccharomyces cerevisiae*: effects of ER protein quality control disruption

Jorg de Ruijter created the high-throughput ELISA method, constructed the yeast deletion strains, performed the screening and cellular clearance experiments, and drafted the manuscript. Alexander Frey conceived of the study, participated in its design, and helped to draft the manuscript.

Publication II: Enhancing antibody folding and secretion by tailoring the *Saccharomyces cerevisiae* endoplasmic reticulum

Jorg de Ruijter carried out the molecular cloning work, performed the screening and cellular clearance experiments, contributed to the mRNA experiments, and drafted the manuscript. Essi Koskela created the background yeast strains, contributed to molecular cloning and the screening experiments, carried out the mRNA experiments, the statistical analysis, and drafted the manuscript. Alexander Frey conceived the study, participated in its design, and helped to draft the manuscript.

Publication III: Screening for novel genes of *Saccharomyces cerevisiae* involved in recombinant antibody production

Jorg de Ruijter created the high-throughput colony-picking method, created the clean deletion strains and verified their expression phenotype, and helped drafting the manuscript. German Jurgens created the yeast deletion strain library, carried out the primary screening experiments and verified the transposon insertion sites. Alexander Frey conceived the study, carried out the secondary screening experiments and drafted the manuscript.

Publication IV: Understanding the metabolic burden of recombinant antibody production in *Saccharomyces cerevisiae* using a quantitative metabolomics approach

Jorg de Ruijter conceived of the study, carried out the yeast cultivations, interpreted the analyzed metabolomics data and drafted the manuscript. Essi Koskela carried out the PAPi analysis and helped drafting the manuscript. Jatin Nandania performed the metabolomics measurement experiments. Alexander Frey cloned the expression constructs, helped to design the study and to draft the manuscript. Vidya Velagapudi carried out the metabolomics data analysis and helped drafting the manuscript.

List of abbreviations

AP	acid phosphatase
CDR	complementarity determining region
CHO	Chinese hamster ovary cells
CPY	carboxypeptidase Y
DNA	deoxyribonucleic acid
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
ERAD	ER associated degradation
ERES	ER exit sites
FAEE	fatty acid ethyl esters
Fab	antibody binding fragment
GFP	green fluorescent protein
Glc	glucose
GlcNAc	N-acetylglucosamine
gRNA	guide RNA
HC	heavy chain
HDSV	heavy density secretory vesicle
HEK	human embryonic kidney cells
HPV	human papillomavirus
HR	homologous recombination
HTP	high throughput
Ig	immunoglobulin
LC	light chain
LDSV	low density secretory vesicle
LLO	lipid linked oligosaccharide
Matα	mating factor α
Man	mannose
ORF	open reading frame
OST	oligosaccharyl transferase
Pdi	protein disulfide isomerase
Ppi	peptidyl-prolyl isomerase
RNA	ribonucleic acid
scFv	single chain variable fragment
SD	synthetic dropout
UPR	unfolded protein response

UPRE	UPR element
wt	wild-type
YAC	yeast artificial chromosome

1. Introduction

From all the microbes used in biotechnology, the yeast *Saccharomyces cerevisiae* is one of the oldest and most frequently used host organisms for the production of a wide range of products. Traditionally, it has been used for centuries in the fermentation processes that are used to make products like bread, beer, and wine. Later, this has evolved with the development of the yeast into a cell factory for the biotechnological industry. The established industrially produced product assortment includes three generations of biofuels, small molecules, and therapeutic proteins. A general description of the characteristics of *S. cerevisiae* as a cell factory is given, followed by an overview of the current state of its utilization in industry.

1.1. Characteristics of *Saccharomyces cerevisiae* as a cell factory

One of the main advantages of *Saccharomyces cerevisiae* enabling the current transition from an old-fashioned workhorse to a modern cell factory is the fact that the organism has a long history of scientific research achievements available. This has generated a tremendous amount of knowledge from a single molecule level up to the cellular level.

The full genomic sequence of *S. cerevisiae* came available in 1996 and showed the yeast to have just over 6000 genes in its 12 megabase genome [1]. As it was the first eukaryote to have its genome completely sequenced, it has been extensively used for gene annotation through functional genomics. This resulted in the characterization of almost 80 % of the functional genome [2]. The knowledge about such a large amount of the genome of *S. cerevisiae* is beneficial for a predictable cellular and metabolic engineering in order to increase its capacity and flexibility as a cell factory.

Exceptionally among the industrially relevant organisms, already for many years there have been convenient methods available for editing the DNA content of *S. cerevisiae*, either by adding DNA in plasmid vectors, or through the manipulation of its genomic DNA. From the methods available for the transformation of DNA into the yeast cells, the lithium acetate method appears to be most widely used. In this method, the yeast cells are readily made competent using lithium acetate ions and foreign DNA is efficiently taken up using a heat shock step [3]. This allows the insertion of plasmids with a very high efficiency of over 10^6 transformants per microgram of DNA [4]. However, plasmid bearing strains require a constant selection pressure to avoid plasmid loss and provide limited control over plasmid copy number, characteristics that are undesirable for industrial production strains. So, a

stable strain with foreign DNA integrated into the genome is advantageous. Through the yeast's high rate of homologous recombination (HR), targeted gene disruption or genomic integration is a one-step process via the transformation of linear DNA containing homologous regions with the target area [5]. However, it should be noted that for polyploid strains, often used in industrial processes, the efficiency of this system to delete or disrupt genes is decreased, as the integration event has to happen in all of the available alleles.

One of the limitations of strains modified with plasmids or genomically integrated genetic cassettes is the limited amount of available auxotrophic and dominant markers, even though their use is improved through Cre recombinase mediated marker recycling [5]. However, the advent of genome engineering using the CRISPR/Cas9 system has made genetic engineering even more versatile. This system uses the Cas9 gene from *Streptococcus pyogenes*, which can function as an RNA-guided endonuclease to generate sequence specific double strand breaks, targeted by the CRISPR designed gRNA (guide RNA) [6]. For knockouts, most commonly a 120 bp double stranded oligonucleotide with the right homologous regions is used to implement a premature stop codon, or to remove the full open reading frame (ORF). For the integration of genes a gene integration cassette with homologous end parts targets it to the right spot in the genomic DNA. The Cas9 protein creates a specific double strand break which allows integration events at very high efficiency without the need for a selective marker, and it can even handle multiple integration events at once. This is especially relevant for polyploid strains, as it was shown that using Cas9 a quadruple auxotrophic mutant strain was created after multiple rounds of targeted disruption [7]. However, in general it has to be kept in mind that designing and cloning of the various gRNA expression cassettes can be time consuming and can thus decrease the cost-effectivity of this method [8]. Nonetheless, the versatility of possible DNA modifications with the CRISPR/Cas9 system making it very interesting for genetic engineering of *Saccharomyces cerevisiae*.

Another limitation in the creation of yeast cell factories has been the limited availability of well characterized endogenous promoters for expression at different strengths. For constitutive promoters, some of the most commonly used ones are: P_{TEF1}, P_{PGK1}, P_{GPD}, P_{CYC1}, and P_{ADH1}, arranged from stronger to weaker expression. Even though they are constitutive promoters, expression levels often still vary depending on the growth phase [9]. Inducible promoters provide targeted gene expression at a specific time during cultivation. Promoters of genes involved in galactose metabolism are the most commonly used for high level gene expression, for example P_{GAL1}, P_{GAL7}, and P_{GAL10}. They are tightly regulated through the presence of glucose and galactose, where galactose gives a strong induction of expression, which is inhibited in the presence of glucose. P_{CUP1} is used for inducible expression at lower levels and is induced in the presence of copper ions in the media [10]. In order to create more versatile expression levels, research has aimed to create engineered and synthetic promoters. Endogenous promoters have been engineered by the addition or removal of regulatory sequences, or through random mutagenesis of the promoter region, in order to get promoters with useful new characteristics [11]. This has lead for example to an increase of promoter strength of 2.5-fold for P_{GPD}, and the creation of a library of new, galactose inducible promoters, that span an almost 50-fold dynamic range of expression levels [12]. An example of the development of a synthetic promoter is the use of the bacterial LexA-DNA-binding domain and corresponding LexA-binding sites in the promoter sequence [13]. This system enabled the expression of multiple genes at highly diverse expression levels,

ranging from almost negligible to very strong using only one synthetic transcription factor and is independent from growth conditions and externally added compounds.

In general, it becomes unpractical to introduce a large number of heterologous genes into *S. cerevisiae* either on plasmids or integrated into the genome, due to a limited availability of selection markers and suitable integration places. One way to approach this problem is the construction of yeast artificial chromosomes (YAC), which can contain DNA in the range of 200-800 kb. Beside the genes of interest, the YACs contain telomeres, auxotrophic markers for selection, and yeast elements for replication and segregation. When using combinatorial cloning approaches, the creation of YACs allowed screening of randomly assembled biosynthetic pathways, which can help in finding the best combination of genes and promoters in complex pathways [14].

The available knowledge and ease of manipulation enable the development of the yeast into a cell factory that can produce a high variety of products. This has been used in cellular and metabolic engineering approaches. In general, the work process can be divided into roughly 5 steps [15]. The obvious first step is the decision of which product the cell factory should produce, followed by which pathways can lead to this product and which of those can be most easily implemented in *S. cerevisiae*. The target genes for this pathway should be selected and inserted into the genome of the yeast, after which methods for regulating expression levels should be designed to achieve optimal gene expression. Finally, fine tuning of gene expression is most often based on data coming from broad spectrum analytical methods like genomics, transcriptomics, proteomics, metabolomics, and fluxomics [15, 16].

1.2. Established and upcoming products made using *Saccharomyces cerevisiae*

Cellular and metabolic engineering methods have been used successfully many times to create different yeast cell factories, with many products also being manufactured on an industrial scale. Some available and upcoming products for biofuels, small molecules, and therapeutic proteins are discussed below.

1.2.1. Biofuels

Bioethanol is the biofuel that has been produced traditionally using *Saccharomyces cerevisiae*. Currently, it is produced the most in the USA, using corn starch as the carbon source, and the second most in Brazil, using sugarcane and molasses as carbon sources. In the production process, the yeast can reach a 93 % efficiency in conversion of the energy from sugars into alcohol [17, 18]. The main advantage of the yeast in this process is that it is a very robust organism that can reach high cell densities, which allows the production process to reach a high volumetric productivity.

Most engineering strategies have been focused on creating strains that are less sensitive to environmental stresses, or that are more versatile and efficient in using new, and more inexpensive, feedstocks [17, 19]. One example is the use of lignocellulosic biomass hydrolysates, which are a relatively cheap source of a complex mixture of different fermentable sugars, but that also contain inhibitors and salts that decrease the yeast's production efficiency. The engineering of the yeast is on one hand done rationally, for example the use of amylase expressing yeast allowed the production of ethanol from raw corn starch and high-yielding rice as carbon sources at a close to 100 % efficiency [20]. On

the other hand, engineering has been done through randomized approaches, like genome shuffling of UV-light mutagenized yeast populations. The improved growth and survival achieved through genome shuffling allowed the yeast to produce ethanol at up to 60 % of the theoretical yield from hard wood spent sulfite liquor [21].

However, as there is a need in the market for improved, high energy biofuels, the yeast is also engineered to change its metabolism into producing products like butanol, sesquiterpenoids and, fatty acid ethyl esters (FAEE).

Butanol is an attractive product as it can be blended at higher percentages with gasoline in biofuels than ethanol. For production, bacterial genes have been inserted into the *S. cerevisiae* genome to create either 1-butanol or isobutanol [22]. After extensive metabolic engineering, a strain has been created that produced just over 80 % of the maximum theoretical yield of isobutanol. And, with titers up to 18.6 g/L, productivity reached comparable levels with traditional bacterial Acetone-Butanol-Ethanol fermentations, but without the undesired byproducts [18, 22].

Sesquiterpenoids are a class of terpenes and are interesting for the biofuel market as they can be used for the replacement of diesel and jet fuel. An *S. cerevisiae* strain has been reported to produce the diesel replacement bisabolene in the gram per liter scale [23].

FAEE are energy rich, which makes them suitable precursors for use in biodiesels. As *S. cerevisiae* does not naturally produce these fatty acid derivatives in large amounts, metabolic pathways have been engineered to increase the carbon flux towards their biosynthesis. This has led to an increase of lipid content of almost 45 % [24], close to some oleaginous yeasts. In another study lipids accumulated up to 17 % of the yeasts cell dry weight, and further strain engineering resulted in a biofuel production yeast cell factory that produces the fatty acid derived biodiesels in modest milligram per liter scale directly from simple sugars [25]. These examples of more refined biofuel production show that *S. cerevisiae* is also an interesting host for the production of next-generation biofuels. Expectations are that production processes will develop beyond laboratory scale, and large companies like Dupont, Gevo, and Amyris are currently investing in obtaining industrially relevant bioproduction processes [26].

1.2.2. Small molecules

S. cerevisiae can metabolize or utilize a great range of sugars, for example glucose, galactose, saccharose, raffinose, and xylose. This has made it a popular host for the (research into) production of sugar alcohols and artificial sweeteners. Xylitol is a sweetener used for example in chewing gum and is favored because it can reduce the risk of developing dental caries and is safe to use for diabetics. Strains expressing xylose reductases from various sources have proven to be able to completely convert a 4 % xylose source into xylitol [27]. Optimization of cofactor availability created strains that look promising in meeting industrial demands for microbial xylitol production, as optimized fed-batch fermentation produced up to 196.2 g/L xylitol [28]. More recent research has been focusing on replacing xylose with more inexpensive, complex substrates like xylan or cellobiose [29, 30].

A different class of sweetening compounds commercially produced in *S. cerevisiae* are steviol glycosides, like the artificial sweetener Stevia produced by the Swiss-based company Evolva. Originally, the product is extracted from the plant *Stevia rebaudiana,* and it is a

mixture of various rebaudioside molecules. For the production of Stevia components using yeast, a strain was engineered with the introduction of a new steviol glycoside transporter, and with modifications of pathways creating new prenyl phosphate precursors. These precursors are modified into steviol, which is then finally glycosylated into the final rebaudioside compound [31]. The various rebaudioside compounds differ in their saccharide moiety and through expression of specific glycosylation enzymes selection of a wanted specific rebaudioside is possible.

A large part of therapeutic small molecules come from natural sources like plants. This makes their production difficult, as often expensive extraction techniques are necessary to harvest the metabolite of interest. Moreover, often the metabolite levels are low in the plant source, meaning that large amounts of the plant needs to be grown, which sometimes is only possible in the right season. To create a more reliable and inexpensive production process, there has been an interest in the heterologous expression of genes in microbial organisms in order to establish alternative sources. Natural compounds of interest from a plant source include flavonoids, isoprenoids, polyketides, and alkaloids [32].

One of the most promising small molecule therapeutic products is the anti-malarial drug Artemisinin, an isoprenoid, for which the natural source is the sweet wormwood plant *Artemisia annua*. As this drug is used to treat malaria over 280 million times a year, prominently in third world countries, there is a need for an inexpensive, reliable source [33]. The Semi-synthetic Artemisinin Project aimed to engineer a microorganism to produce a precursor of the drug followed by convenient chemical conversion into the active final compound. However, production in *Escherichia coli* strains showed shortcomings in strain viability and was able to only produce a precursor molecule that could not be modified further in *E. coli*, so a switch to *S. cerevisiae* was made. The inducible overexpression of 14 genes, including two cytochrome P450 oxidases and a dehydrogenase from *A. annua*, combined with the inducible repression of a squalene synthase, showed to be the winning strategy to create the artemisinic acid precursor. This strain provided a 15-fold improved production titer and reached titers of up to 25 g/L [34]. Together with this microbial precursor production, the company Amyris has developed an easy to use chemical conversion to the final therapeutic molecule, and released all intellectual property rights for it, enabling an inexpensive provision of treatment to developing countries.

Recently, also significant steps forward have been set in the production of benzylisoquinoline alkaloids, which are a group of pharmaceutically relevant alkaloids, including codeine and morphine. One of the first big steps made was the production of one of the common precursors, norcoclaurine, from simple sugars by *S. cerevisiae* [35]. This was followed by the first complete biosynthesis of opioids from simple carbon sources in yeast [36]. Galanie and colleagues produced thebaine (a very important opioid drug precursor) and hydrocodone by expression of 21 and 23 enzyme activities, respectively, from plant, mammalian, bacterial, and yeast sources [36]. However, they stress that many hurdles need to be taken before the process can be scaled up to industrially relevant levels, including new regulations for safekeeping of this new technology. In the same year, Fossati et al. reconstituted a ten-gene pathway to enable the production of codeine and morphine from the thebaine precursor [35]. It has to be noted that also in this study the final titers were still far from commercially relevant. The strains in their work do not yet enable the production of morphine and codeine from simple sugars, but need precursors supplemented to the yeast

cultures. However, these findings have paved the way for the development of yeast based opioid production.

1.2.3. Therapeutic proteins

S. cerevisiae is one of the preferred host organisms for the production of therapeutic molecules, as it meets many requirements for a safe, low cost process, with high productivity. Examples of recombinant protein products produced in *S. cerevisiae* that have already entered the pharmaceutical market are insulin, vaccines, and blood factors like human serum albumin. Several of these products are exceeding yearly sales of 1 billion US dollar [37].

Novo Nordisk is the main producer of insulin and insulin analogs on the market (for example Novolog®, Actrapid®, Levemir®, and Novomix®), and the company commonly uses the yeast *S. cerevisiae* for production of its blockbuster brands. Research has focused on increasing secretion levels through engineering of vesicle transport, secretion leaders, and variants of pre-proinsulin [38–40]. The main products for human blood factors produced in commercial *S. cerevisiae* strains are Recombumin® USP-NF, which is a human albumin, and albufuse®, which is a line of albumin fusion proteins, both are produced by the Novozymes company. The strains have reached high secretion titers of human serum albumin through expression of the protein disulfide isomerase *PDI1* and the chaperone *KAR2*. Moreover, a yeast strain was used in which (a combination of) genes regulating the ATPase cycle of Kar2p were overexpressed to increase production titers by 70 % into the low gram per liter scale [37, 41]. The first commercial interesting strain for production of human transferrins was developed from the strains used for production of Recombumin® and albufuse®. Improvements were made through strain selection after chemical mutagenesis and overexpression of *PDI1*, leading to a ten- and twelve-fold, respectively, increase of secretion of transferrin up to a titer of 2.25 g/L in high density fermentations [42]. The product is now marketed for example as CellPrime® rTransferrin for life science research use.

The production of hepatitis B surface antigen in *S. cerevisiae* has already been achieved almost three decades ago, with titers in the low milligram per liter range [43–45]. This antigen is used in the preparation of the vaccine against hepatitis B, which is marketed for example as Recombivax HB by Merck or as Engerix-B by GSK. Also for this therapeutic protein overexpression of *PDI1* was used to improve titers, which showed to increase titers six-fold up to 74.4 mg/L in fed-batch fermentations [46]. Another vaccine produced in S. *cerevisiae* is Silgard or Gardasil, protecting against human papillomavirus (HPV). The yeast is used to produce L1 protein, which is the major capsid protein of HPV, of HPV types 6, 11, 16, and 18. These proteins can self-assemble into virus-like particles that can trigger immunization but are not able to infect patients as they lack viral DNA [47].

1.3. The yeast secretory pathway

A common problem in recombinant protein production in *Saccharomyces cerevisiae* is that secretion titers remain lower than what the yeast theoretically can secrete. Bottlenecks that decrease the secretion efficiency have been found at several places along the secretory pathway. It is important to know how the secretory pathway functions, in order to be able to understand how it is possible to engineer it to achieve a higher secretion efficiency. Therefore, in this chapter the most important parts of the secretory pathway, and the proteins that play a key role in these processes are discussed. Where applicable, the end of each section describes how that part of the secretory pathway has been manipulated in order to improve the secretion of heterologous proteins.

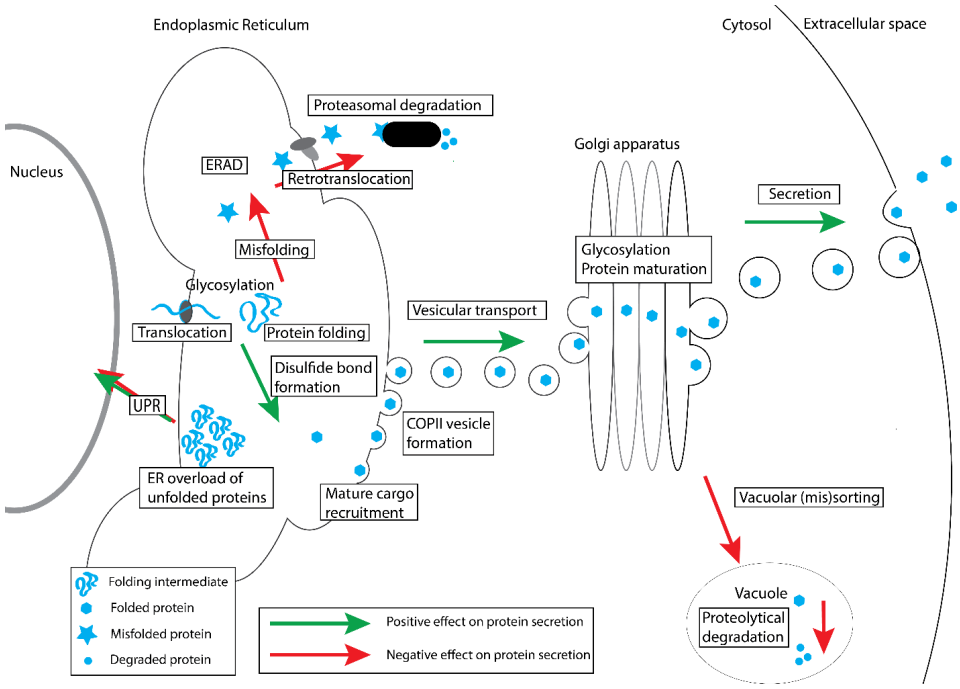


Figure 1 **Overview of the Saccharomyces cerevisiae secretory pathway.** Green arrows show the canonical secretory pathway, starting with polypeptide translocation from the cytosol into the ER-lumen. This is followed by N-glycosylation and chaperone assisted protein folding, and after reaching its native state, the protein is transported to the Golgi system. There, protein maturation occurs, which can include final glycosylation steps and proteolytic cleavage. Finally the protein is secreted into the extracellular space via the cell membrane. Red arrows show processes that can affect secretion negatively, which are the unfolded protein response (UPR) , a cellular response to cope with the stress of unfolded proteins, the ER associated degradation (ERAD) pathway, which degrades misfolding or slowly folding proteins. And finally, missorting from the Golgi towards the vacuole where proteins are degraded by the resident proteases.

The protein secretory pathway in *S. cerevisiae* has been extensively studied and reviewed in literature [38, 48–55]. The canonical pathway is considered to start with translocation of the nascent polypeptide into the lumen of the endoplasmic reticulum (ER) after or during translation. Inside the ER protein folding, in some cases with the help of molecular chaperones, and protein glycosylation take place. After reaching their native state, the

proteins are transported to the Golgi apparatus, and after traversing this, secretory vesicles mediate the final step towards and beyond the cell membrane. An overview of these most important steps in the secretory pathway and how they can influence secretion titers is shown in Figure 1.

1.3.1. The endoplasmic reticulum: the protein folding factory of the cell

The ER is the protein folding factory of the cell and most nascent proteins enter this compartment co-translationally. The importance of the ER as a protein folding factory is highlighted by theoretical estimates that on average around 460 polypeptides per second enter the ER lumen, which amounts to the import of almost 0.75 % of the steady-state ER protein content per minute [56]. The translocation of a polypeptide from the cytosol into the ER requires coordination between several processes and the involved proteins. First, the ribosome that is producing the polypeptide creates the sequence of the signal peptide on the new polypeptide, which is recognized by the signal recognition particle. Together these have to be recognized by the signal recognition receptor, so that finally recruitment to the Sec61 translocation pore complex, through which the ER is entered by the nascent polypeptide, can happen [57, 58]. After leaving the translocation pore at the luminal side, the oligosaccharyl transferase (OST) complex scans the polypeptide for glycosylation sites and, if found, transfers the glycan to the polypeptide. The proteins complete their folding process by reaching their native state, after which they leave the ER. The folding process is assisted by various chaperones and folding enzymes, while other proteins are present to assure that the quality of the proteins is up to par.

1.3.2. N-Glycosylation

Over 350 proteins in *S. cerevisiae* have been validated to be N-linked glycoproteins [59], and the initial glycosylation steps take place in the ER. A schematic overview of these steps is shown in Figure 2. Preceding the transfer of the oligosaccharyl structure to a nascent protein, its $Glc_3Man_9GlcNAc_2$ precursor is created on a lipid carrier. This lipid carrier is the isoprenoid lipid dolichol, which is assembled by a cis-prenyltransferase in the ER-membrane. The dolichol kinase Sec59p then phosphorylates the dolichol and the dolichol-phosphate molecule is then able to receive the first sugar molecule for the buildup of the glycan. Most of the proteins involved in the construction of the glycan moiety are part of the *ALG* gene family, for Asparagine Linked Glycosylation. The first steps of the lipid linked oligosaccharide (LLO) biosynthesis happen on the cytosolic side of the ER-membrane, where the starting point for the oligosaccharide biosynthesis is the addition of the first GlcNAc molecule to the dolichol-phosphate. In total seven enzymes in the cytosol build up a $Man_5GlcNAc_2$ glycan [60]. The next step is the transfer of the glycan tree to the luminal side of the ER, which is postulated to be done by the yeast flippase Rtf1p [61].

In the ER, the next four mannose and three glucose residues are step-wise transferred to the glycan structure in seven additional reactions, hereby creating the final $Glc_3Man_9GlcNAc_2$ structure [60]. The OST complex is the key player that combines glycan biosynthesis with protein biosynthesis [62]. The complex co-translocationally transfers the $Glc_3Man_9GlcNAc_2$ structure to the specific N-glycosylation consensus site (Asn-Xxx-Ser/Thr) of a wide range of nascent polypeptides [63]. The glycan structure is then further modified during the folding process of the protein, first by glucosidase I, which removes the outermost glucose from the glycan tree [64]. The glucosidase II enzyme then cleaves off the second glucose,

which allows the lectin Cne1p to bind to the glycan, and finally also the third glucose is removed by glucosidase II [65]. After removal of the final glucose, a slowly acting mannosidase removes the terminal mannose from the B-branch of the glycan tree, and the $Man_8GlcNAc_2$ serves as the signal for removal from further folding cycles [66].

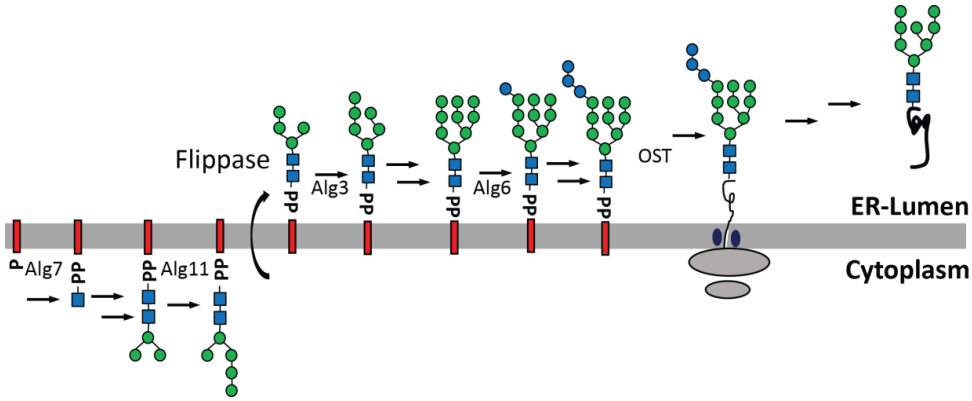


Figure 2 **Schematic overview of N-glycosylation in the ER**. At the cytosolic side of the ER-membrane the lipid linked oligosaccharide is formed through consecutive action of seven glycosylation enzymes to create the $Man_5GlcNAc_2$ glycan. Next, a flippase transfers the glycan moiety into the ER-lumen for further buildup of the general $Glc_3Man_9GlcNAc_2$ N-glycan structure in seven steps. The oligosaccharyl transferase complex transfers the glycan tree to the consensus site for N-glycosylation (Asn-Xxx-Ser/Thr) on a nascent polypeptide entering the ER. During the folding process in the ER, part of the glycan tree is trimmed down as part of the quality control process. When a protein is folded and has the final $Man_8GlcNAc_2$ glycan structure, it is ready for export out of the ER. Red Block: dolichol-P, blue circles: glucose, green circles: mannose, blue squares: GlcNAc.

For many glycoproteins glycans are important for proper folding, as aberrant or missing glycans, due to a defective glycosylation pathway, can lead to a decreased folding and secretion efficiency [67]. The glycan structure allows selective recognition by a variety of proteins like lectins and glycosidases. Partly, these can assist the protein in the folding process, partly the glycan structure also acts as a quality control signal for the cell to assess protein conformation [68]. If the glycan on a protein that has reached its native state is trimmed down to the $Man_8GlcNAc_2$, it won't be subjected to further folding cycles and is ready for export out of the ER. However, certain glycan signals created by further trimming of the glycan tree, combined with unfolded or misfolded patches in the protein, can lead to targeting of the proteins to the ER associated degradation pathway (ERAD), as described further down.

N-linked glycosylation in strain engineering and heterologous protein production

The interest in N-glycosylation in heterologous protein production in *S. cerevisiae* is twofold; first the glycans can play a role in secretion efficiency and protein activity, and second; there is interest in creating strains with so-called humanized glycans for the production of therapeutic proteins. The latter approach is discussed in chapter 1.5.3.

In multiple studies positive and negative effects of N-glycosylation on secretion efficiency have been found. In one study an α-amylase from *Bacillus licheniformis* was expressed in a panel of yeasts with deletions of non-essential genes, and among the deletions identified in the strains with an improved α-amylase production all non-essential N-glycosylation genes

were found. Even though α-amylase is not natively glycosylated in *B. licheniformis*, its amino acid sequence contains six N-glycosylation consensus sites that can be recognized in *S. cerevisiae*. Analysis of the secreted product showed that in the strains with a beneficial deletion an increase in underglycosylated products was secreted in comparison to wild-type. This indicates that yeast N-glycosylation might hamper α-amylase secretion, as the underglycosylated enzymes could possible escape ERAD-mediated degradation. The positive effect of a decreased glycosylation was confirmed through the creation of non-glycosylated α-amylase mutants, which were secreted up to threefold higher than the glycosylated variant [69]. It was hypothesized that through the slow folding process of the enzyme, the glycan structures added by the yeast are modified to target the enzyme for degradation. Although the study for the α-amylase showed an increase in secretion titers, for the production of an exocellulase from *Phanerochaete chrysosporium* glycosylation was shown to mostly affect its enzyme activity. As deletion of 22 glycosylation related genes increased specific enzyme activity at least 1.3-fold, and even up to 6-fold [70].

In contrast to the results above, Sagt and colleagues showed that introduction of N-glycosylation sites in some cases can improve secretion [71]. They describe a case where an engineered cutinase was not readily secreted because of induction of aggregation in the ER through the added hydrophobic residues. After addition of an N-glycosylation site on the N-terminal region of the polypeptide, secretion levels increased fivefold, without altering specific enzyme activity significantly. The authors hypothesize that the introduced N-glycans shielded the hydrophobic patches and thus prevented aggregation [71].

1.3.3. Chaperones and folding enzymes

A plethora of proteins is located in the ER to assist in protein folding and they can be divided in 4 main groups: chaperones and their auxiliary co-chaperones, oxidoreductases like the protein disulfide isomerases (pdi), peptidyl-prolyl-isomerases (ppi), and the yeast homolog to the lectins calnexin and calreticulin (Cne1p).

Chaperones: Kar2p

The main folding enzyme in the ER is the chaperone Kar2p, often also referred to in the name of its mammalian homolog - BiP, both proteins are HSP70 protein family members [72]. Through an interaction with the Sec61 pore complex, the Kar2p protein binds the nascent polypeptide entering the ER-lumen [73]. The Kar2p binding prevents undesirable interactions between hydrophobic regions of nascent polypeptides [74]. The proteins Sil1p, Lhs1p, Jem1p, and Scj1p are the main proteins regulating the activity of Kar2p [41, 75]. Jem1p and Scj1p promote hydrolysis of Kar2p bound ATP. Sil1p and Lhs1p are nucleotide exchange factors that facilitate the release of ADP from Kar2p and provide it with new ATP so that it can cycle through another stage of substrate binding in protein folding or translocation [48, 75]. Additionally, binding of Kar2p to the hydrophobic patches prevents aggregation, and it plays a role in targeting misfolded proteins to ERAD. Kar2p is involved in the UPR through its characteristic binding association with the ER membrane bound Ire1p. When unfolded proteins enter the ER, they are bound by the free pool of available Kar2p. Eventually, when even more unfolded proteins become available in the ER, bound Kar2p will dissociate from Ire1p.This means that the now free luminal domain of Ire1p can also bind unfolded proteins, leading eventually to induction of the UPR, as described below. It was shown that the availability of free Kar2p was drastically decreased when cells experienced endoplasmic reticulum stress [49, 76]. This indicates that the protein acts as a

buffer through modulation of the sensitivity of the UPR to unfolded proteins [77]. For this, of course a high abundance of Kar2p is necessary and this explains why it is one of the most abundant ER-chaperones, with an estimated over 30,000 copies per cell [78]. There is evidence that under stressed conditions the regulation of the Kar2p ATPase activity might be limiting as there is far less Lhs1p and Sil1p, at around 139 and 2420 molecules, respectively. However, a slower Kar2p activity cycle might aid in induction of the UPR, so that a stronger response to the stress causing unfolded proteins is initiated [41, 78].

Oxidoreductases
One essential step for most of the proteins that traverse the secretory pathway, is the oxidation of cysteine residues into disulfide bonds in order to reach the final, stable native state of the protein. The protein disulfide isomerase 1 protein (Pdi1p) is one of the key players in ER oxidative protein folding and is essential for cellular viability. Pdi1p contains two thioredoxin like domains that are active and two that are inactive. The active thioredoxin domains contain an active site that has the CXXC motif, which makes the protein capable of transferring disulfide bonds to nascent proteins. In addition, Pdi1p is also able to reduce and isomerize disulfide bonds if restructuring is necessary for proper protein folding of the substrate [79]. If a protein is incapable of folding correctly, Pdi1p plays a role in targeting it to the ER associated degradation pathway, described below [80]. After transfer of disulfide bonds, Pdi1p needs to be re-oxidized by Ero1p (ER oxidase 1), which contains a flavin domain used for dithiol-disulfide exchange [81]. In absence of Ero1p, Pdi1p cannot properly function and an accumulation of unfolded proteins with incomplete disulfide bonds is the result [82–84]. The other known ER-resident sulfhydryl oxidase Erv2p is, based on *in vitro* characterization, also expected to interact with Pdi1p, but not much is known about its function [85].

Four more members of the PDI protein family have been identified in *S. cerevisiae*: Mpdi1p, Mpdi2p, Eug1p, and Eps1p. However, these proteins are much less characterized and their specific roles are unknown. Eps1p, is assumed to play a role in the ER associated degradation pathway for membrane bound proteins [86]. Mpdi1p was suggested to be involved in disulfide bond formation of glycoproteins through association with calnexin [87]. Finally, Mpdi2p and Eug1p have not been functionally characterized.

Peptidyl-prolyl-isomerases: Cpr5p
S. cerevisiae contains 13 proteins involved in catalyzing the cis-/trans-isomerization of proline peptide bonds. From these, eight belong to the cyclophilin type PPIases, four to the FK506-binding proteins, and one is a parvulin [50]. From these, five can be found in the secretory pathway and play some role in protein folding: Cpr2p, Cpr4p, Cpr5p and Fpr2p/Fkb2p [88]. However, very little is known about their exact role, but it was shown that these proteins are not essential for cell viability, as a combined knockout of the all PPIases did not impair growth. The ER-resident PPIases are upregulated in conditions that lead to protein misfolding, indicating that they have some role in protein folding [88, 89].

The calnexin and calreticulin homolog Cne1p
The *S. cerevisiae* calnexin and calreticulin homolog (Cne1p) is an important protein involved in the early stages of glycoprotein folding in the ER. Just like Kar2p, Cne1p has chaperone functions and aids in folding of its bound protein substrate [90]. It recognizes the $Glc_1Man_9GlcNAc_2$ glycan structure on proteins that are in the process of folding [67]. The

protein was shown to effectively enhance the refolding of denatured protein substrates, but only in presence of the correct glycan structure on the glycoprotein substrate [91]. This means that glucosidase II mediated removal of the terminal glucose molecule can work as a trigger for the release of the bound glycoprotein from calnexin binding, as it vastly decreases calnexin binding efficiency [92]. After release from calnexin, the folding protein either reaches it native state and is exported out of the ER, or is considered terminally misfolding and targeted to the ER associated degradation pathway.

Using folding partners to increase protein folding and secretion
The folding capacity in the ER has been widely regarded as a bottleneck for the efficient production of heterologous proteins in *Saccharomyces cerevisiae* [51, 52, 93–95]. As such, modification of the number of available folding partners in the ER to increase the folding capacity, and thereby increasing secretion levels of the heterologous protein of interest has been an often used approach. However, the outcomes of these studies varied a lot, and most of the outcomes indicated that results are very protein specific and thus generally applicable approaches are elusive so far.

This unpredictability is very well illustrated by the example of modifying Kar2p levels. And indeed for many proteins, like an acid phosphatase from *Schizosaccharomyces pombe,* G-CSF, BPTI, and thaumatin, no positive effect on secretion was found when Kar2p levels were increased [96, 97]. For the first three, however, a decrease of Kar2p levels also diminished their secretion levels on average two- to fourfold [97]. The most prominent increase in productivity achieved with overexpression of Kar2p was obtained with expression of an invertase-bovine prochymosin fusion protein, which resulted in up to 20-fold higher titers [96]. Also the expression of scFv antibody fragments was increased 1.2- to fivefold upon *KAR2* overexpression [98, 99]. In one study on the production of the enzymes B-glucosidase, endoglucanase, and α-amylase, modest increases were found for the first the first two enzymes with *KAR2* overexpression, but in all cases Pdi1p showed more promising results [100].

A different promising approach that involved Kar2p, was to increase the presence of Sil1p, Lhs1p, Jem1p, and Scj1p, which regulate its ATPase cycle. From these, Lhs1p and Jem1p overexpression increased titers of a recombinant human albumin, GM-CSF, and recombinant human transferrin [41]. However, combined overexpression did not yield any synergistic effects, as secretion levels never exceeded the effect of the overexpression of *JEM1* alone.

The formation of disulfide bonds can be considered a limiting factor in recombinant protein production, as overexpression of Pdi1p was in general beneficial for production titers. Highest improvements were a 12-fold increase of human recombinant transferrin, a tenfold higher titer of human platelet derived growth factor B homodimer, and a fourfold increase in titer of the *S. pombe* acid phosphatase [42, 101]. In the case of overexpression of Pdi1p to increase the production of scFv fragments, only a moderate increase in titer was found [99]. Interestingly, it is not only the enzymatic activities of Pdi1p that aids protein folding. Studies performed with either a beta-glucosidase with only one cysteine, or completely without cysteine residues [95, 102], or with expression of a Pdi1p variant devoid of its thioredoxin motif [103], showed an increase in production titers even though in these cases Pdi1p could

not play a role in disulfide bond formation or isomerization. This indicates that Pdi1p also has a chaperone activity and can thus assist in the folding process in a more general way.

In some of the cases describe above, also the combination of Kar2p and Pdi1p overexpression was studied [98, 99, 101]. The data supports a synergistic effect in all three cases, indicating that both proteins assist folding at different stages [99]. It was suggested that Kar2p aids mostly in increasing translocation efficiency and in preventing aggregation of unfolded parts of the proteins, whereas Pdi1p is an active partner in the folding process. *In vitro* studies suggest that Kar2p might capture the folding protein, in that way making the cysteine residues available for Pdi1p to create the disulfide bridges [104].

ERO1 was found in a screen for interesting candidate genes that improve surface targeting of heterologous proteins to the cell wall, using a high throughput screen of yeast cDNA libraries using yeast surface display, allowing the use of rapid flow cytometry analysis. The positive effect was confirmed to also be present for secreted proteins, as the secretion levels of various single-chain T-cell receptors and scFv antibody fragments were becoming higher after *ERO1* overexpression, ranging from an 1.5- up to a 8-fold increase [105]. In the case of a construct that allowed carboxypeptidase A to be secreted, a combination of Kar2p, Pdi1p, and Ero1p showed synergistic effects. From the single overexpressions Kar2p was the most interesting, showing 20 % more secretion than Pdi1p and Ero1p. When all three were combined, titers increased another 36 %, while a combination of Pdi1p and Ero1p led to a relative decrease in titers [106].

Finally, also manipulation of Cne1p levels showed varying results. First, disruption of the *CNE1* gene improved secretion of the glycosylated hen egg white lysozyme, and of a mammalian antitrypsin molecule [107–109]. In contrast, for the expression of a measles surface glycoprotein overexpression of the human calnexin increased secretion 2.5-fold of the soluble glycosylated form, while overexpression of human homologues of Kar2p and various Pdi's didn't improve yields [110].

1.3.4. The unfolded protein response

The folding environment in the ER is crowded with unfolded, folding, and fully folded proteins. To be able to handle this chaos, it is critical for the cell to maintain a proper protein homeostasis. If the ER reaches an abnormal high load of unfolded proteins, the unfolded protein response (UPR) is activated to attenuate stress levels and restore protein homeostasis. It was postulated that the transmembrane protein Ire1p acts as a direct sensor for unfolded proteins [111]. A domain of the luminal side of Ire1p binds directly to unfolded proteins, leading to Ire1p dimerization and subsequent activation of Ire1p [111, 112]. However, some studies suggest that binding to unfolded proteins is not necessary for Ire1p activation [113]. As described above, the ER chaperone Kar2p occupies under unstressed conditions the Ire1p unfolded protein binding site, so that it only becomes available when Kar2p is sequestered from Ire1p [114]. The catalytically active form of Ire1p induces the splicing of the inactive form of the *HAC1* mRNA transcript (*HAC1*^{i}) into its active *HAC1*^{u} form. The *HAC1*^{i} mRNA can't be translated, as part of its intron interacts through base-pairing with the 5' untranslated region of the mRNA, thus effectively preventing translation of the protein product [115].

The *HAC1* protein product is a transcription factor that regulates the response of UPR target genes [116]. Through transcriptional profiling it has been shown that almost 400 genes are

targets for regulation by the UPR. Its target genes are part of processes like protein folding, glycosylation, ER size regulation, ER quality control and protein degradation, and several vesicular transport processes [53, 117–122]. Analyzing the variable responses of the UPR triggered by different sources of stress, Thibault and colleagues found that the stress response is not just a generic on/off switch, but it can be modulated. This means that not only the strength of the UPR is regulated, but regulation includes differential target gene expression based on the source of the stress [123]. Moreover, transcript specific translational regulation was proven through microarray studies, showing that the UPR selectively downregulates genes involved in ribosomal biogenesis, while upregulating various ER folding genes [124]. Additionally, for sustained activity of the UPR the stress response induces transcription of the gene of its own activator *HAC1*. The cells need this autoregulation of transcription of the *HAC1* gene, as strains where this is deactivated become sensitive to prolonged ER-stress through a decrease of available $HAC1^{i}/HAC1^{u}$ mRNA [125].

The transcriptional regulation of UPR target genes is mediated through the so-called unfolded protein response element (UPRE) that is located in the promoter regions of the affected genes [126–128]. The first UPRE sequence was discovered in the promoter of the *KAR2* gene. The necessary and sufficient part of the UPRE sequence was shown to be a palindromic sequence with a one nucleotide spacer in between both three nucleotide long sides [126]. Later, it was shown that there are many more different UPRE sequences to be found, and that they can also be divided into distinct classes based on their structure and length, allowing the variable regulation of the distinct UPR target genes [127, 128].

The UPR in strain engineering and heterologous protein production

It is important to gain insight into the influence of the yeast stress response to unfolded proteins, as the UPR is often induced in yeast strains producing heterologous proteins [99, 129, 130]. Data has suggested that in some cases it is advisable to tune expression levels of the heterologous protein down in order to not activate the UPR, as it can decrease secretion titers [99]. However, using the overall UPR activity to increase protein secretion has also been used, with a constitutive UPR induction through overexpression of the active form of *HAC1* [131]. This resulted in a twofold increase of a native invertase levels, and a 70 % increase of production of an α-amylase from *Bacillus amyloliquefaciens*, but it did not affect secretion of a *Trichoderma reesei* endoglucanase [131]. In the same study, the effect of UPR disruption through inactivation of the *HAC1* was studied, and it showed to decrease secretion of the endoglucanase and the α-amylase by up to 50 and 75 %, respectively. Moreover, in another study the titers of a human insulin precursor and an α-amylase were dropping more severely by over 95 % [132]. These results indicate that the presence of an active UPR response can be necessary for the cells to cope with secretion stress.

The source of the *HAC1* gene for UPR induction does not necessarily need to be *S. cerevisiae*. Using the *T. reesei HAC1* gene, the expression of α-amylase from *B. amyloliquefaciens* was increased 2.4-fold, and a slight increase in *S. cerevisiae* invertase titers was found as well [131]. The approach of overexpression of *HAC1* also showed to be beneficial for the secretion of some heterologous proteins in *Pichia pastoris* and *Aspergillus niger* [120, 133, 134].

1.3.5. The endoplasmic reticulum associated degradation pathway

One of the main protein quality control systems in the ER is the ER associated degradation pathway (ERAD). This process is always active, but its activity is vastly upregulated by the

UPR, while its disruption leads to constitutive UPR activation [117, 122]. In yeast, three distinct ERAD pathways exist, being the ERAD-M (for membrane proteins), ERAD-C (for cytosolic proteins), and the luminal glycoprotein (ERAD-L) pathways [135], highlighting the importance of ERAD for maintaining of the protein homeostasis. Considering the scope of this thesis, the focus will be on the ERAD-L pathway responsible for disposing soluble misfolded glycoproteins. A schematic overview of the ERAD key players and its connection with protein folding and the UPR can be found in Figure 1 of publication I.

The degradation of glycoproteins in ERAD is regulated through recognition of the glycan structure on the protein. Upon entrance into the ER, the $Glc_3Man_9GlcNAc_2$ glycan is transferred onto the nascent polypeptide and during the folding process the three glucoses are trimmed of. Next, the trimming step of the first mannose by mannosidase I is slow, but eventually it will create the $Man_8GlcNAc_2$ glycan, which acts as a signal to remove the glycoprotein from further folding cycles [66]. If a protein is folding very slowly or is misfolding while containing this glycan, its rate of ERAD degradation is increased [136]. From that moment, the Pdi1p/Htm1p protein complex can recognize it as an ERAD target, and start its ERAD-mediated clearance from the ER [137, 138]. Although Pdi1p was shown to assist in oxidative folding of proteins when it's on its own, also by itself it can contribute to ERAD of certain substrates through its chaperone or redox activity [80]. When in complex with Htm1p, the ERAD targeting of misfolded proteins is initiated via a new trimming step by the Htm1p mannosidase, removing the terminal mannose of the C-branch, creating the $Man_7GlcNAc_2$ glycan [139].

Yos9p is an ER resident lectin that has an affinity for the now available terminal α1,6-linked mannose residue [140, 141]. Data has shown that all these trimming and recognition steps are coordinated in a sequential fashion, for example, without Htm1p, Yos9p mediated recognition of ERAD substrates is severely diminished [139, 141]. Recognition by Yos9p leads to targeting of the misfolded protein towards the HRD-ligase complex, which is localized in the ER-membrane and which mediates retrotranslocation to the cytosol [142]. The core of this complex consists of Hrd1p, Hrd3p, Usa1p, and Der1p, and from these proteins, Yos9p binds to Hrd3p. It was suggested that Hrd3p is the complex's subunit that recognizes proteins that are not in their native state and Yos9p makes this more specific by only targeting misfolded glycoproteins to it [143, 144]. The misfolded protein then associates with the close by Der1p, and it was suggested that this association leads to the insertion of the substrate into the translocon complex in the ER-membrane [145].

En route through the membrane the substrate is targeted to the ubiquitin ligase Hrd1p for ubiquitination. It was shown that here Hrd1p and Hrd3p work together in such a way that Hrd3p helps to stabilize Hrd1p and that it regulates Hrd1p's RING domain activity [146], providing a coordination between the ERAD steps on both sides of the ER membrane. The ubiquitination step is performed by the RING domain of Hrd1p, together with the two ubiquitin conjugation enzymes Ubc1p and Ubc7p, that provide the ubiquitin to Hrd1p [122, 147–149]. Ufd1p recognizes the polyubiquitin chain, and then targets the Cdc48p/p97 AAA-ATPase to Hrd1p [150, 151]. This complex ensures the completion of the retrotranslocation step by pulling the degradation substrate out of the ER membrane into the cytosol [147]. The exact step how the substrate finally reaches the proteasome complex for degradation is still unclear, but most likely there is an interaction with other shuttle factors mediating this step [152, 153]. Evidence for this includes the fact that before

degradation the cytosolic enzyme Png1p removes the glycans from the to be degraded protein [154, 155], and Png1p can also bind Rad23p, an ubiquitinated-protein binding protein that transfers those proteins to the proteasome for degradation [156]. So it was suggested that interplay of Png1p and Rad23p can target the ERAD substrate to the proteasome for degradation.

Even though ERAD is constitutively active, it is closely connected with, and upregulated by the UPR [117]. The feedback between the two systems also works in the other direction, as a removal of essential ERAD genes leads to a constitutive induction of the UPR [117, 122]. Data has suggested that if the UPR is non-functional, the ERAD pathway is sufficient to clear the ER form misfolded proteins, under the condition that no further unusual stress is imposed on the cells [117, 119, 122].

1.3.6. Exit from the ER: vesicular transport to the Golgi apparatus

One of the critical steps in maintaining a proper ER-homeostasis is the exit of the fully folded proteins out of the ER for further processing, while keeping the proteins still in the folding process inside the ER. Most of the details of these processes are currently still not fully understood. In general, trafficking of cargo away from the ER towards the Golgi is mediated by COPII vesicles, hence the name vesicular transport. The collection and sorting of cargo proteins into these vesicles takes places at ER exit sites (ERES). The family of Sec proteins plays a vital role, starting with in the assembly of all components of the COPII-vesicles. Through action of the membrane bound Sec16p and Sec12p, the Sar1p protein gets recruited. This protein initiates the formation of the COPII vesicles by recruitment of Sec13p, Sec23p, Sec24p, and Sec31p, together they complete the bud formation [157, 158].

During vesicle bud formation, it is of great importance that cargo proteins are accurately and selectively recruited. For this, as part of the COPII complex, the Sec24p was found to bind selectively to most classes of cargo proteins, having at least three binding sites for cargo proteins [159, 160]. The Sec24p homologs Sfb2p and Sfb3p were shown to even increase the capture of cargo proteins through an overlap of binding affinity, while additionally binding different cargo classes [161]. However, also other methods to recruit fully folded proteins to the vesicles exist. For example, by unspecific recognition of folded proteins through cargo receptors, or cargo anchors, although only few of these have been characterized [50].

It has been shown that the vesicle budding from the ER is one of the processes that is upregulated by the UPR. An increase in transport speed is achieved through stimulation of COPII vesicle formation [118, 162]. This indicates that the UPR regulated reaction to regain a proper protein homeostasis in the ER is not just based on improving proper protein folding in the ER, or on more efficient targeting of misfolded proteins for degradation, but also on ensuring an increased transport of mature cargo proteins out of the ER in order to make more space. Additionally, the secretory pathway is also thought to prevent unnecessary activation of the UPR. Data suggests that, in the absence of stress, one of the small GTPases regulating ER-to-Golgi trafficking, Ypt1p, binds selectively to unspliced *HAC1*^{i} mRNA [163]. This binding ensures that the *HAC1*^{i} mRNA is effectively taken away from getting spliced and activated by Ire1p at the ER membrane. After being trafficked away from the ER an unknown process mediates the degradation of the *HAC1*^{i} mRNA [164]. This makes the UPR and vesicular transport a double feedback loop.

After the vesicles are completely bud off from the ER, they move towards the Golgi apparatus through diffusion. Close to the Golgi apparatus, they find their target membrane through tethering, docking and, finally, they fuse with the Golgi membrane. Tethering to the Golgi requires three key players: the TRAPPI complex, and the Ypt1p and Uso1p proteins. The first step is the direct interaction of one of the TRAPPI subunits with the Sec23p COPII coat protein [165], which is mediated by action of Uso1p and Ypt1p [166]. Next, as that is necessary for membrane fusion, the COPII coat dissociates from the vesicle and *trans*-SNARE pairs are formed to initiate membrane fusion. The SNARE proteins are most commonly divided into v- and t-SNAREs, for vesicle membrane and target membrane, respectively. The proteins contain a characteristic SNARE-motif, which is unstructured when they are monomeric, but forms highly stable helical bundles when they are in complex with each other [167]. This complex formation pulls both membranes closely together, after which they fuse together [168]. Finally, after fusion the cargo is delivered into the Golgi apparatus.

Modification of ER-Golgi vesicular transport
The overexpression of *SLY1*, a regulator the SNARE proteins involved in the Golgi membrane targeting step, was shown to increase the production of *Bacillus* α-amylase, but not for endogenous invertase, or for a human insulin precursor [38]. The authors suggested that the relative large size of the α-amylase might lead to a saturation of the folding capacity in the ER, for which the increase of vesicular transport away from the ER can relieve the folding stress.

1.3.7. Processing in the Golgi apparatus

After the fully folded proteins have been transferred towards the Golgi apparatus, the final steps in post-translational processing can be performed. One of the main processes here is the maturation of the glycans on N-glycosylated proteins. The existing glycan branch is extended with mannose residues in up to 4 consecutive steps. First, Och1p adds one mannose, followed by addition of ten mannose residues by the mannan polymerase I complex, and finally the addition of 40 mannoses by the mannan polymerase II complex [169, 170]. In some cases, Mnn1p, Mnn2p, and Mnn5p can even increase the mannose extension more extensively [171].

On the protein side the Golgi apparatus is where the maturation of some of the proteins through cleavage of their peptide chains takes place. This cleavage can for example activate catalytic activity, or allow a change in protein confirmation so that the protein can achieve binding to its target receptor. For this, the Golgi apparatus contains three resident proteases: Kex1p, Kex2p, and Ste13p. They all have a different polypeptide sequence recognition site, Kex1p is a carboxypeptidase and cleaves before arginine or lysine. Kex2p is an endoprotease and cleaves at arginine-arginine or lysine-arginine. Finally, Ste13p is a dipeptidyl aminopeptidase and cleaves at repeating X-arginine motifs [172].

Golgi apparatus modifications in heterologous protein production
The gene *OCH1,* encoding a key enzyme in the elongation of the glycan outer chain, has been deleted to avoid hypermannosylation of secreted recombinant proteins, either in combination with deletions of *MNN1* and *MNN4,* or with deletions of *MNN1* and *MNN9* [173, 174]. In the former combination, it was reported that, even though the produced

glycans are a mammalian-type high mannose structure, the strains were temperature sensitive, and showed severe growth retardation. After use of a mutagenesis technique, the growth phenotypes were diminished, and the mutant strains showed to be highly efficient secretors of luciferase and human galectin-9, while retaining the mammalian-type N-glycan structure [173]. In the latter study, the triple mutant also showed growth defects, but more importantly a significantly increase in secreted activity of three heterologous enzymes was measured. The authors report that this was not the result of a change in specific enzyme activity, but rather by an increased protein yield. Data showed that the increased secretion was the result of an up-regulation, unrelated to the UPR, of certain components of the secretory pathway, combined with a more efficient release of proteins through a decreased cell wall integrity [174].

Kex2p is a membrane bound endoprotease in the Golgi apparatus that processes the precursor of the mating factor α (Matα) propeptide by cleaving at its Lys-Arg sites [175]. The Matα sequence is often used as a secretion leader sequence for heterologous proteins, and processing of the Matα propeptide is often considered to be a limiting factor in obtaining correctly processed recombinant protein during expression. However, overexpression of *KEX2* has given mixed results, as in one study it did not improve the yield of BPTI, but, when the cleavage site in the secretion signal recognized by Kex2p was optimized in the BPTI sequence, an increased efficiency in processing was found to be beneficial for secretion titers [176]. In a different study, using the Matα signal sequence with sHBsAg as the protein of interest, cell viability was greatly decreased when *KEX2* was strongly overexpressed. In moderate levels, the ratio of cleaved to uncleaved protein found in the culture supernatant was improved, but it did not increase total secretion levels [177].

1.3.8. Post- Golgi apparatus processes: Exocytosis

After maturation in the Golgi apparatus, the proteins are in their final steps of the secretory pathway: the vesicular transport out of the Golgi apparatus towards the cell membrane. For secretion of soluble proteins, this happens in so-called heavy density secretory vesicles (HDSV), while targeting of membrane proteins happens with light density secretory vesicle (LDSV) [178]. These clathrin coated vesicles travel together with motor proteins along cytoskeletal tracks to reach the plasma membrane. After arriving at the plasma membrane, fusion of the secretory vesicle is mediated through SNARE proteins located on both the vesicle and the target membrane. The multisubunit complex involved in this process is called the exocyst, but extensive research nonetheless, there is still much unknown about the exact molecular mechanisms involved in this last trafficking step [179]. In general, the exocyst is thought to form the first connection between the plasma membrane and the secretory vesicle [180]. This might bring the SNARE proteins on each side close enough to interact with each other to form the SNARE complex needed for docking [179]. For example, the Sec6p subunit of the exocyst is known to bind the plasma membrane SNARE protein Sec9p, associated with either the plasma membrane SNARE Sso1p, or with Sso1p-Snc2p (Snc2p is the corresponding vesicle SNARE protein) [181]. Sec9p forms a binary complex together with Sso1p, allowing association with the vesicle SNARE Snc2p [182]. These interactions pull the membranes close enough together so that membrane fusion can occur and the proteins are secreted from the cells.

Improving protein secretion by modifying late vesicular trafficking components

Various approaches have been used to improve vesicular trafficking steps. A straightforward approach is to increase the amounts of the components involved in trafficking towards the plasma membrane. The expression of various heterologous enzymes was successfully increased through the overexpression of the exocytic SNARE genes *SSO1*, *SSO2, SNC1,* and *SEC1* [38, 100, 183, 184]. Interestingly, modification of these late steps of the secretory pathway seemed to provide a more general improvement, as in the studied proteins with improved secretion ranged from an endogenous invertase and a human insulin precursor to enzymes from various microbial sources.

1.3.9. Post- Golgi apparatus processes: protein sorting and missorting

One hurdle often found in the secretory pathway are the alternative transport routes out of the Golgi apparatus that don't lead to secretion, for example towards the vacuole. A way to overcome this hurdle, is to minimize trafficking towards the vacuole, as some heterologous proteins tend to be missorted towards the vacuole for degradation [185]. The genes involved in vacuolar protein sorting are called the *VPS* genes, and most of them have been found in deletion screens for abnormal vacuolar phenotypes, either in vacuolar shape, or protein content, and the latter phenotype is most often exemplified through abnormal secretion of the vacuolar protease CPY. The group of *VPS* genes contains currently over 60 genes, although not all are named with *VPS*. According to the *Saccharomyces* genome database, they have a wide variety of functions and roles, including sorting receptors, AAA-ATPase's, nucleotide exchange factors, SNARE proteins, components of tethering complexes, and various other proteins without a defined function.

Decreasing vacuolar missorting and degradation

The main *VPS* protein connected with the missorting process of heterologous proteins is the transmembrane sorting receptor Vps10p [185, 186]. Deletion of *VPS10* gene was shown to improve secretion of a full-length IgG and a GFP variant, for which the increase was thought to be a result from the suppressed diversion to the vacuole [54, 187]. However, *VPS10* deletion does not completely block missorting to the vacuole, as other pathways exist as well. For example, a *VPS10* deletion strain did not significantly improve secretion of an insulin fusion protein, while deletion of *VPS8, VPS13, VPS35, VPS4*, and *VPS36* proved to increase secretion titers [188]. Nine *VPS* genes were found in the screening of yeast deletion strain collection for enhanced cellulase production. From these, only deletion of *VPS3* and *VPS16* also increased the production of a glucosidase [189]. This increases the evidence that also a lot of these deletions might have protein specific results, as structurally distinct proteins might be degraded in slightly different pathways.

A different way to decrease product loss caused by the vacuole is through deletion of its resident proteases. The main vacuolar protease activity is encoded by the *PEP4* and *PRB1* genes, especially as they are also essential for maturation of other vacuolar proteases, which are protein products from *CPY1*, *CPS1, APE1,* and *DAP2* genes [172]. These vacuolar proteases can limit the yield of recombinant protein production, as they are released in the medium through fermentation stress or cell lysis [190]. Simultaneous deletion of *PEP4* and *PRB1* increased titers of the human interferon-β up to 10-fold [191]. However, in a different study with the same combination of deletions still a high extracellular degradation rate of the produced human serum albumin was observed. As this degradation was absent in cell-free culture supernatant, it was suggested that it was the result of a putative cell membrane

bound protease, only active in acidified media, and unrelated to the vacuolar proteases [192]. The other vacuolar proteases have not been characterized with respect to their influence on heterologous protein production in *S. cerevisiae*.

1.4. Antibodies: structure, folding, and formats

Antibodies are glycoproteins that are natively produced in mammalian B-cells as part of the adaptive immune system. They help protect the host organism against outside threats like microbial pathogens by binding to them, and inactivating them either through neutralization or destruction. Their intrinsic properties that provide them with a very specific and very strong binding affinity to an antigen make them interesting for use in (bio) medical applications. Antibodies are for example used in human diagnostics, and in therapeutical applications, where they can detect and mark tumors [193]. Moreover, in research they are involved in a wide range of applications, like *in vivo* or *in vitro* imaging, in detection assays like ELISA (Enzyme Linked ImmunoSorbent Assay), and in purification or bioseparation applications [194].

1.4.1. Structures and folding of antibodies

In most of the higher vertebrates five types of immunoglobulin (Ig) proteins are produced, being IgM, IgG, IgA, IgD, and IgE. From these, the properties of the IgG molecule and of its variants are discussed, as these are currently the most relevant for biotechnological production. IgG is the least complex of the Ig molecules and is the most abundant antibody in human serum [195], therefore it is also the most researched of the subtypes. The tetrameric IgG molecule has a general "Y-shape" and consists of two heavy chains (HC) and two light chains (LC) (see Figure 3 for a representation of the IgG structure and its domains).

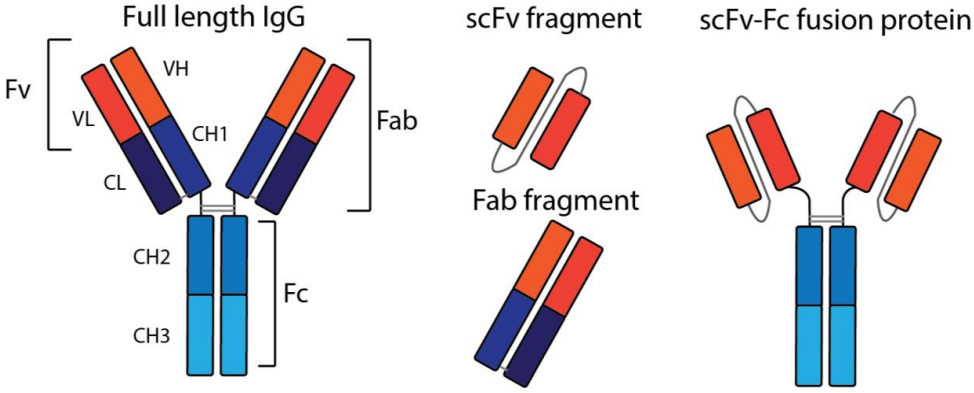


Figure 3 **Full-length IgG structure and domains plus derived recombinant antibody formats**. The full-length IgG molecule is divided into the constant crystallizable fragment (Fc) and the variable antigen binding domain (Fab). The variable (Fv) part consist of the variable domains of the heavy and light chain (VH and VL). The derived fragments shown are the scFv fragment, the Fab fragment, and the scFv-Fc fusion protein. Blue boxes represent the constant regions, and red boxes represent the variable regions.

One end of the fold holds three loops which contain the hypervariable sequences of the antibody that varies between different antibodies and so gives rise to their specific binding affinity to an antigen. It is important to note that the N-terminus and C-terminus are at opposite sides of the fold, so that they can be connected together to form the light and heavy

chains of the antibody [196]. Because of the high variability in amino acid composition in the ends of HC and LC, the N-terminal parts have been named variable domains (V_H and V_L for the HC and LC variable domains, respectively). Parts of the hypervariable regions of these variable domains are the complementarity determining regions (CDR), and these are the regions where the antibodies bind to their antigen. Both V_H and V_L contain three CDRs, and together they constitute the paratope of the antibody.

The full length antibody molecule consists of two regions, the constant crystallizable fragment (F_c), and the antigen binding part, which are connected by the hinge region of the antibody (Figure 3). The antigen binding part can be divided into the antigen binding (F_{ab}), and the variable (F_v) fragments. The main role of the F_C domain is to stabilize the antibody, and to interact with natural killer (NK) cells for antibody dependent cell mediated toxicity. Four classes of IgG molecules (IgG1-4) exists, which structurally vary in the size of the hinge region and in the number of inter HC disulfide bonds [195]. Like all the other types of Ig proteins, the IgG is a glycoprotein, and the glycans are important for its function. It has been shown that removal of the glycan that is most conserved between the different Ig types, at location Asn 297 in the CH2 domain, does not influence antigen binding, antibody stability, or plasma clearance [197]. However, the CH2 domain was shown to be destabilized and therapeutically critical Fc effector functions, like the Fc-ligand interaction required for the clearance of aberrant cells, were lost [197, 198].

With several domains that need to be assembled, together with crucial disulfide bond formation, the folding of a fully functional IgG molecule is a difficult process. The LC can fold independently from HC molecules, and folded LC have also been shown to be secreted separately, without assembling with the HC [199]. The HC however cannot complete its folding process by itself and is thus retained in the ER until it finishes folding after association with the LC. This is mediated by a stable binding of the intrinsically disordered C_H1 domain with the ER chaperone BiP (in yeast the protein is named Kar2p). Since the HC does not undergo the normal cycles of binding and release from BiP, the incompletely oxidized HC is retained in the ER by this interaction [200]. The presence of folded LC molecules is necessary to trigger the release of BiP. So that after forming the covalent linkage with the cysteines in the HC, folding can be completed [201]. This association with LC also enables the rate-limiting isomerization of the proline residues in one of the hinge regions of the HC, making it overall the slowest step in antibody folding [202].

1.4.2. Antibody formats

The IgG molecule has been engineered into various antibody formats for different applications, from which the Fab fragment, the single chain variable fragment (scFv), and the scFv-Fc fusion antibody are shown in Figure 3.

The Fab fragment consist of the first constant and the variable domains of both the heavy and the light chain fragment (Figure 3). Originally, Fab fragments were generated through proteolytic cleavage in the hinge region of a full-length IgG molecule [203]. However, proteolytic generation of Fab fragments is a hard-to-control process, as protocols need to be optimized for each different antibody, and not all proteases are able to cleave all classes of antibodies. For that reason the DNA sequences corresponding to the Fab components have been isolated, and the genes for HC and LC parts of Fab have been expressed independently from each other. Their protein products assemble into the Fab heterodimer, aided and

stabilized by the formation of disulfide and hydrogen bonds [204]. Compared with the full-length IgG, Fab retains specific binding to its target, with an increased tissue penetration. They have been used also for so-called "immunosilent" targets, which cannot be reached by conventional full-length IgG molecules, because the target space is too small. Examples are small cavities in enzymes, or hard to reach antigens hidden in membrane canyons [205]. Alternatively, Fab fragments are used to treat poisonings with potent low mass poisons. The current main use is against poisoning with digoxin, and immediately after administration of the drug the Fab fragments bind the free digoxin, preventing the poison from binding to its target sites. The antibody fragment-toxin complex is then readily secreted in the urine [206].

The scFv fragment is the smallest IgG antibody fragment possible with functional antibody-antigen binding properties. This type of antibody fragments has partially been developed to enable the production of antibody fragments with fully functional antigen binding properties in prokaryotic systems, for a convenient production and screening of antibody variants. The fragment is composed of the variable regions of the heavy and light chains (Figure 3, V_H and V_L, respectively) joined together by a flexible polypeptide linker (Figure 3). Various expression hosts have been shown to be able to generate the scFv fragments for isolation or using surface display, making it an economically interesting alternative as functional fragments can even be produced in relatively inexpensive hosts like the bacterium *E. coli* [207]. In applications, the small size and compact structure offer a more rapid, and more even penetration of tissues and tumors, when compared with the full-length IgG molecules [208]. The fact that they show a more rapid clearance proves to be beneficial for when the fragments are fused together with drugs, as the exposure of healthy tissue to the drugs is lowered [209].

The scFv-Fc fusion protein is another variant of the full-length IgG molecule, where the scFv fragments are genetically fused to the Fc part of the antibody (Figure 3). The presence of the additional Fc part increase the bioavailability of the protein, as through Fc-receptor mediated recycling the serum half-life is greatly increased compared to the scFv fragment [210]. This fusion approach increased for example the potency of one anti human Interleukin-33 antibody in the treatment of allergies [211]. Also, it enabled standard antibody mediated protein purification processes through recognition of the Fc region, comparable to what can be used with full IgG molecules [212]. The great benefit is that the expression and folding process is simplified as assembly of the scFv-Fc fusion protein molecule only requires the homodimerization of one type of polypeptide chain, and thus only a single new gene needs to be introduced into the host organism for expression [212].

1.5. Recombinant antibody production

The wide range of applications of antibody products in diagnostics, treatment and research have created a high need for large amounts of these molecules. In the past three decades, over 22 % of all yearly biopharmaceuticals approved by the FDA were monoclonal antibodies [213, 214]. Looking at sales values, the whole biopharmaceutical product market is having a turnover of at least 100 billion US dollar, of which the top blockbuster antibody product Humira had 11 billion US dollar in sales in 2013 [213]. As such, there is a clear interest in the development and improvement of new and existing expression platforms for the production of recombinant antibody products. In the pharmaceutical industry the most dominant

expression hosts are currently mammalian systems, in particular Chinese hamster ovary (CHO) cells. The market share of approved biopharmaceutical products in the last two decades is over 50 % for all mammalian cells, and over 60 % of the mammalian production is with CHO cells [213]. However, there is a clear interest and scientific research into other expression hosts, such as bacterial, fungal, and plant-based expression platforms, which might increase product yields and quality, and ease downstream processing operations [215].

1.5.1. Mammalian cell systems as a native host

As antibodies are from origin proteins produced in mammalian cells, the most efficient antibody production systems to date are mammalian cell systems, which can reach commercially very interesting titers of up to 10 g/L of antibodies [216]. For example, CHO cells, human embryonic kidney (HEK) cells, and hybridoma cell lines are most commonly used for industrial production of therapeutic antibodies. Most important, these systems provide proper posttranslational modifications, like human-like N-glycosylation, for effective use in human treatment. Additionally, they also show a high production capacity with an attractive yield that can be easily collected from the culture supernatant. However, there are also downsides to these mammalian cell factories. Cultivation can be a big issue, as the cells grow slowly and need expensive, complex media, while the cultivations are threatened by contamination from microbial or viral sources [217–219].

CHO cells are currently still the most used cell type, covering about 70 % of industrially produced recombinant therapeutic proteins, and they have been in use since the first approved recombinant biopharmaceutical in 1986. Thanks to intensive research, they are the most optimized system available, reaching titers of up to 10 g/L for full-length antibodies, and even up to 13 g/L for Fc-fusion proteins [216]. HEK cells are more suitable for quick processes, as transiently transformed HEK cells are able to produce the antibodies within days, compared with extensive cloning and screening methods needed for CHO cells. These transient cell lines are relatively productive and production levels of up to 0.6 g/L have been reported for an scFv-FC antibody fragment [220]. The fact that these cell lines are easily scaled up to 150 liter production volumes makes them very suitable for as well screening of antibody variants, as for the production of grams of antibodies.

Many scientific studies have worked to improve the mammalian cell systems as an expression host. On one hand, research has targeted mostly the gene expression process and on the other hand the protein folding and secretion processes has been studied. On the expression side, one of the main problems is that these cell types yield a highly heterogeneous population after DNA transformation. However, in order to comply with production standards, one single clone needs to be selected for homogeneous industrial production cultivations. As the specific productivity and viability between cells varies highly, selection procedures are needed to select the best performing clones. For this, mostly automated screening setups using flow cytometry and cell sorting have been used [217, 221]. On the other side, extensive genetic engineering has been employed in order to improve the maximum specific productivity of the clones. Part of the cellular response to a high load of unfolded proteins is the overexpression of chaperones and folding enzymes. Various approaches have mimicked this response through overexpression of chaperones, and for PDI, ERO1L, and Erp57 this has led to an increase in product yield. The chaperone BiP is widely overexpressed in the native unfolded protein response, but its engineered

overexpression has not led to increased yields [222]. Also, an increased activation of the general stress response UPR has proven be successful. This was achieved through overexpression of the transcription factor XBP1-s, which showed to impose a generic increase of capacity of the secretory pathway, having a positive effect on the secretion levels of various recombinant proteins [55]. However, it was pointed out in a second study that its overexpression only increased yields in transient cell lines, and not in stable expression lines, indicating that in some stable cell lines the secretory pathway might not necessarily be the limiting factor for production titers [223]. In the aspect of product quality, glycosylation has been scrutinized, as changes in glycan structure compared to human glycan structures can induce an immunogenic response when present in high amounts. Overexpression or deletion of various glycosyltransferases has increased structural homogeneity, and enabled closer mimicking of human structures through removal of non-human sugars and extension of the glycan structures natively produced in CHO cells [224, 225].

Emerging antibody production platforms

Various production platforms have been developed in order to find the most user-friendly and cost-effective production system, or in order to easily study various processes involved in production of the antibody proteins. The organisms used vary from prokaryotic platforms, like the most used bacterium *Escherichia coli*, to eukaryotes like the yeast *Pichia pastoris,* and even plant systems like the tobacco plant *Nicotiana tabacum* have been developed.

1.5.2. Prokaryotic hosts: *Escherichia coli*

Some of the limitations of native prokaryotic expression systems are the absence of a proper oxidative folding environment, like the ER in eukaryotic cells, and the absence of posttranslational modifications, like glycosylation, this limits the complexity of recombinant proteins that can be produced and secreted efficiently. For that reason *E. coli* has been mostly used for the production of ScFv fragments, with various approaches available to increase the yield of functional fragments. Production of antibody fragments in the cytoplasm yields most often non-active aggregates because of the reducing conditions present. Additionally, it has been shown to be inefficient to recover active antibodies through complete denaturation of the aggregates from inclusion bodies, followed by *in vitro* refolding [226]. One approach that yields functional antibody fragments has been the targeting of the protein of interest towards the periplasmic space. This oxidizing environment was shown to be sufficient for correct folding and assembly through disulfide bond formation of Fab fragments [227]. This means however that most Fab fragments need to be isolated from the *E. coli* periplasmic fraction. Other approaches to increase the yield of functional antibodies on a cellular level has been through the overexpression of chaperones and folding enzymes like GroES/L and the PPIase FkPa [228, 229]. On the other hand, an over 100-fold increase in titers can be achieved by scaling up from shake flask experiments to bioreactors. In bioreactors very high cell densities can be reached which helps increase production titers in a similar fashion.

In order to improve the bacterium for recombinant protein production, there has also been extensive research into engineering the *E. coli* for more favorable oxidative folding conditions. The commercially available CyDisCo system allows the formation of native disulfide bonds of heterologous proteins in the cytosol, through expression of a disulfide bond forming enzyme, for example the sulfhydryl oxidase Erv1p [230, 231], often accompanied by a disulfide bond isomerase like PDI. Screening of this system for the

production of correctly folded scFv and Fab fragments derived from a wide range of antibody types, showed an over 90 % success rate. Ten of the scFv, and ten of the Fab fragments tested yielded titers of over 5 mg/L in deep-well plates, and similar concentrations for a selection of tested antibodies in 25 mL cultures in shake flasks [232].

However, even with the native unfavorable oxidative protein folding conditions in *E. coli* there is actually data available for the production of a fully folded full-length IgG molecule. In this study, Makino and colleagues used a high throughput screening of random mutagenized strains combined with an optimization of translation initiation and expression of molecular chaperones [233]. This approach enabled them to produce the IgG molecule with titers of up to 4 mg/L. Although these titers are far from commercially interesting, they could be used for the rapid expression and analysis of libraries of antibodies in antibody discovery programs.

Another approach for the production of full-length IgG molecules with *E. coli* was done using so-called cell-free protein synthesis systems. Here, cell extracts of rapidly dividing *E. coli* strains are prepared for *in vitro* antibody synthesis. The removal of cell walls gives a precise control over cellular reaction parameters which affect protein folding and stability, and that can't be controlled in cell cultures like pH, redox potential, and ionic strength, [234, 235]. This also enabled the addition of separately produced helper proteins like folding enzymes and chaperones. For example, it was found that the addition of the *E. coli* PPI FkpA and the yeast Pdi1p increased titers of IgG molecule trastuzumab up to 1.6 and 1.4 times, respectively [236]. Overall, the cell free systems were shown to be able to produce relatively high titers in a milligram per milliliter scale in very small scale experiments, but concerns are that costs and complexity of putting together the components of the cell-free systems make them not suitable beyond lab scale.

1.5.3. Eukaryotic hosts: Yeasts and fungi

Yeast production systems

Yeasts and fungi provide more possibilities for the production of recombinant antibody fragments than bacteria, as the eukaryotic cell is a more suitable production environment. The major yeast currently used for recombinant antibody production is the methylotrophic yeast *Pichia pastoris*. Its advantages are a good capacity for the production and secretion of recombinant proteins, while it does not secrete a large amount of endogenous proteins, decreasing the complexity of protein purification steps [237]. Additionally, the yeast favors a respiratory growth, and in optimal bioreactor processes this can result in very high cell densities of over 130 g/L cell dry weight. Recombinant protein production is predominantly driven by the alcohol oxidase 1 promoter, which is induced by the presence of methanol, an inexpensive carbon source which can also be used for growth by *P. pastoris* [237]. However, strain improvement is still necessary for achieving higher secretion titers, as for the expression of a Fab it was shown that only 35 % of the target protein was secreted into the culture medium. A small amount (7 %) was inherited to daughter cells, but up to 58 % of the Fab fragment was shown to be degraded in the cells [238]. A follow-up study showed that this degradation took place both by the proteasome and in the vacuole [239], indicating that secretion titers can be improved with comprehensive strain engineering. Currently, two antibody fragments produced by *P. pastoris* are available commercially [219]. Nanobodies ALX-0061 and ALX-0171 are produced by the Belgian company Ablynx, and are used in the

treatment of rheumatoid arthritis and respiratory syncytial virus infection, respectively [240, 241].

Looking at scFv fragments, strain engineering by overexpression of the chaperone BiP doubled previously established titers of a functional scFv fragment from 4 g/L to over 8 g/L in benchtop bioreactors [242]. However, there is a lot of data available that points out that yields tend to decrease when the complexity of the antibody fragments increases [218, 243]. For example production of Fab fragments against the high affinity IgE receptor reached titers around 40 mg/L in shake flasks [244], while the highest titers in fermenters reached around 400 mg/L [245]. One studied reported a direct comparison of the production of a scFv-Fc fusion protein produced by *P. pastoris* and produced by CHO cells. In this case, *P. pastoris* was outperformed by the CHO cells, with final titers of 12.3 and 159.4 mg/L, respectively [246]. Early studies in 1999 into producing active full-length antibodies in *P. pastoris* using shake flasks reached up to 36 mg/L of a functional murine monoclonal antibody against dioxin [247]. In contrast to this, optimization of the production process has proven to be successful for the production of full-length antibody molecules. Potgieter *et al* studied antibody secretion kinetics and produced in optimal conditions over 1.5 g/L IgG in 40-L bioreactors, using a fed-batch protocol [248].

The progress in recombinant antibody production in *S. cerevisiae* is not as far advanced yet as in *Pichia pastoris*. One early study reported the secretion of a functional mouse-human chimeric antibody and its Fab fragment from this yeast in 1988, with titers in the range of 50-80 and 100-200 μg/L, respectively [249]. However, most research on scFv fragment expression showed that secretion titers remained low, because the antibody fragments were retained intracellularly either in the ER, or rerouted to the vacuole for degradation [76, 250]. It was shown that high levels of intracellular scFv led to an activation of the UPR, followed by a decrease of scFv levels, although the decrease in intracellular levels was not leading to an improved secretion [76]. This indicates that it could be beneficial to adjust expression levels in such a way that the UPR is not activated in order to increase secretion titers [76, 129]. In line with this, expression of selective parts of the UPR regulated genes, most often chaperone expression levels, were mimicked in order to obtain higher secretion titers. For example, overexpression of the chaperone Kar2p and a rat PDI showed to increase titers 2- to 8-fold for five different scFv's, with titers up to 20 mg/L in shake flasks, showing also a synergistic effect in co-overexpression of Kar2p and the rat PDI [98]. Similar results were found in a follow-up study where production titers were increased up to 10-fold [251]. In both works authors hypothesized that the positive effect was a result of preventing unfolded scFv of becoming a target for intracellular degradation pathways. One completely different approach to increase scFv expression was engineering of the secretory leader. This strategy proved to be useful, as one of the mutants screened improved scFv secretion up to 16-fold [187]. The new secretory leader showed to be useful as well for expression of full-length antibody, as its secretion was increased 25-fold, with an additional 1.6-fold secretion through overexpression of Pdi1p. Final full length IgG titers reached 1.5 to 9 mg/L in this study [187].

Engineering of Glycosylation in yeasts

One of the downsides of using yeasts is that they tend to hyperglycosylate their (glyco)proteins, while additionally they can glycosylate the proteins at positions not normally found in the native mammalian host [218]. This is unfavorable because it can

decrease the efficiency and effectivity of the glycoprotein when used for human treatment. However, for both *P. pastoris* and *S. cerevisiae* extensive work is done on creating glycoengineered strains with humanized glycans [252–257]. In both the engineering strategies employed by the company GlycoFi, and in the commercially available GlycoSwitch® platform, the *P. pastoris* modifications start with the disruption of the *OCH1* gene, encoding the endogenous glycosyltransferase och1p, followed by the (stepwise) addition of heterologous glycosylation genes, based on which terminal glycan structure is required [254, 258].

Zhang and colleagues compared a glycoengineered *P. pastoris* produced biosimilar to the breast cancer drug trastuzumab (that was produced in CHO cells) [259]. Their data showed that the yeast produced biosimilar could meet the properties of the commercial produced drug in a full spectrum comparison of a preclinical evaluation [259]. Other data has shown that full length IgG1 produced in wild type *P. pastoris* strains had a two to three times faster clearance in mice compared to CHO cell produced IgG. When the same IgG was produced in a glycoengineered strain, the clearance was comparable to CHO cell produced antibodies, while maintaining its antigen-binding affinity [260]. Li and colleagues even showed that by tailoring the specific glycan structures on the expressed IgG improvement of the therapeutic efficacy of the monoclonal antibodies is possible [257]. However, so far no glycoengineered strains are yet available that contain a 100 % homogenous glycan moiety on all glycoproteins. As discussed above, a different approach to circumvent glycosylation heterogeneity or glycosylation at unwanted locations is the production of aglycosylated IgG molecules. The anti-interleukin-6 antibody clazakizumab has successfully been produced in *P. pastoris,* and it has been undergoing clinical trials in treatment of cancer and rheumatoid arthritis patients [261, 262].

One early example of the glycoengineering of *S. cerevisiae* involved overexpression of an α-1,2-mannosidase in a triple *MNN1/MNN4/OCH1* deletion. The strain was shown to produce the $Man_5GlcNAc_2$ glycan, amongst other high mannose glycans [263]. A more recent approach revolved around the modification of an alternative glycan structure to be transferred from the dolichol donor to the nascent polypeptide, starting with the deletion of *ALG11* and *ALG3*, which prevents the glycans from maturing beyond the $Man_3GlcNac_2$ structure. An artificial flippase and a protozoan OST were expressed in the *Δalg3Δalg11* strain in order to get the glycan donor onto the proteins, followed by addition of two GlcNAc residues through human GlcNAc transferase I and II, targeted to the Golgi apparatus [252]. The study faced problems in heterogeneity of the produced N-glycans, possibly caused by residual glycosyltransferase activity. The homogeneity of the glycans was improved in a follow-up study through deletion of *MNN1* and the targeting of a UDP-GlcNAc transporter from *Kluyveromyces lactis* to the Golgi apparatus [253]. However, further optimization of the glycosylation pathway is necessary in order to obtain a completely homogenous glycan signal suitable for the industrial production of therapeutic proteins.

Besides from these examples in the two main yeast expression organisms, production of antibody fragments has also proven to be possible in non-conventional yeast strains like *Hansenula polymorpha, Schizosaccharomyces pombe, Kluyveromyces Lactis and Yarowia lipolytica* [217, 218]. However, these are only minor attempts with low titers and will not be discussed further.

Fungal production systems
Filamentous fungi have been widely used for expression of recombinant proteins in biotechnology, as they are natively secreting high amounts of proteins. For example strains from *Trichoderma* and *Aspergillus* have shown their use in recombinant protein production processes.

Trichoderma reesei has had extensive research on its potential to secrete recombinant proteins, and Nyysönen and colleagues have focused on expression of antibody fragments. In their study they increased production titers of Fab antibody fragments from 1 mg/L to 40 mg/L in shake flasks and to even 150 mg/L in bioreactors, by fusion of the Fab HC to the *T. reesei* cellulase CBHI [264, 265]. After secretion the Fab fragment could be released from the fusion protein by an extracellular protease, yielding a normal functional Fab fragment. Later studies focused on creating improved *T. reesei* production strains through deletion of secreted proteases. Deletion of 7 proteases decreased general protease activity by 96 %, leading to no detectable degradation of an antibody standard incubated in the six protease deletion strain's supernatant [266]. These protease deficient strains were reported to be able to produce full-length antibodies up to 7.6 g/L, and Fab fragments even up to 8.2 g/L [267, 268], making them interesting industrially relevant alternatives to mammalian production systems.

From the genus *Aspergillus, A. niger* and *A. awamori* are used for antibody fragment production. Also in *A. awamori* the fusion of the scFv fragment to a native carrier protein has been used [269–271]. Here, it was shown that the addition of the glucoamylase fusion protein increased titers of a scFv fragment in shake flasks from 10 to 50 mg/L, while scale up to fed-batch fermentations increased titers to 200 mg/L [271]. An optimization study in a stirred tank reactor reached over 100 mg/L when induction was started in the late-exponential phase [269]. The production of glucoamylase fused antibody fragments showed to be more efficient in *A. niger*. Ward *et al.* compared the production of the antibodies trastuzumab and apolizumab in this organism, and shake flask experiments produced 0.9 and 0.2 g/L, respectively [272]. An aglycosylated version of trastuzumab, through an A297Q mutation, was less productive at 0.1 g/L, while its Fab fragment reached 1.2 g/L. The *Aspergillus* produced antibody showed no significant differences to the mammalian produced one, when compared for affinity, binding strength, pharmacokinetics, or effector functions using *in vitro* studies. However, no clinical studies at any stage have been reported at this point [272].

1.5.4. Eukaryotic hosts: plants and plant cell suspension systems

Also plant systems have been developed for the production of monoclonal antibodies, as they as well exhibit some interesting traits. They are found to provide an easy production process with a cost attractive yield and a high production capacity, which gains an increased value through the possibility for unlimited scale up for stable transgenic plant lines. For this, an increase of land for cultivation is the main factor for scale-up, without the need of adjustment of production parameters, as is the case in mammalian and microbial fermentations. Furthermore, there is an improved viral safety when compared with mammalian cells, and production levels stay uniform through the generations. However, as described before with the other systems, also here glycosylation and other posttranslational modifications can cause inconveniences, but during the last two decades many glycoengineering approaches have been reported [273–276]. Additionally, using plants a

specific risk of contamination through soil bacteria or pollen dissemination is present [277]. With respect to humanized glycosylation, the mammalian GlycoDelete system has recently also been introduced into *Arabidopsis* seeds, and it proved to be efficient in creating a suitable glycan species on a heterologous expressed protein [276].

The locations of accumulation of the recombinant proteins vary between species, for example rice, maize, and soybean produce and accumulate the proteins in their seeds. Whereas systems like tobacco and alfalfa accumulate the recombinant protein in their leaves, which makes them interesting as they have a high amount of easily accessible leaf biomass [277]. Overall, there has been significant progress in plant based expression systems of therapeutic antibodies and some of the products have even reached advanced (pre)clinical trial stages of development [278]. One of the products that reached preclinical studies was a novel afucosylated version of the anti-CD20 monoclonal antibody rituximab named BLX-300 [279]. The product was produced using the small aquatic plant *Lemna minor*, which was optimized for N-glycosylation through the expression of RNAi constructs [274]. Gasdaska and colleagues showed that target cell binding and apoptosis induction were similar to the original rituximab, while effector functions suggested that BLX-300 might possibly be more effective and potent than the original [279].

N. tabacum has been successfully used to produce an HBsAg-specific antibody [280]. This antibody is directed against the hepatitis B surface antigen and can thus be used for immunopurification of this antigen for the preparation of vaccines. The production efficiency reached on average 25 mg of antibody/kg of plant biomass, with a purity of over 90 % after processing of the tobacco leaves [280]. The purified product was then successfully used for the immunopurification of recombinant HBsAg, with comparable efficiency to a mouse-derived homolog [281]. The production of the antibody has been approved by the Cuban regulatory authority and is currently produced by the Centre for Genetic Engineering and Biotechnology in Havana, Cuba [282].

Besides the use of full plants for the production of recombinant proteins, there has also been an interest in the use of plant cell suspension cultures. The production can be achieved through either inoculation of cultures from stable transgenic plants or by transforming wild-type cells that are already growing in suspension. Although many recombinant proteins have been produced in cell lines from various plants, antibodies were mostly produced in *N. tabacum* BY-2 tobacco cells, where one of the advantages of cell suspension cultures versus whole plant production was that the cells produced almost no nicotine [283]. Examples are the production of the full size anti-HIV antibody 2G12 at 8 mg/L in seven days [284], and up to 15 mg/L of the monoclonal antibody against HBsAg in ten days [285]. In general, the plant cell suspension cultures show similar limitations to scale-up in bioreactors as what is known from mammalian and microbial fermentations. However, a recent study showed that a scale up from shake flasks to a 200-L orbitally shaken bioreactor did not change the yield parameters in the production of the human monoclonal M12 antibody using BY-2 cells. In both conditions, antibody yields of 20 mg/L were achieved, with an efficient recovery and purification process available [286].

2. Aims of the thesis

The yeast *Saccharomyces cerevisiae* has been successfully employed to manufacture a wide range of products like biofuels, small molecules, and therapeutic proteins. However, for many recombinant proteins secretion turned out to be inefficient and product titers remained low. In this thesis we work on the development of *S. cerevisiae* as an antibody factory to increase its value for use in therapeutic protein production. The aims of this thesis are to gain more insight in factors that influence the production of recombinant antibodies in the yeast *S. cerevisiae*, and, where possible, to use the found insights to create strains that have an increased secretion of a full-length IgG antibody.

First, we aim to identify possible bottlenecks in IgG secretion by selecting a number of genes encoding proteins along the secretory pathway for screening. From these, we selected the most suitable cellular environment for a more extensive screening, the ER folding environment. The production of recombinant proteins induces many changes in the cells, and the unfolded protein response is the main stress response activated when the burden of unfolded proteins in the ER becomes too high. One aim in this thesis is to see if parts of the UPR can be selectively used to increase the IgG production capacity of *S. cerevisiae*. Therefore, we investigate the effects of both the overexpression of selected proteins that are catalyzing folding steps, as well as of the deletion of selected genes encoding proteins involved in different steps of the ER associated degradation pathway.

However, targeted approaches of strain engineering are limiting in their possibilities, as they require knowledge about the function of the studied genes with respect to recombinant protein production. For this reason, one goal of this thesis is to develop a high-throughput screening method that can be utilized to identify genes which influence IgG secretion. We test the approach by screening a random deletion strain library generated by transforming a transposon inactivated genomic library of yeast DNA.

Finally, the production of recombinant proteins has often been observed to impose a metabolic burden to the cells, as the production of recombinant proteins requires the reallocation of cellular resources towards protein synthesis. One goal of this thesis is to gain insights in the effects of recombinant protein production on a metabolite level using a targeted quantitative metabolomics approach.

3. Materials and methods

In this section a brief summary of the materials and methods used in this thesis is given, the more detailed descriptions of the work done can be found in the publications in the appendix.

3.1. Yeast strains

Table 1 **Yeast strains used in this thesis**

Strain	Parental strain	Genotype or genetic modification from parental strain	Source
SS328	N/A	Matα *ade2-101 his3Δ200 lys2-801 ura3-52*	Article I, IV
W303α	N/A	Matα *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	Article II, III
YJR043	SS328	*hrd1Δ::kanMX6* with pEK5(Ura3)	Article I
YJR044	SS328	*yos9Δ::kanMX6* with pEK5(Ura3)	Article I
YJR045	SS328	*hrd3Δ::kanMX6* with pEK5(Ura3)	Article I
YJR046	SS328	*ubc7Δ::kanMX6* with pEK5(Ura3)	Article I
YJR049	SS328	*htm1Δ::kanMX6* with pEK5(Ura3)	Article I
YJR050	SS328	pEK5(Ura3)	Article I
YJR051	SS328	*alg3Δ::kanMX6* with pEK5(Ura3)	Article I
YJR102	SS328	*ire1Δ:: natNT2* with pEK5(Ura3)	Article I
YJR103	SS328	*hrd1Δ::kanMX6, ire1Δ::natNT2* with pEK5(Ura3)	Article I
YJR104	SS328	*hrd3Δ::kanMX6, ire1Δ::natNT2* with pEK5(Ura3)	Article I
YJR105	SS328	*htm1Δ::kanMX6, ire1Δ::natNT2* with pEK5(Ura3)	Article I
YJR106	SS328	*ubc7Δ::kanMX6, ire1Δ::natNT2* with pEK5(Ura3)	Article I
YJR109	SS328	*yos9Δ::kanMX6, ire1Δ::natNT2* with pEK5(Ura3)	Article I
YJR110	SS328	*alg3Δ::kanMX6, ire1Δ::natNT2* with pEK5(Ura3)	Article I
YEK17	W303	*his3Δ::natNT2::(HC+LC of anti CD-20 antibody)*	Article II
YEK18	W303	*his3Δ::natNT2::(HC+LC of anti CD-20 antibody) opi1Δ::loxP*	Article II

3.2. Media and culture conditions

Yeast strains were grown in rich, minimal, or synthetic drop out (SD) medium. Rich media contained 1 % yeast extract, 2 % peptone, and 2 % glucose or galactose, making it YPD or YPGal respectively. Minimal media contained 0.67 % yeast nitrogen base without amino acids *(YNB)* and 2 % glucose or raffinose, with supplementation of necessary amino acids. SD medium was minimal medium supplemented with the drop-out mixture lacking the appropriate amino acids. To induce protein production under control of the galactose inducible promoter, 40 % galactose was added at the appropriate time point to a final concentration ranging from 0.5 to 4.0 %.For screening experiments, cultures were grown in deep-well plates in 1 mL volumes. Shake flask cultivations were done in either 10 mL

volumes in 100 mL flasks or 30 mL volumes in 250 mL flasks. Growth curves were measured in microtiter plate format, either in the Bioscreen analyzer (Growth curves Ltd., Finland) or in the Eon Microplate Spectrophotometer (BioTek, Winooski, USA).

3.3. Plasmids

Table 2 **Protein product expression plasmids used in this thesis**

Name	Promoter	Gene information	Marker	Source
pEK5	*GAL1*	pho5-C2B8-lc and pho5-C2B8-hc	*URA3*	Article I, III
pEK6	*GPD*	pho5-C2B8-lc and pho5-C2B8-hc	*URA3*	This thesis
pEK12	*GAL1*	pho5-C2B8-lc and pho5-C2B8-hc, with NatNT2, *HIS3* locus integrative	*URA3*	Article II
pAF3	*TEF*	pho5-C2B8-lc and pho5-C2B8-hc	*URA3*	This thesis
pAX538	*GAL1*	Matα wt-HyHel-HC and Matα wt-HyHel-LC	*URA3*	Article IV
pAX512	*GAL1*	Matα wt-HyHel-Fv-Fc	*URA3*	Article IV
pAF10	*GAL1*	Matα wt-HyHel-scFv	*URA3*	Article IV
pAX469	*GAL1*	Pho5-HIS	*URA3*	Article III
pDEP17	*Cyc1* (4XUPRE)	NdegY-GFP, integrative into *TRP1* locus of W303 derived strains	*TRP1*	Pincus *et al.*, [77], Article II

Table 3 **Molecular chaperone, folding factor, and transport protein expression plasmids used in this thesis. The source of the plasmids is Article II, excluding the plasmids marked with [a], for which the source is this thesis.**

Name	Promoter	Gene	Marker
pJR001	*TEF*	*PDI1*	*LEU2*
pJR002	*GPD*	*PDI1*	*LEU2*
pJR003	*GAL1*	*PDI1*	*LEU2*
pJR004[a]	*TEF*	*SSO1*	*LEU2*
pJR005[a]	*GPD*	*SSO1*	*LEU2*
pJR006[a]	*GAL1*	*SSO1*	*LEU2*
pJR007	*TEF*	*ERO1*	*LEU2*
pJR008	*GPD*	*ERO1*	*LEU2*
pJR009	*GAL1*	*ERO1*	*LEU2*
pJR010[a]	*TEF*	*SLY1*	*LEU2*
pJR011[a]	*GPD*	*SLY1*	*LEU2*
pJR012[a]	*Gal1*	*SLY1*	*LEU2*
pJR013[a]	*TEF*	*SEC1*	*LEU2*
pJR014[a]	*GPD*	*SEC1*	*LEU2*
pJR015[a]	*Gal1*	*SEC1*	*LEU2*
pJR016	*TEF*	*CPR5*	*LEU2*
pJR017	*GPD*	*CPR5*	*LEU2*
pJR018	*GAL1*	*CPR5*	*LEU2*
pJR021	*PDI1*	*PDI1*	*URA3*
pJR026	*KAR2*	*KAR2*	*URA3*
pJR028	*PDI1*	*KAR2*	*LEU2*
pJR029	*PDI1*	*PDI1*	*LEU2*

Name	Promoter	Gene	Marker
pJR030	*PDI1*	*CPR5*	*LEU2*
pJR032	*KAR2*	*ERO1*	*LEU2*
pJR033	*KAR2*	*PDI1*	*LEU2*
pJR035	*TEF*	*PDI1*	*URA3*
pJR051	*GPD*	*CPR5*	*URA3*
pJR056	*GAL1*	*LHS1*	*URA3*
pJR057	*GPD*	*LHS1*	*URA3*
pJR058	*TEF*	*LHS1*	*URA3*
pJR063	*TEF*	*LHS1*	*LEU2*
pJR065	*KAR2*	*LHS1*	*LEU2*
pJR066	*GAL1*	*SIL1*	*URA3*
pJR067	*GPD*	*SIL1*	*URA3*
pJR070	*KAR2*	*SIL1*	*URA3*
pJR071	*Gal1*	*SIL1*	*LEU2*
pJR074	*PDI1*	*SIL1*	*LEU2*
pJR075	*KAR2*	*SIL1*	*LEU2*
pAF4	*GPD*	*KAR2*	*LEU2*
pAF5	*TEF*	*KAR2*	*LEU2*
pAF6	*GAL1*	*KAR2*	*LEU2*
pEK7	None	None	*LEU2*
pEK17	None	None	*URA3*

3.4. Analytical methods

3.4.1. ELISA

An enzyme linked immunoassay was used to determine antibody titers. The ELISA assay was automated on a HAMILTON Star line liquid handling station. 96-well plates were coated with 100 µL/well of 4.2 µg/mL of goat anti Human IgG (Fc specific) antibody in PBS, with shaking overnight at 4 °C. Plates were washed four times with 200 µl PBT, and blocked for 45 minutes in PBT at 22 °C, with occasional shaking. Serial dilutions of standard antibody were performed in PBT. 200 µl of samples in triplicates or standard antibody in duplicates were added to the wells, and the plates were incubated at room temperature for 90 minutes while shaking. After incubation wells were washed three times with 200 µl of PBT. 100 µL/well of 1:4,000 dilution of goat anti-Human IgG (Fc specific)-peroxidase labelled antibody was added and allowed to bind for 1 hour at room temperature while shaking. After incubation, the wells were washed three times with 200 µL of PBT. For detection, 80 µL/well substrate solution (0.2 mg/mL *o*-phenylenediamine, 3 µL of 30 % H_2O_2 per 10 mL solution in 0.05 M phosphate citrate buffer) was added. After 8 minutes the reaction was quenched by addition of 80 µL/well of 3 M H_2SO_4. Absorbance was read at 490 nm using a BioTek Synergy 2 spectrophotometer. Data evaluation was done with Gen5 software (BioTek).

3.4.2. Acid phosphatase activity assay

Acid phosphatase (AP) activity was measured from clarified culture supernatant using an endpoint method. Samples were measured in triplicates. The assay was started by addition of 100 µl of 20 mM para-nitrophenyl-phosphate (Sigma-Aldrich, Buchs, Switzerland) in sodium acetate buffer, pH 4.2, and the reactions were terminated by addition of 200 µl 2M Na_2CO_3 after 0, 2, 10 and 20 minutes. Blanks contained only substrate but no culture supernatant. Absorbance was read at 405 nm using a BioTek Synergy 2 spectrophotometer. Data evaluation was done with Gen5 software (BioTek).

3.4.3. Cellular clearance assay

Precultures were grown in 5 mL of minimal medium at 30 °C, 220 rpm for 15 hours. Exponentially growing cells were induced with 2 % galactose when they reached an OD_{600} of 0.8, and transferred to 25 °C. After four hours of expression, either 2 % glucose was added to repress the IgG production, or the same volume of water for control samples. Cells were collected zero, two, four, and six hours after addition.

A culture volume equivalent to 10 OD_{600} of cells was clarified by centrifugation, the cell pellet was resuspended in 500 µL lysis buffer (100 mM tris HCl buffer, pH 7.4 containing 1X cOmplete®, EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland), and 1 mM PMSF). The same volume of acid washed glass beads was added, and samples were vortexed for 10 minutes at 4 °C using a Disruptor genie (Scientific industries). After settling of the beads, the supernatant was transferred to a new tube. The glass beads were washed with 500 µL lysis buffer by vortexing the tubes twice for 30 seconds, while keeping them for 30 seconds on ice in between. The combined supernatants were pelleted and the pellets were resuspended in 100 µL SDS-Page sample buffer (62.5 mM Tris-HCl (pH 6.8), 2 % SDS, 10 % glycerol, 5 % β-mercaptoethanol and 0.005 % bromophenolblue). Samples were heated for 5 minutes at 65 °C, after which they were centrifuged for 5 min at 14,000 rcf and supernatants were used for analysis.

10 µL of sample was loaded onto a 12.5 % SDS-polyacrylamide gel and the electrophoresis was run in SDS-tris-glycine buffer. For each sample, replicate gels and blots for the detection of produced recombinant antibody, and for the detection of endogenous tubulin were prepared. After the SDS-PAGE, the separated proteins were transferred to a nitrocellulose membrane using western blotting. A 1:10,000 dilution of anti-human IgG (Fc specific)-peroxidase labelled antibody produced in goat was used for staining. A 1:10,000 dilution of anti-tubulin [EPR13799] produced in rabbit (Abcam, Cambridge, UK) in combination with a 1:10,000 dilution of goat anti-rabbit IgG (Fc specific)-peroxidase labelled was used. Signal detection was done with the ECL western Blotting Detection kit (GE Healthcare, Helsinki, Finland) following the manufacturer's instructions. Analysis and quantification of band intensities were performed using the Bio-Rad Image Lab Software and tubulin as a loading control.

3.4.4. Collection of samples for metabolomics

Yeast cells were inoculated into precultures of 5 ml minimal medium (0.67 % yeast nitrogen base without amino acids, and 2 % raffinose, with supplementation of necessary amino acids). These overnight cultures were used to seed 30 mL of the main cultures at a starting OD_{600}=0.2. After 6 hours of cultivation at 30 °C and 180 rpm, 40 % galactose was added to a final concentration of 4 % to induce protein expression. The cultures were harvested after 18 hours of expression. The fast filtration protocol for harvesting of the yeast cultures was modified from Kim and colleagues [287]. In short, 2 mL of the *S. cerevisiae* cultures was vacuum filtrated through a nylon membrane filter (0.45 µm pore size, 30mm diameter, Whatman, Piscataway, USA). The cell residue was then washed with 10 mL of water, after which the filter with the cells on it was transferred to a 5 mL Eppendorf tube and in the tube flash frozen in liquid nitrogen. Finally, samples were stored at -80 °C until metabolite extraction.

4. Results and discussion

4.1. Determining bottlenecks along the IgG secretory pathway

S. cerevisiae has been shown to be able to produce a wide variety of heterologous proteins from bacterial, other yeast and fungal, and even mammalian origins. However, in most cases the yeast did not reach its full secretory potential as, depending on the source and type of the expressed protein, different bottlenecks were found to exist along the secretory pathway [51].

In order to get more insight in where the limitations are for expression of our IgG molecule we selected a few targets along the secretory pathway to probe for possible bottlenecks. In progressing order down the secretory pathway, we first chose targets in the ER folding environment, with overexpression of *CPR5*, *PDI1*, and *KAR2*. Next were targets in vesicular transport, with overexpression of *SLY1* in the ER to Golgi transport, and of *SSO1* and *SEC1*, which are involved in fusion of vesicles to the plasma membrane. Finally, we tested the contribution of missorting and degradation of the protein of interest through deletion of the vacuolar sorting receptor *VPS10*, deletion of the vacuolar proteases *PEP4* and *PRB1*, and deletion of the mitochondrial endoprotease *CYM1*.

4.1.1. Overexpression of folding enzymes and vesicular trafficking proteins

For an initial screen of how adjustment of the concentrations of ER-resident proteins involved in protein folding affects the secretion of the full-length human monoclonal anti-CD20 IgG molecule, Kar2p, Pdi1p, and Cpr5p were selected and expressed under control of P_{GAL1}, P_{TEF}, and P_{GPD}, ranging from strongest expression to least strong. These three proteins are involved three different aspects of the folding process, Kar2p is expected to aid in a general way through its chaperone function, Pdi1p through catalyzing the formation of disulfide bonds, and finally Cpr5p through catalyzing the isomerization of peptidyl-prolyl bonds.

The secretion titers were measured using ELISA after 24 hours of IgG production with 0.5 % galactose induction, and are shown in Figure 4, together with their parental wild-type strain SS328 as control. It has to be noted that in these experiments, and also in all other secreted IgG titers measured with ELISA in this thesis, that the IgG levels were measured by detecting the Fc part of the IgG molecule with both the capturing and the detection antibodies. This means that possibly also degraded IgG molecule can still be measured by the presence of its Fc part in the culture supernatant. Nonetheless, we work under the assumption that the HC can only be secreted as part of a fully folded full-length IgG molecule, and thus we consider the measured Fc also in that possible case to be part of the secreted IgG titer.

The overexpression of *KAR2* was slightly beneficial for IgG titers, with a maximum increase of 10 % for the *GPD* and *GAL1* promoters. An increased amount of Pdi1p targeted to the ER led in all cases to a decrease in titers. However, the abundance of Cpr5p was shown to be an important limiting factor for IgG folding in the ER. In all cases of *CPR5* overexpression, a 50 to 75 % increase of measured IgG in the culture supernatant was observed. The positive effect of *CPR5* expression indicated that isomerization of peptidyl-prolyl bonds could be a limiting factor in the folding of IgG in *S. cerevisiae*.

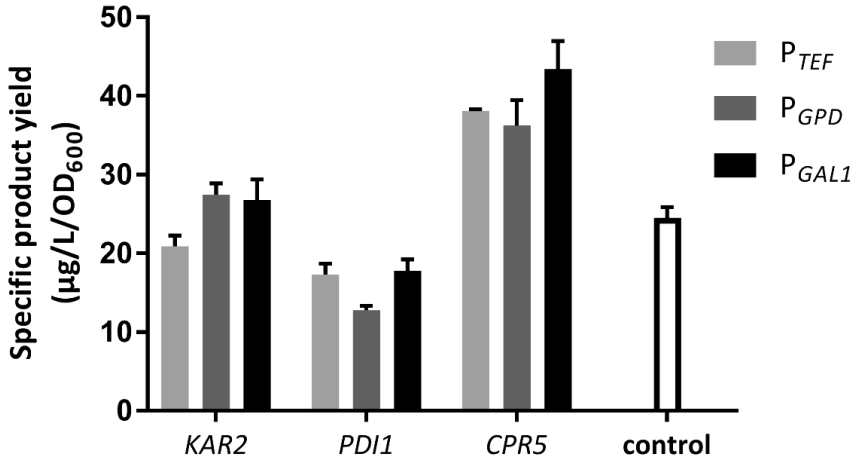


Figure 4 **Antibody production of ER-chaperone overexpression strains.** All strains were grown into the exponential phase in deep-well plates, followed by induction of IgG production with 0.5 % galactose for 24 hours. ELISA was used to measure IgG titers from clarified culture supernatants. IgG titers were normalized for cell densities using the respective OD_{600} values. Error bars represent the standard deviation from three independent cultures, control refers to the wild type SS328 strain.

In theory, one way to relieve the ER from the burden of a high load of secretory proteins would be to increase vesicle trafficking to stimulate the flow of proteins moving away from the ER. We chose the genes *SSO1*, *SLY1*, and *SSO1* for overexpression to study the feasibility of this idea, and each gene was expressed using P_{GAL1}, P_{TEF}, and P_{GPD}. The specific product yields after 24 hours of 0.5 % galactose induction at 30 °C are shown in Figure 5. In general none of the expressed proteins caused a significant improvement in production titers compared to their parental wild type control strain W303α.

Sly1p is a regulator of SNARE proteins involved in targeting the trafficking vesicles to the Golgi apparatus membrane after they are released from the ER. The protein has been shown to accelerate the formation of the SNARE complex *in vitro* [288]. It was already shown before that its overexpression does not improve secretion titers in all cases, but was suggested to be beneficial mostly for larger proteins as they take up more space in the ER [38]. In the case of IgG there appeared to be only a modest 7 % increase of titers, resulting from expression using the *TEF* promoter, suggesting the influence of other factors than just the size of the recombinant protein.

Sec1p and Sso1p are both exocytic SNARE proteins that act in the final steps of the secretory pathway; budding of exocytic vesicles to the plasma membrane. Here, overexpression of *SSO1* decreased secretion titers in all cases, while for *SEC1* there was a small 5 % increase of IgG titers. This finding was in contrast to published data that showed that in general

overexpression of this class of proteins has led to an increase of titers of various heterologous proteins [183, 184, 289, 290]. This could indicate that the main bottleneck for IgG secretion lies at an earlier point in the yeast secretory pathway.

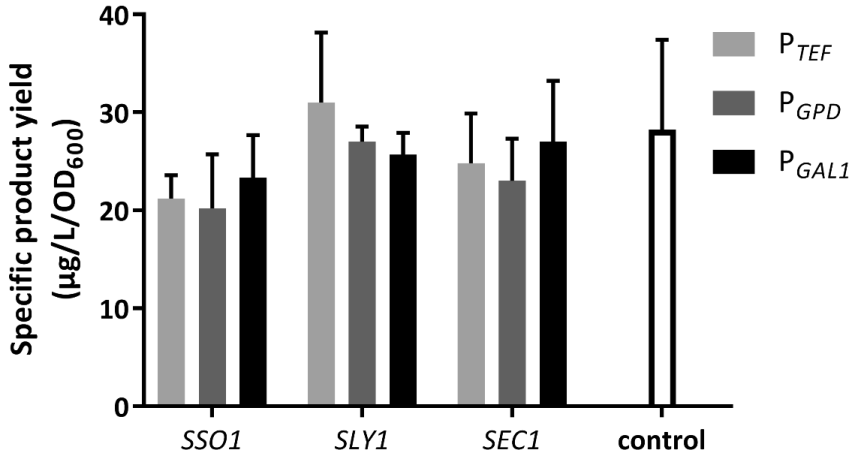


Figure 5 **Antibody production of strains overexpressing vesicular transport components.** All strains were grown into the exponential phase in deep-well plates, followed by induction of IgG production with 0.5 % galactose for 24 hours. ELISA was used to measure IgG titers from clarified culture supernatants, and the IgG titers were normalized for cell densities using the respective OD_{600} values. Error bars represent the standard error from three independent cultures, control refers to the wild type W303α strain.

4.1.2. Targeting protein degradation

One of the possible things that can decrease secretion efficiency at a late stage in the secretory pathway is vacuolar missorting and proteolytic degradation. To evaluate the influence of these processes, we selected three of the involved genes: the vacuolar sorting receptor *VPS10*, and the vacuolar proteases *PEP4* and *PRB1*. In addition to these genes, also the gene *CYM1* was deleted. Unlike the protein products of *PEP4* and *PRB1*, the endoprotease Cym1p localizes to the mitochondrial intermembrane space, so it might not have a direct relation to the secretory pathway [291]. However, strains with a knockout of *CYM1* have shown to enhance the secretion of heterologous peptides like growth hormone, pro-B-type natriuretic peptide and pro-cholecystokinin [292].

In this experiment the P_{GAL1}, P_{TEF}, and P_{GPD1} promoters were used to vary the expression level of the IgG molecule. The specific product yields of the *Δvps10*, *Δpep4*, and *Δcym1* strains, together with the respective wild-type background strain SS328 are shown Figure 6. From the figure it is clear that IgG expression irrespective of the promoter used strongly benefitted from the deletion of the *VPS10* gene, with an over 85 % increase in the IgG titers when expressing IgG from P_{TEF}, and an over 45 % increase in IgG titers when using P_{GPD1}, or P_{GAL1}. Vps10p is a transmembrane receptor residing in the Golgi apparatus and is responsible for targeting CPY to the vacuole. However, data has suggested that this same pathway is used as a salvage pathway for degradation of nonnative proteins through vacuolar degradation [54, 186]. These results showed a stronger improvement of IgG titers by *VPS10* deletion than findings by Rakestraw and colleagues, where the deletion did not significantly increase secretion of an IgG molecule. However, deletion of *VPS13* and *VPS8* yielded similar increases to our result of the *VPS10* deletion, indicating that vacuolar sorting also in their

study was limiting IgG titers, although it must be noted that a different secretion leader was used in their study [187].

There was no significant increase in titers found with deletion of *PEP4,* indicating that it might not be the major protease acting on IgG in the yeast vacuole. Deletion of *CYM1* only resulted in an increase of IgG titers when using P_{GAL1} and P_{GPD1} for expression. This might indicate that only in case of very high expression levels Cym1p is contributing to IgG degradation.

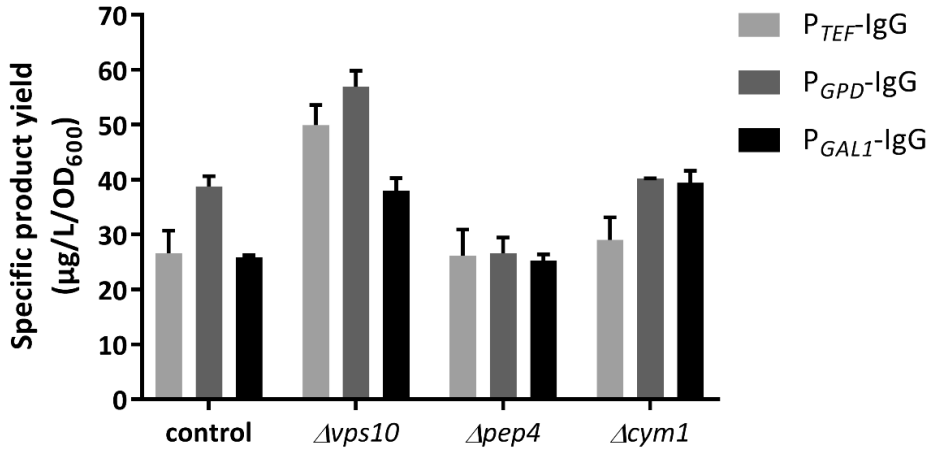


Figure 6 **Antibody production of yeast strains with deletions related to proteolytic degradation**. All strains were analyzed with three different promoters for IgG expression, P_{TEF}, P_{GPD}, and P_{GAL1}. The strains were grown into the exponential phase in 10 mL media in 100 mL shake flasks, followed by induction of IgG production with 0.5 % galactose for the strains with IgG expression under control of the PGAL1 promoter. After 48 hours of IgG expression ELISA was used to measure IgG titers from clarified culture supernatants, and IgG titers were normalized for cell densities. Error bars represent the standard deviation from three to five independent cultures, control refers to wild type SS328.

The unexpected result that prevention of vacuolar missorting did improve titers, but deletion of the main vacuolar protease *PEP4* did not, made us decide to design a new set of experiments. This time, we included the deletion of the other major vacuolar protease *PRB1*, together with the *Δpep4Δprb1* double deletion strain in order to create a strain that has most of the vacuolar protease activity disrupted. Additionally, we studied IgG titers now at 24 and 48 hours after induction, and to investigate the extent of intracellular degradation of IgG cell extracts were prepared as well at both time points (Figure 7).

The inclusion of samples at 24 hours made it apparent that protease deletion created some IgG production phenotypes. After 24 hours, over 50 % more IgG was measured in the supernatant of the *Δpep4* strain than in the wild-type control (Figure 7). For the *Δprb1* and *Δpep4Δprb1* strains there was a 12 % increase at 24 hours. However, after 48 hours a general reduction of the IgG levels was observed indicating that other cellular components can reduce the amount of IgG in the culture supernatant as well. The detected HC in the cell extracts was in both wild-type samples completely absent at its full length size, while after 24 hours it was highly present at similar levels for all three the deletion strains. After 48 hours, the double deletion strain showed still the highest signal intensity for the full length HC. Moreover, also the band pattern created by proteolytic processing of the HC was changing to predominantly higher molecular weight fragments in the deletion strains.

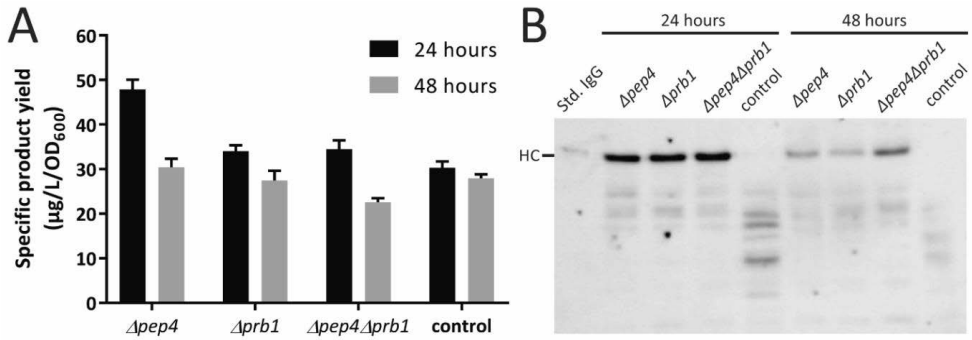


Figure 7 **Analysis of effects of protease deletions on IgG production**. (A) Antibody production of yeast strains with deletions related to vacuolar degradation. All strains were grown into the exponential phase in 10 mL media in 100 mL shake flasks, after which IgG production was induced with 0.5 % galactose. After 24 and 48 hours of IgG expression ELISA was used to measure IgG titers from clarified culture supernatants, IgG titers were normalized for cell densities using the respective OD_{600} values. Error bars represent the standard deviation from three to five independent cultures, the control strain is wild type SS328. (B) Western blot of intracellular IgG. Cell extracts were prepared after 24 and 48 hours of the 4 strains and total protein was separated using SDS-PAGE. After transfer of protein to a nitrocellulose membrane, HC content was visualized using an antibody specific for the Fc region of the IgG.

Compared to the wild-type the three deletion strains showed a decrease or absence of the lowest bands. This could indicate that there was at least a decrease in degradation in these strains. However, after 48 hours it did not lead to a corresponding increase in titers anymore. This could possibly be explained through extracellular degradation or through exhaustion of the inducer.

4.1.3. Targeted screen as starting point for strain improvement strategies

In this first preliminary screen of the secretory pathway we focused on the effects of manipulating the processes protein folding, vesicular transport, and proteolytic degradation on IgG production. Based on literature we selected candidate genes for overexpression and deletion to improve the secretion of IgG from our yeast. However, the results showed that rationally designed strain improvement is not always straightforward.

The vesicular transport step was the only one of the three processes analyzed that showed no significant increases in IgG secretion. This can either mean that this step was at the moment not a bottleneck in IgG secretion, that the proteins chosen for overexpression were not the right ones to improve secretion, or that the expression levels of the selected components were too high so that the vesicular trafficking process is negatively affected.

Concerning the sorting step and proteolytic degradation it became clear that miss-sorting of IgG to the vacuole is a limiting factor, as blocking this step increased titers. The results of deletions of the vacuolar proteases were variable, but indicated the importance of protease activity reduced strains for production.

Our initial screen of proteins overexpressed in the ER consisted of Kar2p, Pdi1p, and Cpr5p. From these, mostly overexpression of Cpr5p, and to some extent Kar2p was beneficial for IgG secretion, while Pdi1p overexpression decreased titers. These results indicated that the folding process in the ER can be considered a major bottleneck for IgG folding and secretion in *S. cerevisiae*.

4.2. Applying a focus on the ER folding environment to improve secretion efficiency

The folding environment in the ER has often been considered to be the first major bottleneck in recombinant protein production [51], so we decided to focus on modifications of the ER to improve protein folding. In general, yeast cells respond to an overload of unfolded proteins in the ER through activation of the UPR, which regulates expression levels of close to 400 genes [122]. As this affects many cellular processes, it can impose an additional burden to the cells if it happens during heterologous protein expression. This implies that a complete activation of the UPR could in the end not be the most beneficial approach to create an improved cell factory [129]. For this reason we strove to selectively mimic parts of the UPR related to the ER to create an improved folding environment, without the cellular burden of a full UPR activation.

4.2.1. Studying the UPR: ER size expansion through upregulation of lipid biosynthesis

One of the most straightforward approaches to create a less crowded ER is to increase the ER functional folding space through expansion of the ER membrane. During the UPR, the volume of the ER is increased over 1.5-fold through the Ino2p and Ino4p dependent induction of lipid biosynthesis genes [121, 293]. The transcription factors Ino2p and Ino4p work in a heterodimer complex to activate the transcription of the phospholipid synthesis genes. Their function is inhibited by Opi1p, which binds to Ino2p, and so prevents formation of the transcription activating complex Ino2p/Ino4p. Removal of the repressing Opi1p protein leads to a constitutively activated Ino2/4 complex [121, 294, 295]. *OPI1* deletion increases the size of the ER by approximately 50 %, and this is an increase comparable to a UPR induced size increase of the ER. However, with the *OPI1* deletion the increase is uncoupled from induction of the UPR, and additionally it was shown to alleviate ER stress when compared to a wild type strain [121].

We decided to see if an expansion of the ER preceding heterologous protein production would lead to an improvement of IgG secretion. For this, we first created a modified parental IgG production strain by integrating the galactose inducible expression cassette for the full-length IgG molecule into the *HIS3* locus of the yeast genome. Thereafter, a marker-free deletion of the *OPI1* gene was created [296]. Results from experiments for characterization of both production strain backgrounds are shown in Figure 8.

As the changes in regulation of lipid biosynthesis might have an effect on growth performance in different media, growth curves were measured for 40 hours in both complex (YPD and YPGal), and in synthetic defined media (SD and SGal) (Figure 8A and B, respectively), under normal cultivation and under protein production conditions. Under normal growth conditions, in YPD and SD media, no defects in growth were observed, although the wild-type reached slightly higher final OD_{600} values (top panels of Figure 8). When IgG expression was induced due to the presence of galactose, in YPGal and SGal media (lower panels in Figure 8), the *Δopi1* strain displayed delayed and diminished growth characteristics.

The differences observed were most prominent in rich media, where the maximum growth rate decreased to almost half: from 0.110 h^{-1} for wt to 0.065 h^{-1} for the *OPI1*-deletion strain, barely reaching its stationary phase during the 40 hours of growth. In contrast, in SGal media, the corresponding maximum growth rates were 0.280 h^{-1} and 0.266 h^{-1}, respectively, although the *Δopi1* strain did display a prolonged lag time.

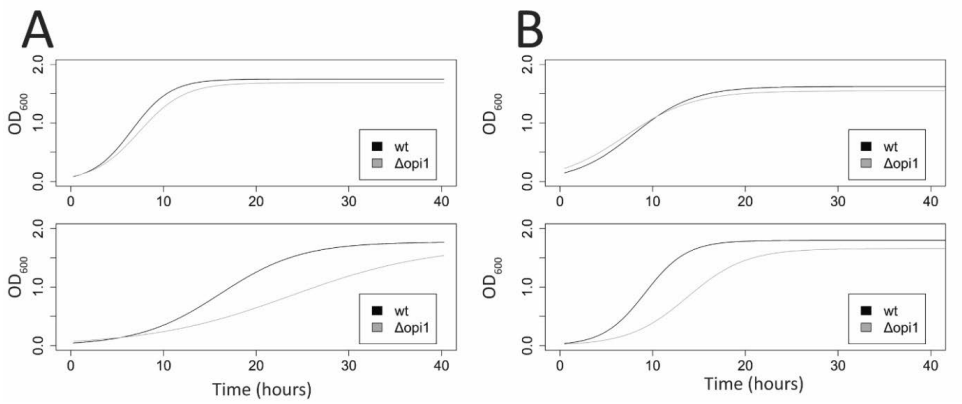


Figure 8 **Growth of wild-type and Δopi1 deletion strains in liquid media.** Cultures with exponentially growing cells were diluted to OD_{600}=0.02 in fresh media and grown with continuous shaking in microtiter plates for 24 hours at 30 °C. OD_{600} was measured every 30 minutes, and the data shown represents the average of 8 replicates per growth curve. Upper panels represent growth under non-inducing conditions, and lower panels represent growth under protein production inducing conditions, both in rich and selective media (A and B, respectively).

Next, the secretory capacity of both strains was compared by measuring IgG titers after production at 20, 25, and 30 °C (Figure 9A), to see if the strains show difference in production at different temperatures. The increase in ER volume had a clear positive effect on IgG titers, as the *Δopi1* strain was outperforming the wild-type in each condition. The differences between the two strains increased with increasing temperature, leading to an up to threefold higher IgG titer for the *Δopi1* strain at 30 °C. As the *Δopi1* strain showed a decreased cell density under production conditions, the normalization of IgG titers to cell densities increased the differences even further, with a corresponding increase in specific product yield of up to 4.8-fold compared to the wild-type. A similar approach to manipulate the size of the ER was successfully used for the production of membrane

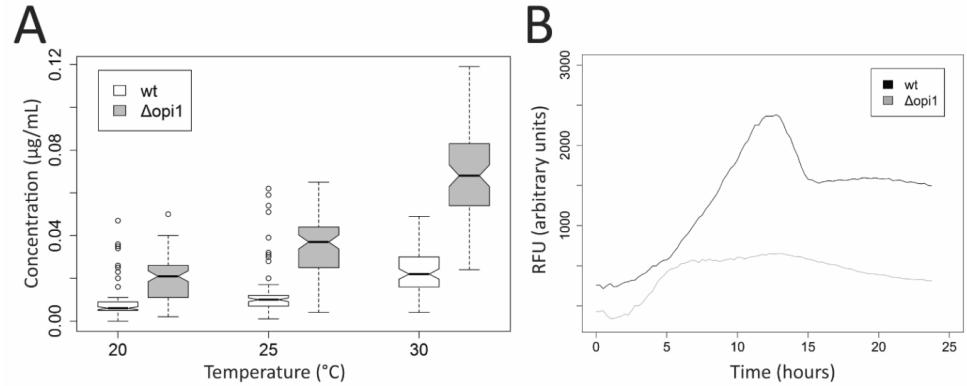


Figure 9 **IgG production characteristics of wild-type and Δopi1 strains.** (A) Notched box plot representation of all the measured antibody concentrations for the wild-type and *Δopi1* strains. The data for each box consists of 82 or 84 measurement points. (B) Fluorescence of GFP under control of an UPR responsive promoter was recorded in the IgG expressing wild-type and Δopi1 strains. The fluorescence values are corrected for background and cell densities.

proteins in the yeast *Yarrowia lipolytica*. There, a rerouting of metabolic fluxes from storage lipids to phospholipid biosynthesis led to an increased ER membrane, which was accompanied by an improvement of membrane protein accumulation through the increased space for membrane insertion [297].

It was our goal to create a better production strain by decreasing the burden of heterologous protein production stress through mimicking a selective part of the UPR, therefore we tried to gain insight in the relative level of UPR induction in these strains. For this, we integrated a UPR reporter cassette into the genome of both strains. This cassette consists of a GFP gene under control of a crippled *CYC1* promoter fused to four UPRE sequences, which activate GFP expression when the UPR is activated [77]. The magnitude of the UPR in both strains upon induction of IgG production is shown in Figure 9B. It is interesting to see that the initial signal increase happened at the same rate in both strains. However, in the wild-type strain the signal increased until it reached its peak strength after approximately 13 hours. In contrast, the signal reached in the *Δopi1* strain already after five hours a plateau value, indicating that the stress was more quickly resolved. Finally, the UPR signal persisted in wt until the culture reached the stationary growth phase, after which it decreased to a stationary level.

Overall, the enlarged ER in the *Δopi1* strain improved the IgG production titers up to 4.8-fold, while greatly reducing the unfolded protein response. This makes the *Δopi1* strain a suitable starting point for further strain improvements.

4.2.2. Studying the UPR: selective overexpression of folding enzymes

After successfully increasing the conformational folding space of the ER, and thereby increasing IgG secretion titers, we decided to study which ER-resident proteins could selectively increase the folding efficiency of the IgG molecule. For this, the set of ER proteins described before (Kar2p, Pdi1p, and Cpr5p) was complemented with the thiol oxidase Ero1p, and with the Kar2p nucleotide exchange factors Lhs1p and Sil1p.

In order to get a more broad range of expression levels, the three promoters used before: P_{GAL1}, P_{TEF}, and P_{GPD1}, were complemented with P_{KAR2} and P_{PDI1}. These two promoters from the yeast genes *KAR2* and *PDI1* have different basal expression levels, which are upregulated by induction of the UPR [126]. In total, 26 plasmids from the six selected genes with up to five different promoters were successfully constructed. 52 yeast strains with a single selected gene expression plasmid were created to test the effect of each helper plasmid – promoter combination by itself. Based on the performances of these single expression strains, 67 extra yeast strains were created that contained a combination of two different helper protein expressing plasmids. This approach was used to search for synergistic interactions between proteins of a different function. All strains contained always two plasmids with the *LEU2* and *URA3* marker enabling the use of the identical media throughout all experiments.

An overview of the screening process is given in Figure 10. Each strain was cultivated in three biological replicates, originating from three different transformants. In each of these cultivations, the colony was inoculated into a varying position of the deep-well plates to minimize bias caused by plate position. The two background strains, wild-type and *Δopi1,* were included in every transformation, and, during expression cultivations, in every condition on every plate, to serve as a control for transformation efficiency and for proper expression induction.

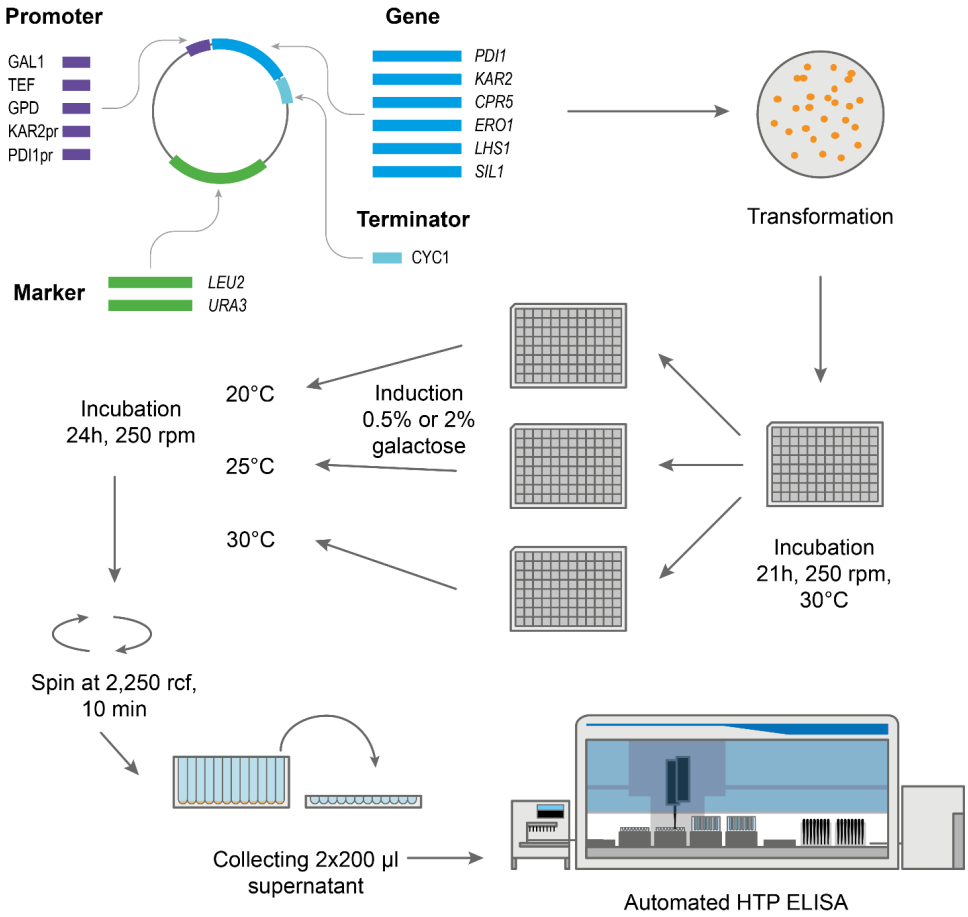


Figure 10 **Schematic representation of the targeted screening setup.** The plasmid library of selected genes was created by combining the coding regions of the six genes, with the five different promoters and a vector backbone either containing a *LEU2* or *URA3* selective marker, all plasmids contain the *CYC1* terminator. The plasmids were transformed into wt and *Δopi1* background strains, which both have a single-copy of the coding region for light and heavy chain genes, under control of the *GAL1* promoter, integrated into the *HIS3* locus of their genome. Strains were inoculated from plates to 1ml of liquid media in 96-deep-well plates and grown for 21 hours at 30 °C. The precultures were diluted 1:4 in fresh media in three plates and grown for 5.5 hours at 30 °C, after which antibody expression was induced with 0.5 or 2.0 % galactose. Antibody was expressed for 24 hours at 20, 25, and 30 °C, after which final cell densities were measured. Antibody titers were determined from clarified supernatants using a high throughput (HTP) ELISA on an automated liquid handling station using two technical replicates; each strain was analyzed in three independent biological replicates.

A graph showing an overview of all around 3000 data points measured in this screen is shown in Figure 3 of Publication II. The data showed that for cell density, antibody titer, and specific product yield, the two strain backgrounds showed distinct populations. The *Δopi1* strains reached generally higher antibody titers and lower cell densities, which manifested in higher specific product yields. The general increase in antibody titer was also observed for the background strains in Figure 9A. In general, OD values and IgG titers increased with temperature, but the increase in titers was more strain dependent than cell density

dependent. Remarkably, an increase in induction strength led to a decrease in IgG titers, with an average antibody concentration for 2 % galactose being 20.7 µg/L, and for 0.5 % galactose 25.4 µg/L (in wild-type background). However, there were no significant differences observed for the specific product yields, which were 20.1 and 21.0 µg/L/OD_{600}, respectively. For this reason, most of the analysis is done with respect to 0.5 % galactose induction, as this yielded higher secretion titers.

4.2.3. Effects of folding enzyme overexpression on IgG secretion

As expected, the IgG titers varied depending on which chaperone or folding catalyst was expressed, as well with the promoter used for its expression. Figure 11 reports the fold-change relative to the respective strain background. The colors and numbers in each cell represent the fold-change of the specific product yield relative to the parental background strain in the same condition. In general, the expressed elements created similar effects in both strain backgrounds, but with slightly smaller relative increases in the *Δopi1* strains. In all the experiments combined, when comparing effects to the respective strain backgrounds, we identified 49 cases with an increase in final antibody titers of over 50 %, and 71 cases with an over 50 % increase in specific product yield.

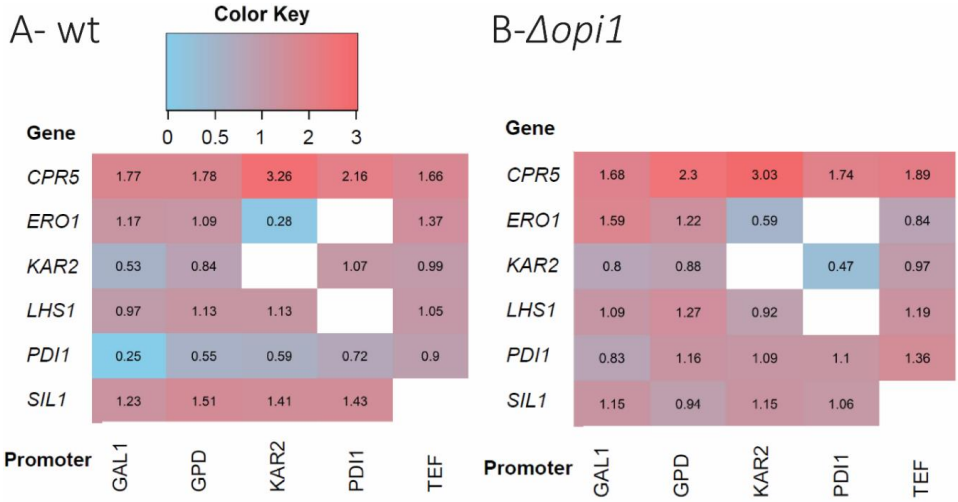


Figure 11 **Effects of overexpression of a single protein on antibody secretion.** A heatmap representing the change in specific product yield of each gene-promoter overexpression relative to the strain background (wt in A, *Δopi1* in B). The colors and numbers of each cell represent the fold-change in specific product yield caused by the specific gene-promoter combination for the yeast strains grown at 30 °C and induced with 0.5 % galactose.

One of the rate-limiting steps in the folding process of full-length antibodies is the isomerization of certain proline residues, and it was shown that *in vitro* the antibody domain folding could be catalyzed by the addition of a peptidyl-prolyl isomerase [201, 202]. Our data confirmed that this step is also rate limiting in full-length antibody assembly in the yeast ER, as, from the proteins analyzed, overexpression of the PPIase Cpr5p was the most beneficial for increasing IgG titers, reaching a 1.65- to 3.26-fold improvement. The use of overexpression of PPIases to improve recombinant protein production has so far only been studied in *E. coli* strains. In those experiments expression of the periplasmic FkpA protein was shown to increase the solubility of a single chain antibody and Fab fragments [229, 298].

The overexpression of *KAR2* showed similar results as those that were found in our preliminary targeted screen. Even though its expression has shown to be beneficial for production of scFv fragments [98, 99, 251], for the production of IgG all but one condition actually decreased IgG titers. Only in the wild type background, *KAR2* expression under P_{PDI1} and 0.5 % galactose led to a small 7 % increase in titers, while in the same condition in the *OPI1* deletion background it led to a more than 50 % decrease. On average, the negative effect of *KAR2* overexpression seemed to be slightly bigger with higher IgG induction levels. This could be related to the point that Kar2p binds to Ire1p in order to desensitize Ire1p to low levels of unfolded protein stress, and under normal conditions this promotes the deactivation of Ire1p to inhibit its splicing of *HAC1* mRNA, which activates the UPR [77, 299]. In that sense, overexpression of Kar2p could lead to a further desensitization of Ire1p to the unfolded IgG molecules and thus a delay of UPR activation. In general, up to some level this UPR activation could be beneficial for IgG secretion, as it helps to prevent an accumulation of unfolded IgG in the ER.

Another consideration with Kar2p is that it is one of the most abundant protein in the ER, with an estimated amount of around 30,000 molecules per cell, so that overexpression might not change that amount relatively much [78]. However, two proteins that are the main regulators of its activity cycle, Lhs1p and Sil1p, are only present at around 140 and 2400 proteins in the ER, respectively. For that reason, we tried to improve the Kar2p activity cycle through overexpression of both proteins. Our data showed that the hypothesis of regulating Kar2p activity had promising results, as especially Sil1p overexpression showed an increase in IgG levels, with up to 1.5-fold improvement, and also Lhs1p increased titers by up to 27 %. Remarkably, while Kar2p overexpression gave worse results at high IgG induction, with Sil1p and Lhs1p that condition gave the best results. This increase of IgG titers through regulation of Kar2p activity and not Kar2p abundance is supported by data that showed that in a mammalian system, the Kar2p homolog BiP does not cycle away efficiently from both the unassembled heavy and light chain molecules [200, 300], which might stall protein folding at high levels of Kar2p in the ER. These results indicate that an important strategy in strain improvement for recombinant protein production is to rather consider improving the regulation and activity of an important protein, than to increase its abundance. The expression of these two co-chaperones have already been successfully used to increase titers of human transferrin and recombinant human albumin, although fold-changes were slightly lower than in our data [41].

In the wild-type background overexpression of *PDI1* proved again to lead to a significant decrease in IgG titers, even though its expression has increased titers of various heterologous proteins before [98–102, 251]. One possible cause could be a disruption of the cellular redox balance in the ER through an increase of Pdi1p, which was not matched by an increase of its redox partners [51]. Additionally, evidence has been found that Pdi1p facilitates the targeting of certain substrates to the ERAD pathway, either through its chaperone activity, or through its redox activity [80], which could contribute to an increased IgG degradation. And also here, as in the case of Lhs1p and Sil1p, the effects of overexpression were slightly more positive in the *OPI1* deletion strains, with an up to almost 40 % increase in titers with P_{TEF}, while the highest overexpression levels with P_{GAL1} still gave a significant decrease. It has been shown that the size increase of the ER in the *Δopi1* strain does not lead to an increased production of ER resident chaperones, effectively leading to a diluted ER. More importantly, the same study showed that, unlike in wild type strains, the concentrations of the important ER-resident proteins Kar2p, Lhs1p, and Pdi1p, do not increase when the UPR is activated [121]. This could mean that in the *OPI1* deletion background the negative effects from too

high concentrations of some of the proteins that we overexpressed are less pronounced due to a lower final concentration as there is no additional activation of transcription from their genomic DNA copy through UPR induction. However, it would require further studies into the levels of these proteins under various levels of stress to be able to determine the exact effect.

Finally, the overexpression of the thiol oxidase Ero1p showed as well that the oxidative balance is an important factor for protein folding, as the secretion levels of IgG were varying greatly depending on which promoter was used. Ero1p is important for oxidative protein folding, as it provides Pdi1p with disulfide bonds for transfer to folding proteins [81], and the activation and inactivation of Ero1p has been considered crucial for ER homeostasis [82]. It has been shown that Pdi1p acts as the main regulator for Ero1p, so when there is sufficient reduced Pdi1p in the ER it reduces the Ero1p regulatory disulfide bonds to get Ero1p into an active state. If Ero1p has oxidized a sufficient amount of Pdi1p, Ero1p will auto-oxidize its regulatory disulfide bonds to avoid overoxidation of the ER [82]. This mechanism to protect the ER from overoxidation through Ero1p function could be a reason why Ero1p overexpression is in general more beneficial for IgG titers than Pdi1p overexpression.

4.2.4. Combining overexpression of two folding elements

From the single overexpression studies it was clear that improvement of one aspect of the folding process was very beneficial in some of the cases. The fact that modification of various parts of the folding process could each by themselves increase the folding efficiency, made us wonder if it would be possible to further optimize the system by combining overexpression of two folding factors at the same time in the same strain. For this, we used a two-faced rational approach, which was on one hand based on known functionalities and interactions of the proteins, which were used to predict complementary actions, and on the other hand to find new interactions between the proteins beneficial for IgG folding. An overview of the effects of the different combinations used on specific product yield can be found in the appendix with publication II.

Some of the strains with overexpression of two different folding elements showed an increased IgG titer when compared with their respective background strain. However, in general none of the selected pairs showed beneficial synergistic or combinatorial effects with respect to the effects from their singular overexpressions. For example, as we noticed that for the single overexpression studies it was more successful to regulate Kar2p activity through expression of Sil1p and Lhs1p, rather than Kar2p overexpression, we decided to study their interactions. For this, we generated strains with either Kar2p and Lhs1p, Kar2p and Sil1p, or with Lhs1p and Sil1p, with a variation of the available promoters. However, from the close to 100 cultivations of all these strains at the different temperatures and induction conditions, only ten cultivations portrayed a positive result compared to the strain background. Unfortunately, none of them exceeded the improvement of a single expression of one of these factors.

Two examples of these combinations in both strain backgrounds are shown in Figure 12A and 12B, for wild type and the *OPI1*-deletion strains, respectively. The figure shows that overexpression of *LHS1* and *KAR2* under control of P_{GAL1} were by themselves not significantly beneficial for IgG titers as in these cases, Lhs1p did not significantly increase titers and Kar2p even led to a decrease in titers. The strain which had both proteins expressed under this promoter showed even a further decreases in titer, suggesting a negative synergistic effect. One of the possible cause for this could be the fact that these strains had now in total four genes expressed under the highly active *GAL1* promoter. This

could lead for example to a too high amount of expressed protein, which is thought to be toxic for cells [301]. Also, as all four proteins are targeted to the ER, it could decrease folding efficiency through overloading of the ER.

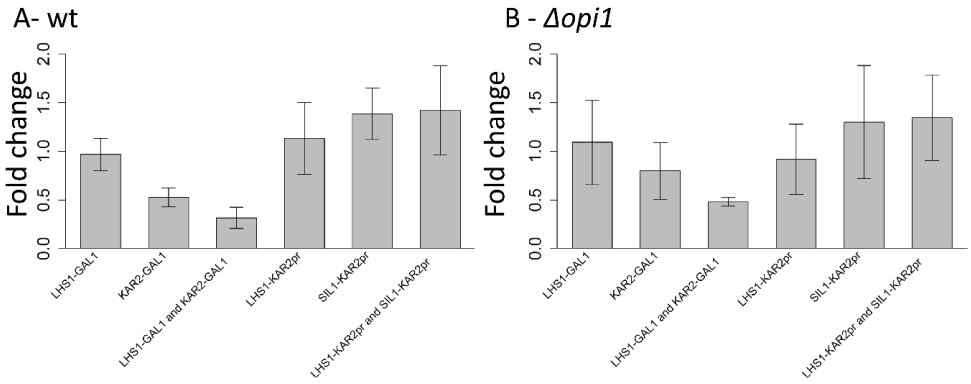


Figure 12 **Fold changes in specific product yield of two-gene combinations compared to overexpression of the respective single genes.** Data is show in wild type (A) and in Δopi1 (B). The bar plots show the relative specific product yield, normalized to the respective background strains, for the selected genes and their combination in wild-type and *Δopi1* yeast strains. Error bars represent the standard deviation of expression cultures with 0.5 % galactose at 30 °C.

The case of overexpression of *LHS1* and *SIL1* under control of P_{KAR2} provided a different image. However, even though this combined overexpression was one of the most beneficial ones, leading to an up to 1.5-fold increase of titers, it still was in the same order of magnitude as the effect of overexpressing Sil1p by itself. This indicates that Sil1p is probably the main protein catalyzing the ATP-cycle of Kar2p.

One other pair of interacting proteins selected was Pdi1p and Ero1p, because the proteins are both involved in the formation of disulfide bonds in the ER. In all promoter-gene combinations a decreased level of IgG titers was observed. This might indicate that the problem lies not directly in the activity of these proteins, but it could be related to the general redox-state in the ER. One possibility is that the pool of glutathione in the ER is insufficient to keep up with the increased amount of Pdi1p and Ero1p, as the ratios of reduced and oxidized glutathione are important for Ero1p and Pdi1p activation [82]. Also combining Pdi1p overexpression with Kar2p overexpression did not lead to improvements relative to the single overexpressions, even though previous studies did find a synergistic effect on the expression of scFv fragments [98, 99].

Finally, we analyzed if Cpr5p, the most beneficial single overexpression Cpr5p, showed synergistic interactions for IgG titers together with the other proteins analyzed in this screen. Even though some of these combinations yielded the highest titer improvements in these experiments compared to the respective strain backgrounds, none of them exceeded the relative fold-changes caused by overexpression of only *CPR5*. This is illustrated by the combined expression of *CPR5,* and either *LHS1* or *SIL1*, all under control of P_{KAR2}, which generated both an around 2.3-fold increase at 30 °C. This is however still significantly lower than the observed over threefold increase by expression of *CPR5* alone.

Overall, the strategy of combining the expression of different chaperones and folding catalysts to increase the potency of the folding environment was not more successful than expression of single factors. Possible explanations could be that there are still other elements missing, like for example the co-factor glutathione for an improved functioning of Pdi1p and Ero1p. Other options are that the combinations are not optimal, but that other proteins should be screened to find the best partners. For example, the experiments with Lhs1p and Sil1p could be extended with Jem1p or Scj1p, which are both also involved in Kar2p regulation. The other possibility would be to study a broader range of expression levels, as high level protein expression of various proteins might create a significant competition for cellular resources between high level protein production and growth [302–304]. This could be an explanation for the decreased cell densities we have measured in some of the cultures of double overexpression stains.

4.2.5. The ER as successful starting point for the creation of better production strains

The overall outcome of this targeted screen was that overexpression of *CPR5* proved to be very successful, as it yielded the highest amounts of IgG secreted per cell under all the promoters used. An overview of the ten strains with the highest specific product yields can be seen in Table 4, and this analysis put *CPR5* in all top five positions. If the overexpression was combined with the deletion of *OPI1*, the specific product yield could be increased over tenfold when compared with the wild-type strain.

Table 4 **List of strains with the highest specific product yields**[(a)]

Added elements	Strain	Specific product yield (mg/L/OD_{600})	Relative frequency[(b)]	P-value[(c)]	Fold-change[(d)]
CPR5-P_{GPD}	*Δopi1*	0.2027	0.2	0.000002	10.22
CPR5-P_{KAR2}	*Δopi1*	0.1992	0.15	0.000159	10.15
CPR5-P_{GAL1}	*Δopi1*	0.1579	0.1	0.000560	7.99
CPR5-P_{TEF}	*Δopi1*	0.1525	0.075	0.000036	7.66
CPR5-P_{PDI1}	*Δopi1*	0.1424	0.05	0.007624	7.15
ERO1-P_{GAL1}	*Δopi1*	0.1226	0.05	0.020502	6.13
SIL1-P_{GAL1}	*Δopi1*	0.1223	0.05	0.021128	5.53
PDI1-P_{TEF}	*Δopi1*	0.1208	0.075	0.279237	6.09
LHS1-P_{GPD}	*Δopi1*	0.1163	0.05	0.299870	5.88
LHS1-P_{KAR2} + *SIL1*-P_{KAR2}	*Δopi1*	0.1139	0	0.011316	5.73
none	*Δopi1*	0.0886	0.075	N/A	4.47
none	wt	0.0199	0	N/A	1

(a) Data represents the mean value of experiments conducted with 0.5% and 2% galactose induction at 30 °C
(b) Relative frequency in the 95th percentile of *Δopi1*-strains
(c) Calculated with the nonparametric equivalent of t-test, Wilcoxon signed rank test using the *Δopi1*-strain without added elements as the reference
(d) Averages of the fold-changes were calculated relative to wild-type

Displayed in the table are the strains with the highest specific productivity, based on a combined evaluation of the production results from 0.5 % and 2.0 % galactose, to mitigate the effect of induction levels. In order to ensure that the strains we found to be the highest producers were not statistical outliers, we conducted frequency analysis. With the frequency analysis we determined which strains were most enriched amongst the lowest (5^{th} percentile), and the highest (95^{th} percentile) five percent of all measured specific product yields in all temperatures. Many of the expressed proteins with specific promoters were found in the same percentiles of the two strain backgrounds at all temperatures used, which indicates that the effect is really protein dependent and not a result from background strain or cultivation temperature.

In our targeted screen we significantly increased the expression of IgG, and reached a reproducible specific product yield of 200 µg/L/OD_{600} for the *Δopi1 + CPR5-$P_{GPD,}$* which corresponded to an absolute antibody concentration of 126 µg/L at 0.5 % induction. Additionally, individual replicates showed titers reaching up to 300 µg/L/OD_{600}. However, when comparing production to commonly used industrial platforms like mammalian cell lines, the titers were still very modest. Seeing that we used an industrially relevant full-length IgG molecule as model protein for secretion efficiency, a tenfold increase in secretion through engineering of only aspect of protein folding should not be underestimated. This production strain is a promising starting point for further optimization of other genetic targets to improve protein secretion efficiency. Additionally, deep-well plate cultivations are far from optimal conditions for protein expression and process optimization is expected to improve titers significantly further.

4.2.6. Studying the UPR: disruption of the ER associated degradation pathway

Seeing the positive results that can be achieved by mimicking parts of the UPR that promote protein folding, we started to wonder about the influence of the ER protein quality control machinery on IgG secretion. For glycoproteins the main quality control pathway in the ER is the ER associated degradation pathway, which targets misfolded glycoproteins. This targeting is achieved through a combined glycan and protein signal. When terminally misfolded, the glycoproteins are targeted for retrotranslocation to the cytosol followed by proteasomal degradation. However, it is known that through the nature of this selection process also slowly folding glycoproteins can be unnecessarily targeted for degradation. In *P. pastoris*, it was found that expression of the Fab3H6 antibody fragment led to a significant decrease of product yield because proteasomal activities were increased by 20 % [239].This possibly puts also in our *S. cerevisiae* production system the slowly folding IgG molecule at risk. Especially when taking into account that ERAD is a process that is constitutively active, and that the expression of its components are vastly upregulated by the UPR, which increases the chance of ERAD mediated degradation of IgG components under production conditions. Unfortunately, previous studies aiming to gain more insight in the mechanisms of ERAD had always used model substrates designed to be prone to misfolding, like CPY* and pRA*. However, for the creation of improved production strains for heterologous proteins it is absolutely necessary to evaluate ERAD using the real protein substrates.

Therefore, we decided to study if the activity of ERAD is limiting the secretion levels of IgG through deletion of various ERAD components, with and without an additional disruption of the UPR. The genes selected from ERAD were: *HTM1, YOS9, HRD1, HRD3,* and *UBC7*, additionally the *ALG3* gene involved in N-glycosylation was deleted, and the induction of the UPR was disrupted by deletion of *IRE1*. The main roles of these proteins are shown schematically in Figure 1 of Publication I, but in short Htm1p generates the glycan signal that starts the targeting of the glycoprotein substrate for ERAD. This signal is recognized by Yos9p, which brings the degradation substrate to the retrotranslocation and ubiquitination complexes in the ER membrane of which Hrd1p, Hrd3p, and Ubc7p are members. Deletion of *ALG3* leads to an aberrant glycoform on the glycoprotein, which might allow escape from degradation by ERAD. As Ire1p is the protein that splices the inactive *HAC1* mRNA, which then activates the unfolded protein response dependent regulation of close to 400 genes, deletion of *IRE1* will lead to an abolished UPR.

The genes *HTM1* and *YOS9* were selected for deletion to study the effect of disrupting parts of the ERAD targeting step, *HRD1, HRD3,* and *UBC7* were selected to study the effect of disruption of parts of the retrotranslocation pore complex, and *ALG3* was selected to study

if a glycoprotein with a pruned glycan form can escape ERAD target. Finally, disruption of the UPR by *IRE1* deletion was included to study the effects when an accumulation of IgG cannot be rescued by induction of the UPR.

Physiological effects of deletion strains.
Since the disruption of protein quality control processes might decrease viability of the yeast strains, we studied the effects of the deletions on growth (Figure 13). No differences in growth behavior were observed and all strains reached the stationary phase after approximately 10 hours of cultivation, while the maximum growth rate was reached between 2.5 and four hours of cultivation. For a quantitative comparison μ_{max} values were calculated, which ranged from 0.505 h^{-1} to 0.581 h^{-1} for the strains in the wild-type background and from 0.497 h^{-1} to 0.562 h^{-1} for the strains that had a deletion of *IRE1*. Statistical analysis with single factor ANOVA did not detect significant differences between the μ_{max} values of these strains. For the strains bearing deletion of *UBC7, IRE1*, and *HRD1*, it had been reported previously that growth was not affected under normal conditions [122, 305, 306], just like the disruption of putative ERAD components in *Aspergillus niger* [307].

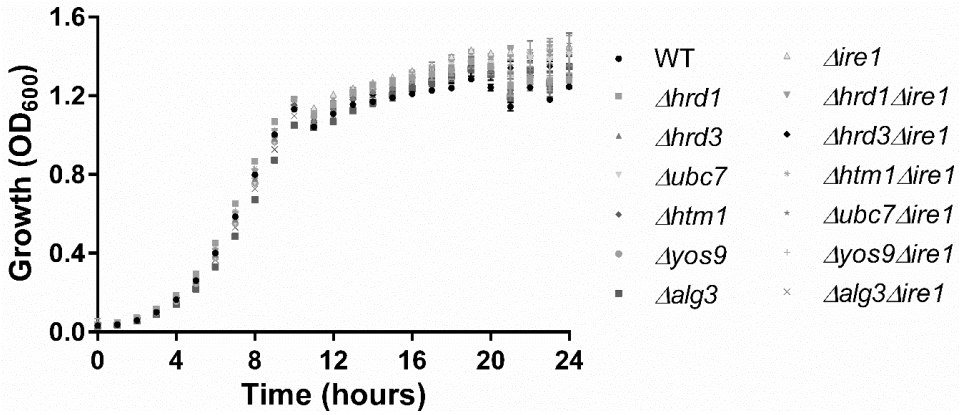


Figure 13 **Growth of ERAD deletion strains in liquid media**. Cultures with exponentially growing cells were diluted to OD_{600}=0.1 in fresh YPD media and grown in microtiter plates for 24 hours at 30 °C with continuous shaking. OD_{600} was measured every 30 minutes and the data shown represents the average of four replicates, with an average % standard error ranging from 1.60 to 4.15 %.

Since the production of heterologous proteins can pose a burden on cellular processes we extended the analysis of effects of the deletions to IgG producing strains. Cultures of exponentially growing strains containing the expression plasmid for a human IgG were spotted in serial dilutions on solid media and incubated at 25, 30, and 37 °C, the latter temperature was chosen to analyze the effect of a high temperature as a source of stress. All strains grew indistinguishable from each other on non-inducing rich and selective media (YPD and SD-URA, respectively), confirming the results of the growth curves. However, some of the strains showed decreased growth under inducing conditions (SGal - Ura) (Figure 14).

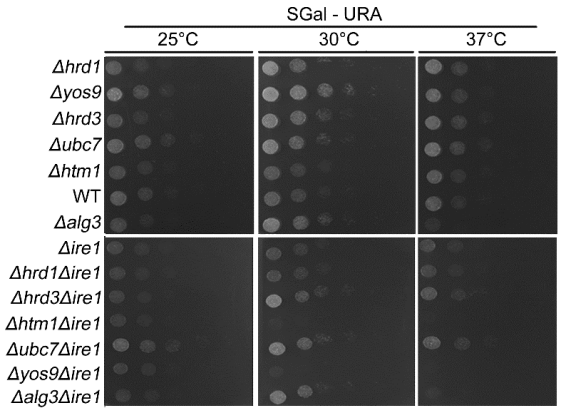


Figure 14 **Growth analysis of ERAD deletion strains on inducing, solid media at different temperatures**. Serial fivefold dilutions of exponentially growing cells were spotted on SGal – URA plates. The plates were incubated at 25, 30, or 37 °C for 3 days.

Most apparent were the strong decreases in growth of the *Δhtm1Δire1* and *Δyos9Δire1* strains compared to both the wt, as well as the *Δire1* parental strains. Most clearly observable at 30 and 37 °C, these two strains were not able to grow. Remarkably, the *Δubc7Δire1* and *Δhrd3Δire1* strains appeared to be growing more efficient than their parental strains. The *Δalg3* and *Δalg3Δire1* strains showed an absence of growth at 37 °C when plated on selective media. However, this was expected to be unrelated to UPR and ERAD influences, as it has been described in literature to be a result of a decreased viability through a changed cell wall composition [308].

IgG secretion in ERAD and UPR mutant strains

Based on the results from the spotting assays, it was decided to decrease the temperatures of the cultivations from 30 °C to 25 °C at the moment that IgG expression was induced by galactose. Figure 15 shows the specific antibody yield after 24 hours of IgG expression with 4 % galactose at 25 °C. The IgG levels were normalized for cell densities, and final OD readings of the cultures varied by 13 %.

Removing proteins from different stages of the ERAD pathway led to different phenotypes. Only removal of Htm1p led to an increase of antibody titers of around 15 %. The increase of titers in the *HTM1* deletion strain might be caused by a possible escape of IgG from ERAD through a delayed recognition as an ERAD substrate, which could give the slowly folding IgG protein more time to reach its native state. The removal of the mannose from the glycan by Htm1p has been shown to be required for efficient binding of ERAD target proteins by Yos9p [139, 141]. Removal of Yos9p decreased the titers to 18 % of wild-type levels and also deletion of *HRD1, HRD3,* and *UBC7*, all three involved in the retrotranslocation step of ERAD decreased by up to 30 % compared to wild-type. A set of experiments testing the influence of the deletion of ERAD components on the secretion levels of an overexpressed endogenous yeast acid phosphatase did not lead to any significant changes in measured titers (data not shown).

Overall, in all strains where the ERAD substrate can be marked for degradation by Htm1p, a removal of ERAD components led to a decrease in IgG titers. So, in order to exclude a rescue of accumulating IgG through induction of UPR parts that stimulate protein folding, we created a *Δire1* strain background that is unable to mount the UPR.

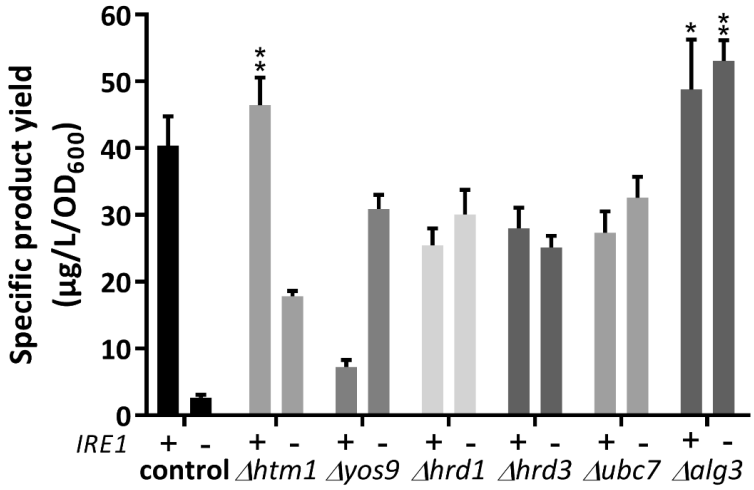


Figure 15 **Antibody production of ERAD deletion strains**. All strains were grown into the exponential phase in 96 well-plate format after which IgG expression was induced with 4 % galactose for 24 hours. ELISA was used to measure IgG titers in clarified culture supernatants, and IgG titers were normalized for cell densities using the respective OD_{600} values. Error bars represent the standard error from nine independent cultures. *P<0.05; **P<0.01; (P-values are calculated for strains with an improved specific product yield versus wild-type control using Student's two-tailed t test). Presence of the *IRE1* gene is marked with + and -.

Abolishing the UPR through *Δire1* deletion was detrimental for IgG secretion, as the secreted IgG levels were reduced to 7 % of wild-type. A similar manipulation of the UPR, but through deletion of *HAC1*, showed similar detrimental effects on the expression of α-amylase [131]. Remarkably, this phenotype was partially rescued when the deletions of the ERAD components were introduced into the *Δire1* strain background. In the *Δhrd1Δire1, Δhrd3Δire1,* and *Δubc7Δire1* strains, secretion levels did not significantly decrease compared to the single ERAD deletions strains. Even though titers in the *Δhtm1Δire1* strain were still seven times higher than the *Δire1* strain, it was still an almost 2/3 decrease compared with the *Δhtm1* strain. Interestingly, in the *Δyos9Δire1* strain titers showed an increase compared to *Δyos9,* reaching 76 % of wild-type levels.

The deletion of *ALG3* was the only one to increase protein titers in both the wild-type, and in the *Δire1* strain backgrounds. The deletion did not show to be affected by an abolished UPR, as there was no sign of a decreased titer in the *Δalg3Δire1* strain. In the wild type background IgG titers reached 120 % of wild type levels, and in the *Δire1* background the titers reached even higher levels of up to 130 % of wild type levels. In principal, the *ALG3* deletion strain should not be able to produce the glycan which is normally recognized as the canonical ERAD signal by Yos9p. However, conflicting data has been published using artificial ERAD substrates. First, it was shown that in an *Δalg3* strain the ERAD substrate CPY* was accumulating in the ER [136], showing that the present glycan form allows escape from ERAD degradation. However, there is evidence that the glycan produced in this *Δalg3* strain is also recognized by Yos9p [137].

4.2.7. Development of a cellular clearance assay

Although the measured IgG titers show if a deletion of a gene, an overexpression of a protein, or a change in cultivation conditions, have a positive or negative impact on secretion efficiency, such experiments do not provide any information about the underlying

mechanism. Therefore, there is a need for more in-depth characterization of strain modifications and how they affect IgG secretion, in order to be able to engineer the secretory pathway in a more efficient way. For this reason, we developed a cellular clearance assay to see how the speed of clearance of IgG is different in the various strains. The general idea was to induce IgG production in the strains for a fixed amount of time, then inhibit the production, and from that point on analyze changes in the cellular IgG content over time.

To establish the assay we considered what would be the best conditions to use. Our commonly used production system already allows the specific induction of IgG expression through the galactose inducible promoter. In similar assays, often cycloheximide is used to inhibit translation. However, this might create conditions that indirectly affect IgG processing. For this reason, we decided to analyze if addition of glucose would give satisfactory results, as this selectively inhibits the galactose promoter. Results of these first experiments using the wild-type yeast strain are shown in Figure 16. IgG expression was induced with addition of galactose in exponentially growing cultures. After four hours of expression protein production was inhibited by the addition of 2 % glucose, 200 µg/mL cycloheximide, or a combination of both. For the control experiment the same volume of water was used as a mock treatment. The intracellular IgG content was visualized after zero, two, four, and six hours, using an antibody specific for the HC, while equal loading of gels was verified using an anti-tubulin antibody. All signal intensities were digitally quantified, and the tubulin signal was used to correct the IgG signals for loading differences.

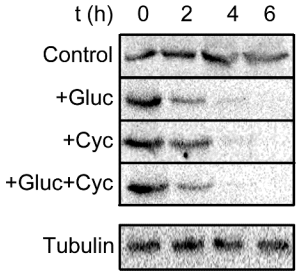


Figure 16 **Establishing the cellular clearance assay**. Antibody expression was induced in exponentially growing wild-type yeast cultures with galactose for four hours. After this the cultures were treated with glucose (gluc), cycloheximide (cyc), both, or water (control) to inhibit protein expression. After zero, two, four, and six hours cell extracts from ten OD_{600} units of cells were prepared from the cultures and analyzed using SDS-PAGE and western blot for intracellular IgG content. A representative blot for tubulin, used as loading, is shown.

The control sample showed that in the mock treatment IgG production continued during the time course of the experiment. From the different inhibition treatments, the decrease of the signal was highest in glucose alone and in glucose in combination with cycloheximide, with a signal half-life of 1.36 and 1.18 hours, respectively. The treatment with cycloheximide alone led to a slower IgG clearance, with an IgG signal half-life of 1.88 hours. This decrease in cellular processing indicates that a general inhibition of protein synthesis by cycloheximide also affects the processing of IgG in a negative way. For this reason, we decided to rely solely on the inhibitory effect of glucose to specifically repress only IgG expression, as we were interested in analyzing the processing of IgG with least unspecific interference.

4.2.8. Cellular clearance of ERAD and UPR mutant strains

In the current model of glycoprotein processing in the yeast ER, the glycoprotein can either reach its native state quickly, followed by transport out of the ER towards the Golgi

apparatus, or it can stay in an intermediate folding state in the ER indefinitely, or it can be misfolded and degraded via the ERAD pathway. Since it has been proposed that also slowly folding proteins can become targets for ERAD degradation, we decided to study if the found differences in productivity were correlated to differences in clearance speed from the ER. In the analysis we worked under the assumption that no differences affecting IgG secretion exist between the strains once the IgG has been successfully folded. This made it possible to assume that changes observed in the IgG clearance speed between the different strains were a result of a change in the rate of ERAD mediated protein degradation.

First, the full set of 14 strains used in the ERAD studies was analyzed for differences in cellular clearance. Blots from five selected strains, together with a representative anti-tubulin blot, are shown in Figure 17. Also here, all strains kept producing IgG during the experiment in the mock treatment. In the wild-type IgG levels decreased quickly and no signals were detected anymore after 6 hours. While in general in the strains not shown in Figure 17 followed the trend of the background strains, the *Δubc7, Δhtm1Δire1,* and *Δyos9Δire1* strains showed a slower decrease of IgG signals, indicating that IgG processing was retarded.

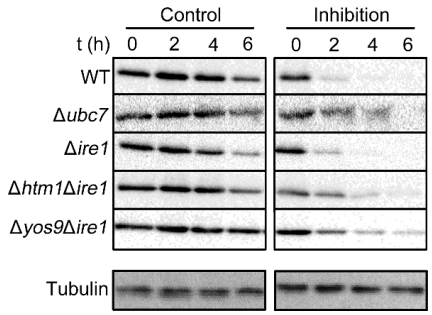


Figure 17 **Analysis of IgG clearance of ERAD deletion strains using Western Blot**. Antibody expression was induced in exponentially growing wild-type yeast cultures with galactose for four hours. After which glucose was added to inhibit IgG expression, zero, two, four, and six hours later cell extracts were prepared from the cultures and analyzed using SDS-PAGE and western blot for intracellular IgG content. A representative blot for tubulin, used as loading, is shown for control and inhibition.

In order to be able to distinguish differences in clearance between strains more clearly clearance curves were prepared. For this, the intensities of the IgG signals on the western blot were quantified and normalized for loading differences using their respective tubulin signals. After this, band intensities were set to be 100 % at time point zero to create the clearance curves shown in Figure 18A and 18B.

In general, the single deletion strains in Figure 18A showed a higher variability compared to the double deletion strains in Figure 18B. The wild-type strain showed in all cases the fastest decrease of IgG levels from the cell extracts, and for the *Δire1* background strain a highly similar clearance was observed. Additionally, from the strains with an additional deletion the *Δhtm1, Δire1, Δhrd1Δire1, Δhrd3Δire1,* and *Δalg3Δire1* strains showed similar clearance kinetics to the background strains. On average, each of these strains had only 15 to 25 % of the IgG signal left after two hours, decreasing further to approximately 5% after four hours, and finally only negligible signals below 2 % were left after six hours.

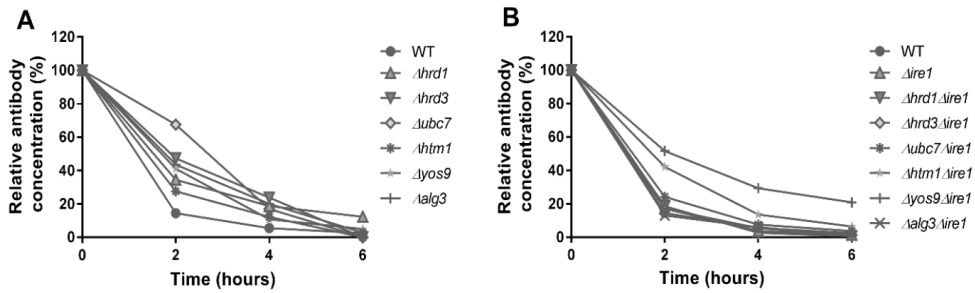


Figure 18 **Quantitative analysis of IgG clearance.** Cultures of strains in the wild-type background (A) and the *IRE1*-deletion background were analyzed for their cellular clearance of IgG. Western blots of cell extracts from the cellular clearance assay were stained with anti-IgG and anti-tubulin antibody (Figure 17), after which Image Lab software was used for quantification of IgG and tubulin signal levels. Data represents the average of two blots normalized for loading differences using the tubulin signals, % is relative to the normalized IgG signal at zero hours.

In the set of single deletion strains, the *Δhrd1* and *Δubc7* strains showed clearance patterns that were different from the other strains. For the *Δhrd1* strain, clearance started off similar to the rest, but in the final time point still 12 % of the IgG was left, which was more than double the amount of the average of the other strains. Deletion of *UBC7* led to an initial decrease in clearance speed, as after two hours into the clearance assay, still 68 % of the IgG was left. However, after six hours the relative cellular load became similar to the other strains. Interestingly, in the *Δubc7Δire1* strain only a slight initial decrease in clearance speed was observed. Both of these proteins are part of the HRD-ligase complex and for both deletion of *UBC7* and *HRD1*, a delayed degradation of ERAD substrates was found earlier [122, 142, 148]. Additionally, in a *Δhrd1* strain an ERAD substrate was shown to be retained at increased concentrations in the ER [309], which might contribute to the increased amount of IgG left at the end of the clearance assay in this strain.

The strongest delay in clearance was observed in the *Δyos9Δire1* strain, as this strains failed to deplete the cells of all the IgG within the six hours after the glucose induced inhibition of IgG expression and had 21 % of the IgG left. For the *Δhtm1Δire1* a similar slow initial clearance was observed (after two hours there was 42 % left, compared with 51 % for the *Δyos9Δire1* strain), but after six hours the strain had only 6 % left of the initial IgG amount, just slightly higher than the other strains in the *IRE1* deletion background. Htm1p and Yos9p function in the early stage of ERAD and are responsible for generation and recognition of the ERAD-glycan signal, respectively. And deletion of these proteins might diminish the efficiency ERAD targeting of substrates, leading to a prolonged ER residence time. However, Yos9p is known to also be able to slowly recognize unfolded proteins without the correct glycan signal, based solely on hydrophobic patches and it can target them for retrotranslocation and degradation [142]. This alternative slow way to recognize unfolded proteins was supported by a decreased clearance speed for this strain, which still reached similar low IgG levels in the end of the assay compared to the other strains. When looking at the titers, we saw that in the strain unable to mount the UPR (*Δhtm1Δire1*) to support protein folding, IgG secretion titers were decreased compared to the *Δhtm1* strain. In contrast, removal of Yos9p removed the main targeting step for retrotranslocation and thus increased the ER residence time of ERAD substrates. The reduced clearance speed that we observed for the IgG molecule had also already been reported earlier for artificial ERAD substrates [139, 141].

4.2.9. Cellular clearance of chaperone strains

After the analysis of the various ERAD deletion strains showed differences in IgG clearance, we decided to evaluate if our strains with a modified folding environment also show differences in ER-clearance. For this, we made a selection of five strains: first, wt and *Δopi1*, representing both strain backgrounds used in that part of the study. Next, we selected the highest producers from both backgrounds: wt with the *CPR5*-P_{KAR2} plasmid, and *Δopi1* with the *CPR5*-P_{GPD} plasmid. And finally, we selected one of the strains that negatively affected productivity, the *Δopi1* strain carrying the *KAR2*-P_{GAL1} and the *LHS1*-P_{GAL1} plasmids.

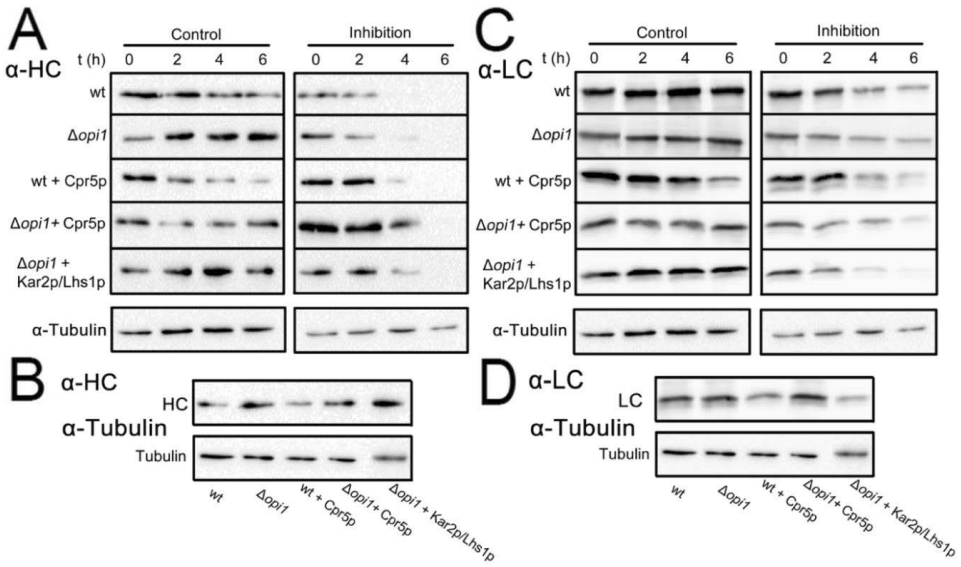


Figure 19 **Analysis of IgG clearance in strains with a modified ER environment using western blot.** Antibody expression was induced in exponentially growing wild-type yeast cultures with galactose for four hours. After which glucose was added to inhibit IgG expression, zero, two, four, and six hours later cell extracts were prepared from the cultures and analyzed using SDS-PAGE and Western blot for intracellular HC (A) and LC (C) content. A representative blot for tubulin, used as loading, is shown. Samples from time point zero of all strains were loaded on one gel, and analyzed for their intracellular HC (B) and LC (D) content, with their respective tubulin signals shown as loading controls.

The experiments were performed as described above, with two additions in the analytical part. First, to get a comparison of the initial relative intracellular IgG load between the strains, blots were prepared that contained the cell extracts from each strain at time point zero of the clearance assay. Second, all the samples were analyzed also for light chain content, in addition to the heavy chain and tubulin blots. Four sets of the blots are shown in Figure 19. The control blots showed that in the mock treatment all strains kept producing both HC and LC, while in the samples from the glucose repressed cultures, LC and HC content decreased during the assay. No HC signal was left after 6 hours, while most of the samples still showed residual amounts of LC at that time point.

The clearance curves were calculated based on the HC and LC signal strengths from the blots after correction for loading with the respective tubulin signals and are shown in Figure 20. A first clear observation was that HC and LC follow a different clearance pattern from each other. This could be expected as the heavy chain needs the light chain in order to complete

its folding process and to be secreted as the full-length IgG molecule. In contrast, the light chain can be secreted as a separate entity as soon as it is folded into its native form [201].

The comparison of the relative load of HC and LC from all strains (Figure 19B and 19D, respectively), showed a notable increase of intracellular HC in the *Δopi1* strains compared to the wt strains, which was accompanied by a slightly delayed clearance. The *Δopi1* strain had an over 75 % higher initial HC load compared to the wt strain and the selected highest producer in the *Δopi1* background had more than two times the HC load of the selected best producing wt strain. In CHO cells it was shown that a high intracellular content of HC can be considered a molecular marker characterizing efficient producing clones [310]. In the same study LC content in the cells was shown to be a less significant molecular marker, while for heterohybridoma cells intracellular LC levels had a very strong correlation with strain productivity [311]. Interestingly, in our strains the differences in initial LC load were relatively small, but the *Δopi1* derived strains showed a delayed clearance and higher residual LC amounts, compared with the respective wt strains. This could indicate that the availability of an excess of LC is favorable for the successful assembly and secretion of full-length IgG molecules.

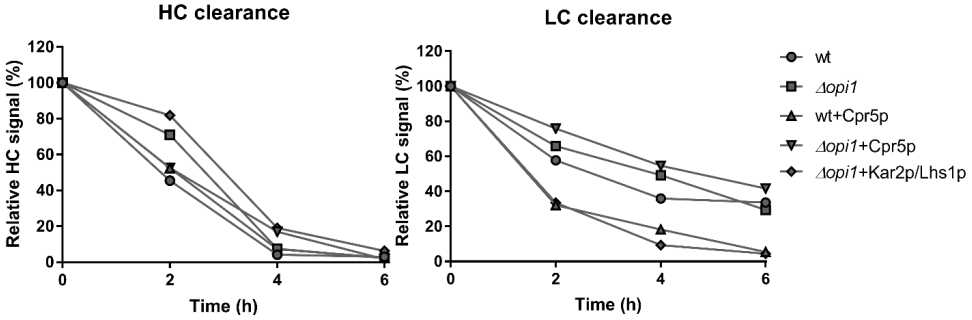


Figure 20 **Quantitative analysis of HC (A) and LC (B) clearance**. Blots of cell extracts from the cellular clearance assay were stained with anti-HC, anti-LC, and anti-tubulin antibody (Figure 19), after which Image Lab software was used for quantification of HC, LC and tubulin signal levels. Data represents the average of two or three blots normalized for loading differences using the tubulin signals, % is relative to the normalized IgG signal at zero hours.

The Δopi1 strain with the *KAR2*-P_{GAL1} and the *LHS1*-P_{GAL1} plasmids was included to see if the decreased IgG secretion titer phenotype was reflected in a clearance phenotype. The strain showed a relatively low initial load of HC and LC, even after correction for loading differences. For the clearance, it showed a decreased clearance of HC and an increased clearance of LC compared to the other *Δopi1* background strains. This indicates that a slow clearance of LC can be beneficial for secretion, while the observed fast clearance might hamper it. The decreased clearance of HC can be a result of the high level overexpression of Kar2p, as it is known to bind tightly to HC and has difficulties releasing it [312]. An overflow of Kar2p might prevent the LC from associating with the HC and inadvertently promoting the secretion of separate LC molecules.

This data suggest that the availability of HC and LC is important for efficient full-length IgG secretion and that distortion of this balance might decrease efficiency of the secretory system. Expression of chaperones can increase the speed of protein folding in a crucial way to improve secretion titers, but they can be the cause of unpredictable effects as their

function might be subunit specific. This can disturb the beneficial balance between the HC and LC availability, leading to a decrease of secretion titers.

4.3. Development of a randomized functional genomics approach to screen novel genetic targets

Even though our targeted approach was shown to be effective in increasing the secretion of full-length IgG molecules from our yeast strains, we realized that this approach will become limiting on the long run. This comes from the fact that every genetic engineering target chosen requires knowledge about its relation with the protein for which the secretion needs to be increased. This is further limited by the point that, even though over 80 % of the 6000 genes from *Saccharomyces cerevisiae* are characterized, still close to 20 % are without a known function [2]. For this reason, a good complementary approach to a targeted screen is to use a genome-wide approach to screen libraries of yeast strains for efficient producer strains. However, even though the effects of strain engineering are often target protein specific, a large part of screening methods use artificial product as a model protein. Examples are easily measured fluorescent proteins like GFP or luciferase [313], or enzymes like β-galactosidase or acid phosphatase [314]. Another used approach is the use of fusion proteins as model proteins, for example in yeast surface display [315, 316].

The ease of genetic manipulation of *S. cerevisiae* permits to straightforwardly screen genome-wide libraries for proteins affecting secretory capacity. Therefore, we screened a transposon mediated mutagenized yeast deletion strain library for the secretion levels of the full length human IgG antibody. Interesting clones were contrasted for expression of a heterologous protein and an endogenous protein by measuring IgG and acid phosphatase secretion, respectively. After this, the genetic disruption caused by the transposon was determined. Finally, targeted deletion strains were made of two of the identified transposon insertions sites to confirm the expression phenotypes.

4.3.1. Establishing strains and methods for library screening

Generally, screening procedures show a bias towards the (model) protein used for screening, to overcome this limitation, we sought to create a screening method using the full-length IgG molecule to increases the relevance of the results. First, we assessed the background biological variability of the screening procedure, in order to be able to determine what could be considered suitable hits for further analysis, with a deviation far enough from the average of the library clones expression level. For this, we grew replicate cultures of the parental W303α strain, instead of using different transposon mutated transformants from the screening library. The parental strain, containing the galactose inducible IgG expression plasmid, was inoculated into 72 wells of a deep-well plate, and IgG titers were measured using automated ELISA after 24 hours of expression. On average the clones produced 34.8 ± 7.50 μg/L, with a calculated Z-score over the plate of $3.17*10^{-5}$, where most variation was caused by only a few replicates deviating further from the average. This data confirmed that the variations coming from sample handling and small variations in growth conditions over the plate were small enough to justify the use of this parental strain background and cultivation setup for the screening method.

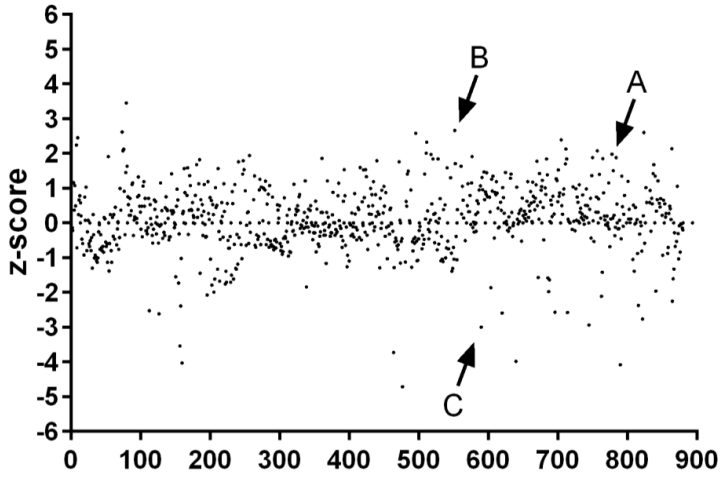


Figure 21 **Z-score overview of first 900 clones analyzed for IgG expression**. IgG titers of the transformants were determined using an ELISA assay after 24 hours of induction. The IgG expression of the transformants showed a normal contribution and hits were selected based on a Z-score ≥ ± 2.0. The clones A, B, and C, selected for further analysis, are marked with an arrow.

The large scale mutagenesis of the yeast genome was done using a bacterial transposon plasmid library created and provided by Kumar and colleagues [317]. They estimated that this insertion library contains in total over 300,000 independent insertions, corresponding to 50 genome equivalents of DNA. Their screening of the transposon library showed it to be unbiased in selection of genomic targets, and it was successfully used in a screening for a genome-wide function analysis [317].

For our library screen, the obtained Tn7 mutagenized yeast insertional library was propagated in *E. coli*, and the plasmids were isolated using a midi prep kit. The purified plasmid was digested with *Not*I to release the transposon mutagenized yeast genomic DNA. After purification of the linearized DNA fragments they were transformed into competent W303α yeast cells that carried a plasmid for the galactose inducible expression of the full-length human IgG molecule. We picked 900 transformants for IgG expression cultures in deep-well plates. After calculations of Z-scores for each transformant in their respective expression plates, so that all transformants can be compared irrespective of the plate they were grown in (Figure 21), we defined a Z-score of 2.0 as the minimum criteria for clones to be included for confirmation analysis. Eighty transformants were reanalyzed to confirm the primary screening results. For the first round of verification of the screening setup we included clones both with an increased, and with a decreased IgG titer, among them also the strains as A, B, and C, which were later characterized more in-depth (Figure 21).

4.3.2. Characterization of selected hits

Clones A, B, and C, that were selected from the initial screen based on their expression phenotype, were complemented with four randomly picked strains, without a clear phenotype in the primary screen, to serve as controls. In the secondary round of screening, the strains were analyzed for growth, expression of IgG, and expression of a yeast endogenous acid phosphatase (AP).

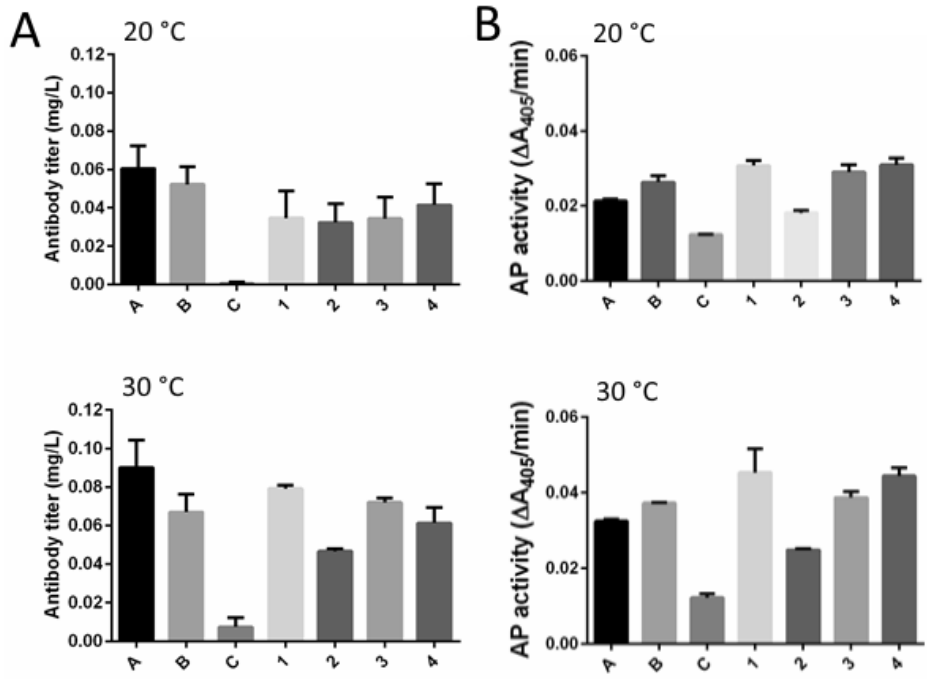


Figure 22 **Secretion of IgG (A) and AP activity (B) of candidate and control strains**. Strains were grown at 20 (top) or 30 °C (bottom) and protein expression was induced with 0.5 % galactose for 24 hours. Data represents the average and standard deviation of two to four experiments with four replicate cultures.

To evaluate the expression phenotype, all seven strains were transformed with the IgG expression plasmid or the yeast AP expression plasmid. All strains were grown in the 96 deep-well plate format, with either 0.5 or 2.0 % galactose to induce protein production, and grown at either 20, or 30 °C. Samples were taken 24 hours after induction to measure IgG titers and AP activity from clarified supernatants. The data from 0.5 % galactose induction at 20 and 30 °C is shown in Figure 22, the other data can be found in publication III, figures 3 and 4.

In general, the secondary screen confirmed the IgG expression phenotypes of the primary screen. Clone A produced the highest IgG titers, followed by clone B, while the absence of IgG production in clone C was confirmed under all tested conditions (Figure 22A). The increased production in strains A and B was most prominent at the lower temperature, compared with the four control strains, with lower differences at 30 °C. Remarkably, strain C did show production of AP, even though it was strongly reduced compared to all the other strains. This indicates that production of endogenous protein was less affected than heterologous protein production in this strain. For strains A and B, there was no increase in AP activity observed compared to the 4 control strains.

4.3.3. Identification and verification of gene disruptions

Genomic DNA was isolated from strains A, B, and C for identification of the genomic integration sites using vectorette PCR. The found genomic integration sites are summarized in Table 5, and in Figure 23 a schematic overview of the integration sites, together with the surrounding loci, is shown. In clone A, the transposon was integrated into the ORF of *VPS30*,

in clone B the integration site was in the ORF of *TAR1*, and finally in clone C the integration site was located just upstream of the *HEM13* ORF. Among the identified genes, the biological functions of *VPS30* and *HEM13* are relatively well characterized, but the role of *TAR1* is not well established yet.

Table 5 **Genomic integrations of clones A, B, and C from screening**

Strain	Disrupted gene[(a)]	Function[(a)]
Clone A	*VPS30/YPL120W*	Subunit of the phosphatidylinositol 3 kinase complexes I and II, required for overflow degradation of misfolded proteins when ERAD is saturated.
Clone B	*TAR1/YLR154W-C*	Protein potentially involved in regulation of respiratory metabolism, located in the mitochondria
Clone C	*HEM13/YDR044W*	Coproporphyrinogen III oxidases, oxygen requiring enzyme that catalyzes the sixth step in the heme biosynthetic pathway

[(a)]Annotation from the *Saccharomyces* Genome Database

In order to completely verify the relationship between the disruption of the genes caused by the insertion of transposon and the observed expression phenotypes, yeast strains with clean deletion of the found ORF´s were prepared. From these, deletion of *HEM13* proved to be problematic, as no viable colonies were found after transformation of the knockout cassette in four commonly used laboratory strains (W303, SS328, CEN.PK113-7D, and BJ3500). Literature revealed that previously *HEM13* deletion strains displayed very slow growth at various temperatures, and are often not viable at all [318, 319]. However, mutations and partial deletions were viable with less severe growth defects [320], indicating a partial inactivation of *HEM13* expression in strain C. One other option was shown to be the addition of heme into the media in order to compensate for the disruption of the heme biosynthetic pathway caused by *HEM13* deletion [320]. Due to the combination of an unfavorable expression phenotype together with the, for biotechnological applications, undesirable need for specific media additions, we decided to exclude the *HEM13* deletion from further analysis.

Together with their parental W303α wild type strain, the *Δvps30* and *Δtar1* strains were transformed with the galactose inducible IgG and AP expression plasmids. IgG titers and AP activities were determined after 24 hours of protein expression at 30 °C, in deep-well plate and shake flask format (Figure 24). The data showed that both in plate and shake flask cultures the strains behaved similarly with respect to their expression phenotypes. With respect to IgG secretion titers, the *Δvps30* strain had a significantly increased specific product yield that increased on average by 100 % with respect to the wild type. In contrast, in the *Δtar1* strain only a slight increase was observed in shake flasks, but not in the plate cultivations. This strain showed however the highest increase in AP productivity of all three strains, although the *Δvps30* was also significantly improved with respect to the wild-type background.

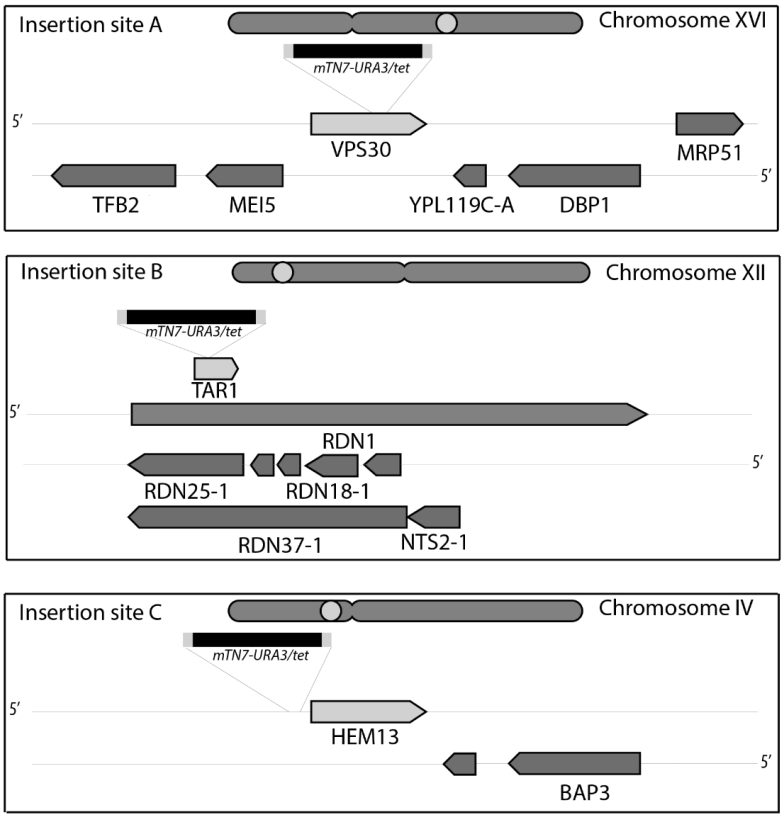


Figure 23 **Characterization of the integration sites of the transposon from strain A, B, and C in the yeast genomic DNA**. Vectorette PCR was used with digested yeast genomic DNA to amplify the region around the inserted transposon. The exact integration site was found using BLAST on the sequence data, the surrounding genetic loci are shown in addition.

This data shows that the screening setup did what we designed it for, to find genes that improve the secretion of IgG. The contrasting phenotypes we found between IgG and AP expression show the importance of contrasting the secretion levels of different proteins in order to get a more general idea about the nature of the effect of a gene deletion on protein secretion. In some cases knowledge about the functionality of the delete genes can provide more information about the underlying mechanism that improve the protein secretion. However, for the deletion of *TAR1* it was difficult to indicate how it causes changes in protein secretion, as not much is known about the exact function of its protein product. The gene is located on the anti-sense strand of the multi-copy 25S rRNA gene, and Tar1p was shown to localize to the mitochondria [321, 322]. The protein seems to have a role in maintaining the oxidative phosphorylation capacity of the yeast cell under respiratory conditions [323]. However, it is unclear how its disruption is connected to the improved secretion of AP.

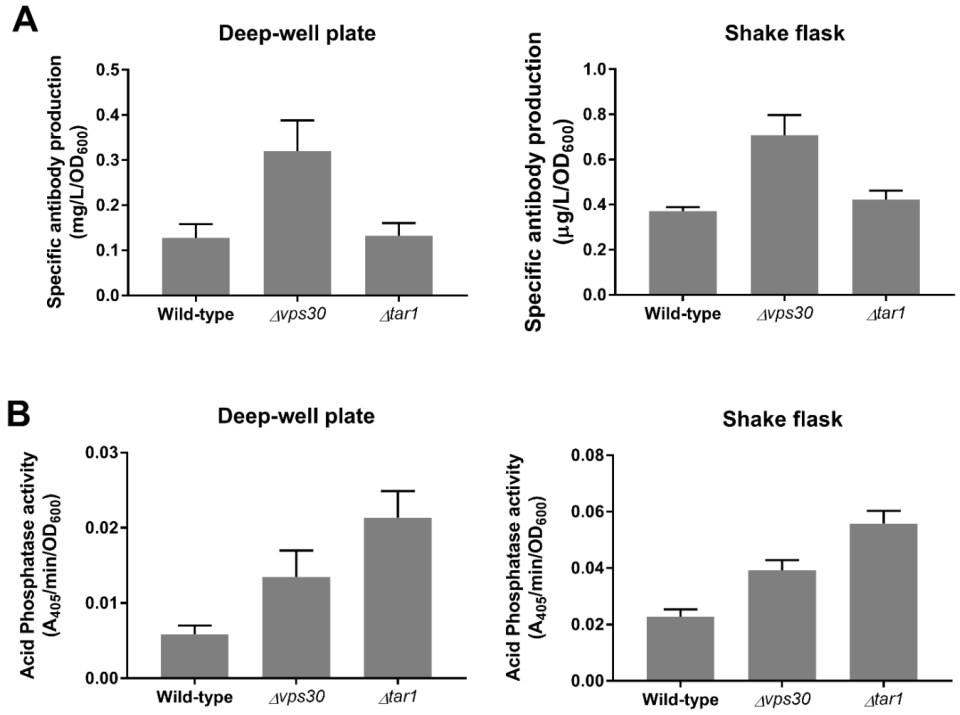


Figure 24 **IgG and AP production of clean knockouts of selected strains from the library screening**. Protein expression was induced for 24 hours with 0.5 % galactose, both in deep-well plates as well as in shake flask cultivation. The cultivations were followed by determination of IgG titers using ELISA or AP activity using a colorimetric assay. Data represents the averages and standard deviations from five to eight biological replicates.

VPS30 on the other hand has been well documented, the gene encodes a protein that is part of the vacuolar protein sorting receptor family. Vps30p was shown to be involved in the CPY to vacuole protein sorting pathway, and mutations in and deletion of the gene showed to lead to missorting of the vacuolar carboxypeptidase Y (CPY), followed by its secretion into the extracellular space [324]. It showed that its CPY expression phenotype was indirectly caused by an impaired recycling of Vps10p. The Vps10p protein is the cargo receptor for CPY to the early endosome, and it is normally recycled back to the Golgi apparatus in a Vps30p mediated process. However, when Vps30p is removed from the process, Vps10p accumulates in the endosome, and is eventually transferred to the vacuole. The CPY that is now accumulating in the Golgi apparatus will then eventually be secreted [324]. It was shown that several heterologous proteins are targets of this CPY to vacuole pathway, leading to unwanted degradation, and giving an indication of how IgG titers are positively affected by *VPS30* deletion [54, 187, 188, 325]. Later, it was shown that this degradation pathway is a back-up for ERAD targets that escape ER degradation. Kruse and colleagues showed that the ERAD target A1PiZ (the Z variant of the human α-1 proteinase inhibitor) is escaping vacuolar degradation when the *VPS30* gene is inactivated [326]. In addition, another effect of *VPS30* deletion was a low constitutive activation of the UPR, which reached levels comparable to wild-type cells under tunicamycin stress [326]. This low level activation of the UPR before stress from the IgG expression is induced might aid the cells to be able to more efficiently secrete the IgG molecules.

4.3.4. Differences between the screening rounds

In general, the expression phenotypes of the Δ*vps30* and *Δtar1* strains were more distinct in the final strain characterization, than in the secondary screen. This is related to the point that in the final screen the deletion strains were compared with the laboratory wild-type background strain, which contained no further modifications than bearing the IgG expression plasmid. However, the four transformants selected as controls from the primary screen contained insertions of the transposon mutagenesis library. Although they did not exhibit a clear expression phenotype, it is still possible that they were secreting IgG slightly more efficient than the W303 wild type.

The expression of IgG and AP for the Δ*vps30* and *Δtar1* strains was studied in deep-well plates and in shake flasks. No significant differences in the relative titer increases compared with the wild type were observed between both cultivation systems. This indicates that the positive effects of the deletions are transferable to the slightly bigger production scale in the shake flask. As the cultivation conditions between deep-well plate and shake flasks are quite different it is important to consider if the deletion phenotypes are preserved in the different growth formats. Through a significant increase in specific IgG yield and specific AP activity it was clear that the shake flask provided relatively better growth conditions, with better shaking providing better aeration and mixing of media components.

4.4. Gaining insight in the metabolic burden of heterologous protein production

In many studies it has been observed that recombinant protein production in microbial hosts leads to significant changes in the cell. For example, recombinant protein production is prone to activate the UPR if the load of unfolded proteins in the ER becomes too high [122]. The UPR is known to regulate close to 400 genes [117], meaning that the transcriptome and proteome of the cells changes significantly. For example, chaperones and folding factors were found to be upregulated [293]. Amongst the changes triggered by protein overexpression, are also alterations at the metabolome level, and it is known that recombinant protein production is a significant metabolic burden to the host cell. This burden originates from a redirection of resources from normal cellular processes towards the production of the recombinant protein [327]. For *S. cerevisiae* this metabolic burden was shown to lead to reduction in the maximum specific growth rate, a decreased biomass yield, and a lower respiratory capacity [76, 303, 327–329]. Noting that many cellular processes are changed when recombinant protein production is induced, it is to be expected that a shift in the direction of cellular resources to accommodate elevated protein production also alters the levels of intracellular metabolites. So far, analysis of the effects of the metabolic burden has been mostly restricted to parameters like growth rate, biomass yield, carbon source consumption, and byproduct formation [328, 330]. Modeling methods have provided insights in some of the metabolic flux distributions in particular in the central carbon metabolism under recombinant protein production conditions [302, 331]. However, the changes in intracellular metabolite levels caused by recombinant protein production have often been neglected, even though they could provide possible new leads for metabolic engineering of production strains.

4.4.1. Changes in growth characteristics for yeast strains expressing different antibody variants

We observed differences in growth characteristics during cultivations of strains producing a scFv fragment, a scFv-Fc fusion protein, and a full-length IgG molecule, the respective recombinant strains are from here on referred to as scFv strain, Fusion strain, and IgG strain. In order to quantify the growth changes caused by the induction of antibody production we recorded growth curves. Without protein production no clear differences in growth patterns were observed (Figure 25A). And even though the differences between the μ_{max} values were statistically significant, they were rather small. However, when galactose was added to the media to induce protein production, all expression strains showed a lag period, followed by a decreased growth rate compared to the wild type control (Figure 25B and Table 6). Interestingly, the μ_{max} values decreased more when the size of the expressed antibody variant increased, down to a μ_{max} of only half of the wild-type control for the IgG strain (Table 6). The three proteins produced differ in the amount of Ig-fold domains, 2 for scFv, 8 for the scFv-Fc fusion protein, and 12 for the full-length IgG. The Ig-fold is not natively found in yeast, and that contributes to the slow folding and maturation of the antibody molecules. It is noteworthy to mention, that protein folding was proposed to be the most energy consuming component of the yeast secretory machinery [78], and the lengthened folding process for the larger antibody molecules might contribute to the increased metabolic burden observed in these strains.

Table 6 **Growth rates of expression strains**

Strain	Antibody (fragment)	Size	Ig folds	μ_{max}[(a)] (raffinose)	μ_{max}[(a)] (raffinose + galactose)
WT	WT control	N/A	N/A	0.161±0.00143	0.095±0.000958
ScFv	ScFv fragment	26.1 kDa	2	0.151±0.000852*	0.087±0.000715**
Fusion	ScFv-Fc fusion	106.0 kDa	8	0.152±0.000663*	0.060±0.000409***
IgG	Full IgG	144.5 kDa	12	0.148±0.00066*	0.048±0.000534***

(a) *P<0.05; **P<0.01; *** P<0.001 (versus WT in the same condition. By Student's two-tailed t test)

4.4.2. Expression of antibody variants leads to distinct intracellular metabolic profiles

Seeing the large differences in growth rates when protein production was induced, we wondered if these changes were reflected in changes in intracellular metabolite levels in the recombinant strains. To analyze the intracellular metabolite profiles of the different expression strains, we used a targeted (semi) quantitative mass spectrometry based metabolomics method to measure the concentrations of 76 metabolites from the wild-type, and from the three recombinant strains under protein expression inducing conditions.

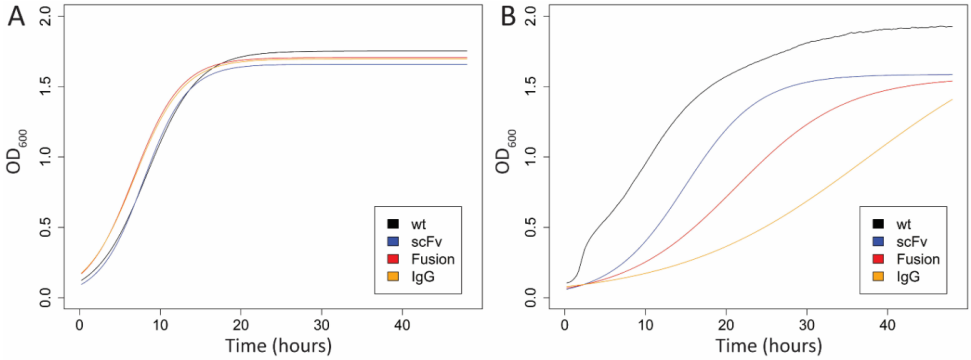


Figure 25 **Growth curves of strains expressing different antibody variants.** Cultures with exponentially growing cells were diluted to OD_{600}=0.02 in fresh media without (A), or with galactose (B) to induce protein expression, and grown in microtiter plates for 48 hours at 30 °C with continuous shaking. OD_{600} was measured every 30 minutes and the data is here represented by a logistic growth curve fitted to the average of four replicates per curve, except for wt in (B), because the curve was not applicable with the model.

Unsupervised multivariate analysis, Principal Component Analysis (PCA), showed that the four strains formed distinct populations based on the metabolite profiles. The scFv and IgG strains were more similar to each other than to the Fusion strain or to the wild-type (Figure 26A). Supervised multivariate analysis, Partial Least Squares Discriminant Analysis (PLSDA), identified the top variables contributing to the group separation, and Figure 26B-D shows an overview of the 20 metabolites most contributing to the differences between the recombinant strains and the wild-type. The metabolites that were determined to be contributing most to the separations between the recombinant strains and the wild-type were predominantly involved in amino acid metabolism (for example ornithine, proline, and arginine) and redox metabolism (for example GABA, glutathione, and succinate).

4.4.3. Recombinant protein production alters the pool of free amino acids

The expression of the antibody variants induced changes in concentrations of all amino acids that were measured. We sought for a possible correlation between differences in the amino acid composition of the expressed antibody variants with the changes in amino acid levels in the recombinant strains. For this, we compared the amino acid compositions of the antibody variants, with the average amino acid composition of *S. cerevisiae* that was published by Karlin et al. [332]. The main differences were found to be a decrease of arginine, asparagine, and aspartate, and an increase of serine, threonine, and valine in the recombinant proteins (Figure 27A).

Next, we calculated the log2 fold-changes of the pool of intracellular free amino acids in recombinant strains respective to the wild-type, and sought for possible correlations. We mainly observed a decrease in concentration in the strains expressing the antibody variants (for 13 out of 18 amino acids). The changes ranged from an over two fold increase in concentration for methionine and alanine to an over threefold decrease for tyrosine and arginine (Figure 27B). However, no clear correlation was observed between the changes in free amino acid levels and the differences in amino acid composition of the expressed proteins compared with wild-type or relative to each other. The large changes in amino acid levels suggest that it could be a target to improve production titers. Although genetic

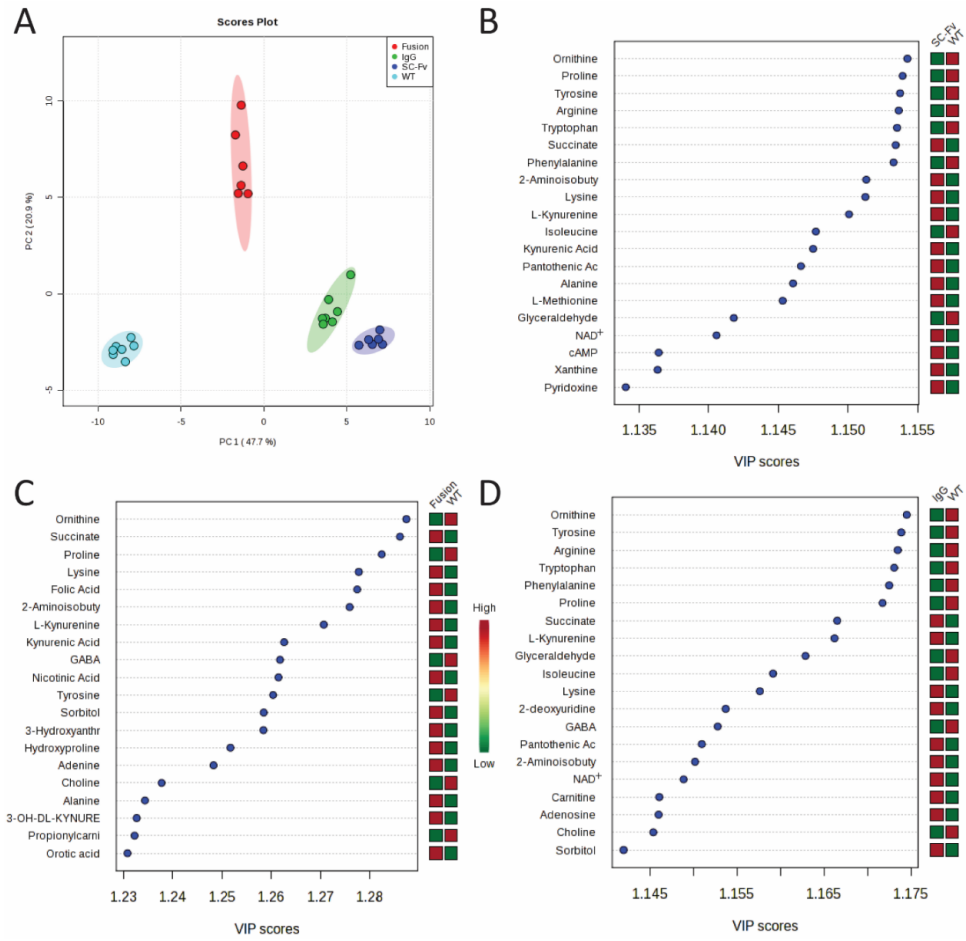


Figure 26 **Recombinant protein expression inflicts changes on the metabolome**. (A) PCA scores plot from all metabolomics samples of the four different strains, showing a clear clustering within samples from the same strain as a separation between samples from the different strains. (B-D) The top 20 metabolites are ranked for their contribution in group separation in the PLSDA-VIP plots, comparing the scFv strain (B), the Fusion strain (C), and the IgG strain (D) with the wild-type.

engineering of metabolic pathways is expected to be complex, as many amino acids also act as precursors for different metabolic pathways, supplementation of certain combinations of amino acids to culture medium was proven to be successful to increase the production of a recombinant xylanases [333].

4.4.4. Recombinant protein production has an impact on metabolites involved in redox metabolism

The production of recombinant antibodies comes with the need for formation of disulfide bonds so that the proteins can reach their native folding state. This process consumes reduced glutathione when disulfide bonds are broken, and that can lead to oxidative stress in the ER [132]. The concentration of glutathione was decreased 0.94-, 0.62-, and 1.39-fold in the scFv, the Fusion, and the IgG strain, respectively. Glutathione was also reported to

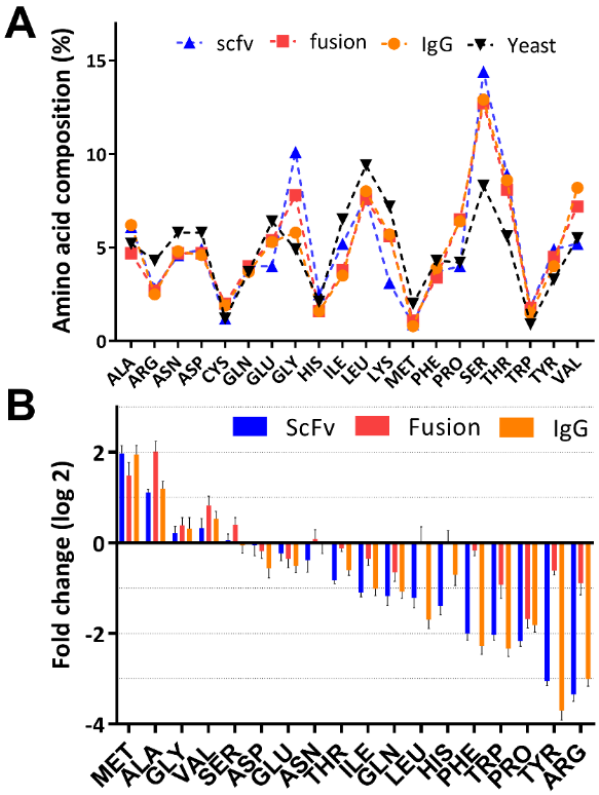


Figure 27 **Amino acid composition analysis.** (A) Overview of amino acid composition of the expressed antibody variants and an average amino acid composition of yeast proteins. (B) Fold changes of concentrations of amino acids relative to wild-type after induction of expression of the respective antibody variants.

be able to buffer oxidative stress in the ER [334], so the decrease in glutathione we observed suggests that the production of the antibody fragments disturbed the redox balance in the ER

In all recombinant strains GABA showed a decrease in concentrations when compared with wild-type, and that decrease became stronger with an increase of the size of the antibody variant. Also, GABA was one of the determining metabolites contributing to the group separation in all strains (Figure 26B-D). The metabolite was decreased 3.55-fold in the scFv strain, 4.42-fold in the Fusion strain, and 4.84-fold in the IgG strain. It was shown that GABA can play a role in oxidative stress tolerance by acting as a buffer for cellular redox changes [335], this happens through a conversion from tryptophan to GABA, followed by a conversion to succinate. In our data we found that the tryptophan concentrations were decreased up to 2.33-fold, and the succinate concentrations were increased up to 4-fold in the recombinant strains. This indicates a possible increased flux through this metabolic pathway to buffer the changes in the cellular redox state resulting from recombinant antibody production.

NAD^+ is a key player in electron transport in many cellular reactions, the metabolite is either synthesized by nicotinate scavenging, or through *de novo* biosynthesis from tryptophan [336]. The concentration of NAD^+ was increased 1.41-, 1.01-, and 0.88-fold in the scFv, the Fusion, and the IgG strains, respectively. The scavenging pathway utilizes nicotinic acid for NAD^+ regeneration, nicotinic acid was increased 3.19-, 1.15-, and 1.91- fold in the scFv, the Fusion, and the IgG strain, respectively. For the *de novo* biosynthesis pathway, the amino acid tryptophan was one of the amino acids with the most decreased levels. In contrast, downstream from tryptophan in the metabolic pathway the metabolites L-kynurenine and 3-hydroxyanthranilic acid showed increased concentrations. For L-kynurenine this was 2.51-, 4.06-, and 2.55- fold, and for 3-hydroxyanthranilic acid this was 0.47-, 1.46-, and 0.19-fold, both in the scFv, the Fusion, and the IgG strain, respectively.

4.4.5. The cellular burden is connected to slow IgG folding and maturation

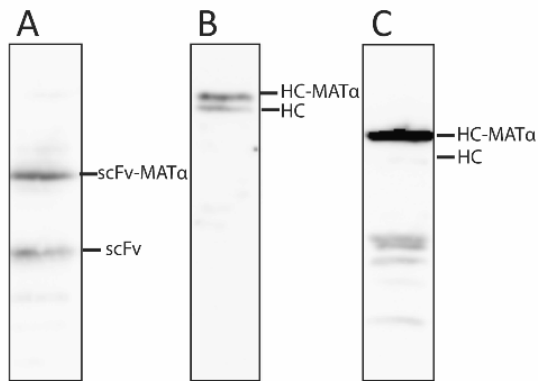


Figure 28 **Western blots of cell extracts from the antibody variant expressing strains.** After 18 hours of protein expression cell extracts were prepared from strains expressing the scFv fragment (A), the scFv-Fc fusion protein (B), and the full-length IgG molecule (c). The scFv fragment was probed using a mouse anti-tetra his antibody together with a rabbit anti-mouse peroxidase labeled antibody. The Fc portion of the scFv-Fc fusion protein and the full-length IgG were probed with a goat anti-human (Fc-specific)-peroxidase labelled antibody.

Western blot analysis of cell extracts from the expression cultures showed for all three recombinant strains the protein signals at the expected apparent molecular sizes, displaying the polypeptides with, and without the Matα propeptide (Figure 28). The presences of polypeptides with the Matα propeptide indicates that part of the polypeptides is accumulated in the ER and Golgi apparatus during the folding and protein maturation process. The proportion of unprocessed polypeptides was the highest in the IgG strain, suggesting that the folding process is slowest for the largest antibody variant with the most Ig-folds. The IgG strain also displayed the lowest maximum growth rate, indicating that the cellular burden of the slow folding process is significant. However, even though we found connections between the changes in metabolite levels and cellular processes involved in the recombinant protein production, the changes in maximum growth rates can only be partially explained by the metabolic burden, as induction of the UPR is expected to put an additional stress on the cells.

In this thesis, we showed that optimization of aspects of the secretory pathway, like protein folding and trafficking steps, increased the secretion efficiency of IgG. In our data, the overexpression of *CPR5* was most beneficial for IgG titers. The isomerization of certain

proline residues is one of the slowest steps in the folding of the IgG heavy chain fragment and this step is catalyzed by the Cpr5 peptidyl-prolyl isomerase [201, 202]. The optimization of one of the rate limiting steps in IgG folding reduces the negative effects of this bottlenecks in the secretory pathway and leads to a reduction in the cellular burden from recombinant protein production.

5. Conclusions and future prospects

In this thesis we sought to identify genetic factors that influence the secretion of a full-length IgG molecule from the yeast *Saccharomyces cerevisiae*. We found genes involved in protein folding, N-glycosylation, vacuolar sorting, and proteolytic degradation, that increased or decreased IgG production after their overexpression or deletion. An overview of all the genes studied and their functions, together with their effect on IgG secretion is shown in Table 7 and Table 8, for deletions and overexpressions, respectively.

By studying a selection of genes that are regulated by the UPR, and that affect the protein folding process in the ER we aimed to harness parts of the cells natural stress response to unfolded proteins to increase the folding efficiency of the IgG molecule. We increased the functional folding space of the ER through deletion of the *OPI1* gene, which showed to increase IgG titers by up to 4.8-fold. The overexpressed protein most beneficial for IgG titers in this study was the peptidyl-prolyl isomerase Cpr5p, with an increase of IgG titers of up to 3.2-fold. The overall best performing strain was the *Δopi1* strain with *CPR5* under control of P_{GPD}, this strain secreted over ten times more IgG than the wild type control strain.

Deletion of genes encoding components of protein quality control system ERAD did not negatively affect strain fitness of the strains under non-stressed conditions. However, of the strains tested only deletion of *HTM1* improved IgG titers, by 1.15-fold. Creation of a glycan-form that could possibly escape ERAD through deletion of *ALG3* also improved IgG titers, by 1.20-fold. In order to gain more insight in how the deletions affect the folding process in the ER a cellular clearance assay was developed. The assay allowed us to distinguish differences in the cellular clearance of IgG between some of the ERAD deletion strains.

We successfully developed a high throughput method for screening a transposon mediated yeast deletion strain library, and identified genes influencing IgG secretion. The data showed that clear IgG secretion phenotypes could be distinguished in the primary screen. After confirmation of the transposon insertion site of two selected transformants the resulting *Δvps30* and *Δtar1* strains showed to improve IgG secretion by up to 2.5-fold and up to 1.13-fold, respectively. The genes were also shown to improve the secretion of a control protein, the endogenous yeast acid phosphatase by up to 1.75-fold for *Δvps30*, and up to 2.5-fold for *Δtar1*. *VPS30* was a known target in strain engineering, but the gene *TAR1* was previously not connected with protein secretion.

Expression of a scFv, a scFv-Fc fusion antibody, and a full-length IgG was found to impose an increasing burden on *S. cerevisiae* that was inversely correlated to the growth rate with an increased size and complexity of the antibody variant. Using a targeted (semi) quantitative metabolomics approach we showed that all three production strains formed distinct populations based on the levels of selected metabolites. The most significant

changes were found to be related to metabolites involved in amino acid and redox metabolism.

Table 7 **Effects of gene deletions on specific IgG yield**

Deletion	Function	Fold change[(a)]
Δalg3	α-1,3-mannosyltransferase involved in the synthesis of the dolichol-linked oligosaccharide donor for N-glycosylation	1.21
Δcym1	Lysine specific metalloprotease of the mitochondrial intermembrane space	1.52
Δhtm1	ER α-1,2-exomannosidase, involved in the glycan trimming step of ERAD	1.15
Δhrd1	Ubiquitin-protein ligase, required for ERAD of misfolded proteins	0.62
Δhrd3	HRD-complex membrane protein with a central role in ERAD	0.69
Δire1	Transmembrane protein that mediates the UPR	0.07
Δopi1	Transcriptional regulator of phospholipid genes	4.8
Δpep4	Vacuolar proteinase A	1.58
Δprb1	Vacuolar proteinase B	1.12
Δpep4 Δprb1	Vacuolar proteinase A and B	1.14
Δtar1	Potentially involved in regulation of respiratory metabolism	1.13
Δubc7	Ubiquitin conjugating enzyme involved in ERAD	0.68
Δvps10	Vacuolar transmembrane sorting receptor	1.47
Δvps30	Subunit of PTdIns 3-kinase complexes I and II, involved in autophagy and vacuolar protein sorting	2.51
Δyos9	ER quality control lectin involved in ERAD	0.18

(a) Fold change of specific product yield relative to the respective wild type strain

Table 8 **Effects of gene overexpression on specific IgG yield**

Gene	Function	Promoter induced fold change[(a)]				
		P_{TEF}	P_{GPD}	P_{GAL1}	P_{KAR2}	P_{PDI1}
CPR5	ER peptidyl-prolyl cis-trans isomerase	1.66	1.78	1.77	**3.26**	2.16
ERO1	ER thiol oxidase	**1.37**	1.09	1.17	0.28	N.D.
KAR2	ER molecular chaperone	0.99	0.84	0.53	N.D.	**1.07**
LHS1	ER nucleotide exchange factor for Kar2p	1.05	**1.13**	0.97	1.13	N.D.
PDI1	ER protein disulfide isomerase	0.9	0.55	0.25	0.59	0.72
SEC1	Involved in docking and fusion of exocytic vesicles	0.88	0.82	0.96	N.D.	N.D.
SIL1	ER nucleotide exchange factor for Kar2p	N.D.	**1.51**	1.23	1.41	1.43
SLY1	Involved in ER/Golgi vesicle trafficking	**1.11**	0.96	0.91	N.D.	N.D.
SSO1	Plasma membrane t-SNARE	0.76	0.72	0.83	N.D.	N.D.

(a) Fold change of specific product yield relative to the respective wild type strain background for each of the promoters used. N.D. = not determined

5.1. Further targeted strain improvement

In this thesis we focused on improving the secretion of a full-length IgG molecule, and have identified genetic targets that do so. However, it would be very interesting to determine the universal applicability of these targets on the secretion of a broader range of recombinant proteins. In many studies it was observed that the strain modifications that improved secretion efficiency for one specific protein cannot be easily used to generate the same positive effect for the secretion of other recombinant proteins [51, 290]. The protein folding, processing, and secretory pathways form a complex network, and each of these needs to be specifically tuned to accommodate the characteristics of the target protein, for example protein size, glycosylation sites, disulfide bonds, hydrophobic elements, dimerization, amongst others. It has been shown that even small differences in amino acid sequences of a protein can have significant consequences for secretion levels [337]. In order to analyze this, additional recombinant proteins relevant for production in industrial processes need to be selected to analyze if the strain improvements found in this thesis are also beneficial for other classes of proteins.

Furthermore, in this thesis we have shown that mimicking selective parts of the UPR can be beneficial for improved IgG titers. In that respect, it could be interesting to further continue the research into the effect of the protein quality control system ERAD, as this was shown to contribute to degradation of up to 60 % of a Fab3H6 antibody fragment produced in *P. pastoris* [238, 239]. In this thesis, we focused merely on the impact of disrupting ERAD to avoid unwanted degradation, which led mostly to a decrease in IgG titers. However, there is a possibility that overexpression of selective ERAD parts could improve the quantity and quality of the produced IgG. Most interesting would be to increase the presence of components of the retrotranslocation pore complex, as an increased removal of ERAD substrates from the ER might increase the folding space for other proteins. The analysis of the influence of ERAD on product titers becomes especially interesting with the rise of glycoengineered strains for industrial biopharmaceutical production, as the change in glycan structure on the glycoproteins in these strains might alter their affinity for glycan dependent folding and degradation processes. An indication for the effect of glycan engineering was found in this thesis, where deletion of the *ALG3* gene increased IgG titers, and this is a deletion used in some of the glycan engineering strategies [252, 253].

Finally, in this thesis we found genetic targets at various parts of the secretory pathway that by themselves could significantly improve IgG secretion. There is a possibility that engineering one part of the secretory pathway not just improves protein secretion, but also creates a new bottleneck or moves the existing bottleneck to a new place in the secretory pathway. In that sense it would be interesting to create an IgG production strain that has been optimized for production at several stages of the secretory pathway, in order to see if modifications of different parts show a synergistic effect when they are combined. For example it would be interesting to see if the positive expression phenotype of modifying the ER environment by deletion of *OPI1* can be further improved by modification of a later step in the secretory pathway. The diminishing of vacuolar degradation through additional deletion of the *VPS30* gene we found in the randomized functional genomics approach would be an interesting candidate for this.

5.2. Deletion library screen improvements

For the yeast deletion strain library screening method it would be beneficial if the amount of information that is obtained in the first round of screening is increased. One option to achieve this would be simultaneous overexpression of a second target protein in the library strain, already in the first round of screening. This could be either a second heterologous protein in order to analyze the effect of the deletion on different classes of proteins [316]. Or, another option is the overexpression of an endogenous *S. cerevisiae* protein, like the yeast acid phosphatase, to characterize the effect on heterologous versus endogenous protein expression. In this thesis, we have analyzed the AP expression of selected clones in a secondary screening round. In initial experiments, the genetic cassette encoding the AP was integrated into the IgG expressing parental strain from the library screening, and this allowed us to analyze the expression phenotypes of IgG and AP simultaneously in the primary screening round (unpublished data). The enzyme's activity can be relatively easily measured in a high-throughput activity assay using a colorimetric substrate and allows distinguishing of different expression phenotypes of the library transformants when compared with the relative IgG expression levels.

There are also possibilities to include reporter assays that provide information about the state of cellular processes that influence protein secretion. After implementation of the AP expression, we also integrated the UPR-reporter construct (that has been used in publication II) into this double expression strain, and tested its suitability for identifying ER-stress phenotypes in the library transformants. This enabled the measurement of the fluorescence of GFP as a relative measure for how high the stress caused by unfolded proteins is (unpublished data). Another fluorescent readout option could be a reporter for the redox state of the ER [338].

When through combination of the GFP UPR-reporter with the IgG and AP expression a strain with a multiplex readout is created, reporting heterologous protein expression, endogenous protein expression, and induction strength of the UPR. This could enable a more versatile readout to analyze the effects of deletions in the background strain, allowing to select interesting clones based on more information.

One other possible approach to increase the use of the screening approach, would be to utilize an already optimized production strain as the parental strain for this mutagenesis screen, for example the *OPI1* deletion strain with an integrated cassette for the overexpression of the *CPR5* gene under control of the GPD promoter. In this way it would be possible to identify genes that can further improve an already significantly optimized secretory process.

5.3. Towards more versatile industrial yeast cell factories

When many genes of interest that improve secretion efficiency have been selected for overexpression, the burden their expression imposes on the cell has to be considered. For example, it has to be taken into account that for industrial scale production strains, the expression of multiple helper proteins in a plasmid based system is not feasible. This is due to possible variations in plasmid copy numbers and corresponding expression levels, and also due to an undesirable requirement for a constant multiplex selection pressure for the various plasmids. Furthermore, yeast strains tend to more easily lose their plasmids in larger scale cultivations due to long cultivation times, as dead cells provide amino acids to the

media after lysis, and due to use of rich or undefined media [339, 340].In general, stable integration into the yeast genome is a viable option for a limited number of genes, but the most versatile solution would be the construction of yeast artificial chromosomes (YAC). These synthetic chromosomes can accommodate DNA up to 800 kb providing capacity for many genetic elements to be expressed. YACs containing on average around 50 different heterologous expression cassettes were successfully used in the random assembly of biosynthetic pathways to produce various flavonoid compounds [14].

After extensive strain engineering, it can be expected that multiple genes, encoding proteins that increase the secretion efficiency of the target protein, have been selected for overexpression. When these are overexpressed at the same time a secure fine tuning of expression levels becomes paramount to avoid saturating the cell with the overexpressed proteins. Balancing of expression levels becomes especially important when the protein products interact with each other in a stoichiometric way. It is quite probable that native yeast promoters don't offer enough possibilities for adjustment of expression levels and a switch to synthetic promoters could be necessary. These rationally designed promoters often use DNA binding molecules from other organisms, together with different numbers of binding sequences in the promoter region to regulate expression levels [13].

Besides all strain engineering steps, also bioprocess engineering will play an important role in the development of *S. cerevisiae* as an antibody factory. To determine the current full production potential of *S. cerevisiae*, it would be interesting to use our IgG production strain with the highest titers, or a to be developed strain with a more extensive engineering of the secretory pathway, and analyze the maximum IgG titers that can be reached with bioprocess engineering. The experimental setups we used for IgG production were favorable for screening purposes, but had far from optimal growth conditions. Especially in the deep-well plates oxygen transfer is limiting and the batch-phase conditions might result in non-optimal carbon source concentrations when the cultivation time proceeds. To be able to reach the true potential of these *S. cerevisiae* strains as antibody factories, their expression cultivations would need to be scaled up to bioreactor systems. For example, a fed-batch bioreactor setup that allows full control over reaction parameters; the feed allows continuous control over carbon source levels necessary for growth and induction level of protein expression, while stirring speed and aeration create optimal oxygen levels, and can lead to corresponding improved growth rates. Finally, when process conditions have been optimized, and the highest possible productivity is reached, a fair comparison of the real value of *S. cerevisiae* as an antibody factory can be made with the current established mammalian systems. It has to be noted that for CHO cells over 25 years of research has been invested in order to get the optimal production cell lines and conditions that lead to the current high IgG titers [341]. In comparison there has been less than a decade of research on recombinant full-length IgG production in *S. cerevisiae*, but with the current progress we believe that the yeast for sure has potential.

Finally, there is the question of transferability of the knowledge obtained in our *S. cerevisiae* production system into other, less characterized, microbial expression hosts. For example the methylotrophic yeast *P. pastoris* is an often used host for the production of heterologous proteins. This is because it benefits from the possibility to grow on relatively inexpensive methanol as its sole carbon source and protein expression inducer, it secretes low amounts of native proteins, and it is able to grow into very high cell densities, thereby increasing the volumetric protein yield. However, genetically, *P. pastoris* is less well characterized than *S. cerevisiae*, and that makes it less suitable for targeted engineering strategies. Additionally, genetic manipulation is less efficient, what makes it less suitable for large scale

genetic engineering studies. Seeing that *P. pastoris* and *S. cerevisiae* are relatively similar, it can be assumed that there is a possibility that similar effects can be reached when using the successful engineering strategies from *S. cerevisiae* in *P. pastoris*. For example, *P. pastoris* homologs of the overexpressed or deleted genes in *S. cerevisiae* can be used to improve heterologous protein production. Additionally, it even has been shown to be possible to use *S. cerevisiae* proteins in *P. pastoris* to create an improved cell factory [342].

Overall, many targets for strain and process engineering are available to increase the IgG secretion from *S. cerevisiae*. Although the IgG titers are currently still rather modest compared to established antibody production systems like CHO cells, we do believe that *S. cerevisiae* has the potential to become a relevant antibody factory.

6. References

[1] A. Goffeau, B. G. Barrell, H. Bussey, R. W. Davis, B. Dujon, H. Feldmann, F. Galibert, J. D. Hoheisel, C. Jacq, M. Johnston, E. J. Louis, H. W. Mewes, Y. Murakami, P. Philippsen, H. Tettelin, and S. G. Oliver, "Life with 6000 genes.," *Science*, vol. 274, no. 5287, pp. 546, 563–567, Oct. 1996.

[2] L. Peña-Castillo and T. R. Hughes, "Why are there still over 1000 uncharacterized yeast genes?," *Genetics*, vol. 176, no. 1, pp. 7–14, May 2007.

[3] S. Kawai, W. Hashimoto, and K. Murata, "Transformation of *Saccharomyces cerevisiae* and other fungi: methods and possible underlying mechanism.," *Bioeng. Bugs*, vol. 1, no. 6, pp. 395–403, Jan. 2010.

[4] D. Gietz, A. St Jean, R. A. Woods, and R. H. Schiestl, "Improved method for high efficiency transformation of intact yeast cells.," *Nucleic Acids Res.*, vol. 20, no. 6, p. 1425, Mar. 1992.

[5] J. H. Hegemann and S. B. Heick, "Delete and repeat: a comprehensive toolkit for sequential gene knockout in the budding yeast *Saccharomyces cerevisiae*.," *Methods Mol. Biol.*, vol. 765, pp. 189–206, Jan. 2011.

[6] J. E. DiCarlo, J. E. Norville, P. Mali, X. Rios, J. Aach, and G. M. Church, "Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems.," *Nucleic Acids Res.*, vol. 41, no. 7, pp. 4336–4343, Apr. 2013.

[7] G. C. Zhang, I. I. Kong, H. Kim, J. J. Liu, J. H. D. Cate, and Y. S. Jin, "Construction of a quadruple auxotrophic mutant of an industrial polyploid *Saccharomyces cerevisiae* strain by using RNA-guided Cas9 nuclease," *Appl. Environ. Microbiol.*, vol. 80, no. 24, pp. 7694–7701, Dec. 2014.

[8] T. Jakočiūnas, M. K. Jensen, and J. D. Keasling, "CRISPR/Cas9 advances engineering of microbial cell factories," *Metab. Eng.*, vol. 34, pp. 44–59, Dec. 2015.

[9] S. Partow, V. Siewers, S. Bjorn, J. Nielsen, and J. Maury, "Characterization of different promoters for designing a new expression vector in *Saccharomyces cerevisiae*," *Yeast*, vol. 27, no. 11, pp. 955–964, Nov. 2010.

[10] B. Peng, T. C. Williams, M. Henry, L. K. Nielsen, and C. E. Vickers, "Controlling heterologous gene expression in yeast cell factories on different carbon substrates and across the diauxic shift: a comparison of yeast promoter activities.," *Microb. Cell Fact.*, vol. 14, no. 1, p. 91, 2015.

[11] G. Hubmann, J. M. Thevelein, and E. Nevoigt, "Natural and modifi ed promoters for tailored metabolic engineering of the yeast S*accharomyces cerevisiae*," *Methods Mol. Biol.*, vol. 1152, pp. 17–42, 2014.

[12] J. Blazeck, R. Garg, B. Reed, and H. S. Alper, "Controlling promoter strength and regulation in *Saccharomyces cerevisiae* using synthetic hybrid promoters.," *Biotechnol. Bioeng.*, vol. 109, no. 11, pp. 2884–2895, Nov. 2012.

[13] A. Rantasalo, E. Czeizler, R. Virtanen, J. Rousu, H. Lähdesmäki, M. Penttilä, J. Jäntti, and D. Mojzita, "Synthetic Transcription Amplifier System for Orthogonal Control of Gene Expression in *Saccharomyces cerevisiae*.," *PLoS One*, vol. 11, no. 2, p. e0148320, Jan. 2016.

[14] M. Naesby, S. V. Nielsen, C. A. Nielsen, T. Green, T. O. Tange, E. Simón, P. Knechtle, A. Hansson, M. S. Schwab, O. Titiz, C. Folly, R. E. Archila, M. Maver, S. van Sint Fiet, T. Boussemghoune, M. Janes, A. S. S. Kumar, S. P. Sonkar, P. P. Mitra, V. A. K. Benjamin, N. Korrapati, I. Suman, E. H. Hansen, T. Thybo, N. Goldsmith, and A. S. Sorensen, "Yeast artificial chromosomes employed for random assembly of biosynthetic pathways and production of diverse compounds in *Saccharomyces cerevisiae*.," *Microb. Cell Fact.*, vol. 8, p. 45, 2009.

[15] I.-K. Kim, A. Roldão, V. Siewers, and J. Nielsen, "A systems-level approach for metabolic engineering of yeast cell factories," *FEMS Yeast Res.*, vol. 12, no. 2, pp. 228–248, Mar. 2012.
[16] D. Coudreuse, "Insights from synthetic yeasts.," *Yeast*, vol. 33, pp. 483–492 May 2016.
[17] H. V Amorim, M. L. Lopes, J. V. de Castro Oliveira, M. S. Buckeridge, and G. H. Goldman, "Scientific challenges of bioethanol production in Brazil.," *Appl. Microbiol. Biotechnol.*, vol. 91, no. 5, pp. 1267–1275, Sep. 2011.
[18] U. Petrovič, "Next-generation biofuels: a new challenge for yeast.," *Yeast*, vol. 32, no. 9, pp. 583–593, Sep. 2015.
[19] J. O. Westman and C. J. Franzén, "Current progress in high cell density yeast bioprocesses for bioethanol production.," *Biotechnol. J.*, vol. 10, no. 8, pp. 1185–1195, Aug. 2015.
[20] R. Yamada, S. ichi Yamakawa, T. Tanaka, C. Ogino, H. Fukuda, and A. Kondo, "Direct and efficient ethanol production from high-yielding rice using a *Saccharomyces cerevisiae* strain that express amylases," *Enzyme Microb. Technol.*, vol. 48, no. 4–5, pp. 393–396, Apr. 2011.
[21] D. Pinel, F. D'Aoust, S. B. del Cardayre, P. K. Bajwa, H. Lee, and V. J. J. Martin, "*Saccharomyces cerevisiae* genome shuffling through recursive population mating leads to improved tolerance to spent sulfite liquor," *Appl. Environ. Microbiol.*, vol. 77, no. 14, pp. 4736–4743, Jul. 2011.
[22] N. A. Buijs, V. Siewers, and J. Nielsen, "Advanced biofuel production by the yeast *Saccharomyces cerevisiae*.," *Curr. Opin. Chem. Biol.*, vol. 17, no. 3, pp. 480–488, Jun. 2013.
[23] P. P. Peralta-Yahya, M. Ouellet, R. Chan, A. Mukhopadhyay, J. D. Keasling, and T. S. Lee, "Identification and microbial production of a terpene-based advanced biofuel.," *Nat. Commun.*, vol. 2, p. 483, Jan. 2011.
[24] Y. Kamisaka, K. Kimura, H. Uemura, and M. Yamaoka, "Overexpression of the active diacylglycerol acyltransferase variant transforms *Saccharomyces cerevisiae* into an oleaginous yeast.," *Appl. Microbiol. Biotechnol.*, vol. 97, no. 16, pp. 7345–7355, Aug. 2013.
[25] W. Runguphan and J. D. Keasling, "Metabolic engineering of *Saccharomyces cerevisiae* for production of fatty acid-derived biofuels and chemicals.," *Metab. Eng.*, vol. 21, pp. 103–113, Jan. 2014.
[26] K.-K. Hong and J. Nielsen, "Metabolic engineering of *Saccharomyces cerevisiae*: a key cell factory platform for future biorefineries.," *Cell. Mol. Life Sci.*, vol. 69, no. 16, pp. 2671–2690, Aug. 2012.
[27] S. M. Pratter, T. Eixelsberger, and B. Nidetzky, "Systematic strain construction and process development: Xylitol production by *Saccharomyces cerevisiae* expressing *Candida tenuis* xylose reductase in wild-type or mutant form.," *Bioresour. Technol.*, vol. 198, pp. 732–738, Dec. 2015.
[28] J.-H. Jo, S.-Y. Oh, H.-S. Lee, Y.-C. Park, and J.-H. Seo, "Dual utilization of NADPH and NADH cofactors enhances xylitol production in engineered *Saccharomyces cerevisiae*.," *Biotechnol. J.*, vol. 10, no. 12, pp. 1935–1943, Dec. 2015.
[29] Z. Li, H. Qu, C. Li, and X. Zhou, "Direct and efficient xylitol production from xylan by *Saccharomyces cerevisiae* through transcriptional level and fermentation processing optimizations.," *Bioresour. Technol.*, vol. 149, pp. 413–419, Dec. 2013.
[30] E. J. Oh, S.-J. Ha, S. Rin Kim, W.-H. Lee, J. M. Galazka, J. H. D. Cate, and Y.-S. Jin, "Enhanced xylitol production through simultaneous co-utilization of cellobiose and xylose by engineered *Saccharomyces cerevisiae*.," *Metab. Eng.*, vol. 15, pp. 226–234, Jan. 2013.
[31] E. Simon, I. N. Anderson, M. D. Mikkelsen, J. Hansen, and V. Douchin, "Efficient Production of Steviol Glycosides in Recombinant Hosts," 17-Dec-2015.
[32] B. Huang, J. Guo, B. Yi, X. Yu, L. Sun, and W. Chen, "Heterologous production of secondary metabolites as pharmaceuticals in Saccharomyces cerevisiae.," *Biotechnol. Lett.*, vol. 30, no. 7, pp. 1121–1137, Jul. 2008.
[33] C. J. Paddon and J. D. Keasling, "Semi-synthetic artemisinin: a model for the use of synthetic biology in pharmaceutical development.," *Nat. Rev. Microbiol.*, vol. 12, no. 5, pp. 355–367, May 2014.
[34] C. J. Paddon, P. J. Westfall, D. J. Pitera, K. Benjamin, K. Fisher, D. McPhee, M. D. Leavell, A. Tai, A. Main, D. Eng, D. R. Polichuk, K. H. Teoh, D. W. Reed, T. Treynor, J. Lenihan, M. Fleck, S. Bajad, G. Dang, D. Dengrove, D. Diola, G. Dorin, K. W. Ellens, S. Fickes, J. Galazzo, S. P. Gaucher, T. Geistlinger, R. Henry, M. Hepp, T. Horning, T. Iqbal, H. Jiang, L. Kizer, B. Lieu, D. Melis, N. Moss, R. Regentin, S. Secrest, H. Tsuruta, R. Vazquez, L. F. Westblade, L. Xu, M. Yu, Y. Zhang, L. Zhao, J. Lievense, P. S. Covello, J. D. Keasling, K. K. Reiling, N. S. Renninger, and J. D. Newman, "High-level semi-synthetic production of the potent antimalarial

artemisinin.," *Nature*, vol. 496, no. 7446, pp. 528–532, Apr. 2013.
[35] E. Fossati, L. Narcross, A. Ekins, J.-P. Falgueyret, and V. J. J. Martin, "Synthesis of Morphinan Alkaloids in *Saccharomyces cerevisiae*.," *PLoS One*, vol. 10, no. 4, p. e0124459, Jan. 2015.
[36] S. Galanie, K. Thodey, I. J. Trenchard, M. Filsinger Interrante, and C. D. Smolke, "Complete biosynthesis of opioids in yeast," *Science*, vol. 349, no. 6252, pp. 1095–1100, Aug. 2015.
[37] M. Huang, J. Bao, and J. Nielsen, "Biopharmaceutical protein production by *Saccharomyces cerevisiae* : current state and future prospects," *Pharm. Bioprocess.*, vol. 2, no. 2, pp. 167–182, Apr. 2014.
[38] J. Hou, K. Tyo, Z. Liu, D. Petranovic, and J. Nielsen, "Engineering of vesicle trafficking improves heterologous protein secretion in *Saccharomyces cerevisiae*.," *Metab. Eng.*, vol. 14, no. 2, pp. 120–127, Mar. 2012.
[39] T. Kjeldsen, "Yeast secretory expression of insulin precursors.," *Appl. Microbiol. Biotechnol.*, vol. 54, no. 3, pp. 277–286, Sep. 2000.
[40] N. A. Baeshen, M. N. Baeshen, A. Sheikh, R. S. Bora, M. M. M. Ahmed, H. A. I. Ramadan, K. S. Saini, and E. M. Redwan, "Cell factories for insulin production.," *Microb. Cell Fact.*, vol. 13, no. 1, p. 141, Jan. 2014.
[41] T. Payne, C. Finnis, L. R. Evans, D. J. Mead, S. V Avery, D. B. Archer, and D. Sleep, "Modulation of chaperone gene expression in mutagenized *Saccharomyces cerevisiae* strains developed for recombinant human albumin production results in increased production of multiple heterologous proteins.," *Appl. Environ. Microbiol.*, vol. 74, no. 24, pp. 7759–7766, Dec. 2008.
[42] C. J. A. Finnis, T. Payne, J. Hay, N. Dodsworth, D. Wilkinson, P. Morton, M. J. Saxton, D. J. Tooth, R. W. Evans, H. Goldenberg, B. Scheiber-Mojdehkar, N. Ternes, and D. Sleep, "High-level production of animal-free recombinant transferrin from *Saccharomyces cerevisiae*.," *Microb. Cell Fact.*, vol. 9, p. 87, Jan. 2010.
[43] G. A. Bitter, K. M. Egan, W. N. Burnette, B. Samal, J. C. Fieschko, D. L. Peterson, M. R. Downing, J. Wypych, and K. E. Langley, "Hepatitis B vaccine produced in yeast.," *J. Med. Virol.*, vol. 25, no. 2, pp. 123–140, Jun. 1988.
[44] J. H. Hsieh, K. Y. Shih, H. F. Kung, M. Shiang, L. Y. Lee, M. H. Meng, C. C. Chang, H. M. Lin, S. C. Shih, S. Y. Lee, T. Y. Chow, T. Y. Feng, T. T. Kuo, and K. B. Choo, "Controlled fed-batch fermentation of recombinant *Saccharomyces cerevisiae* to produce hepatitis B surface antigen.," *Biotechnol. Bioeng.*, vol. 32, no. 3, pp. 334–340, Jul. 1988.
[45] A. Miyanohara, A. Toh-e, C. Nozaki, F. Hamada, N. Ohtomo, and K. Matsubara, "Expression of hepatitis B surface antigen gene in yeast.," *Proc. Natl. Acad. Sci.*, vol. 80, no. 1, pp. 1–5, Jan. 1983.
[46] E.-J. Kim, Y.-K. Park, H.-K. Lim, Y.-C. Park, and J.-H. Seo, "Expression of hepatitis B surface antigen S domain in recombinant *Saccharomyces cerevisiae* using GAL1 promoter.," *J. Biotechnol.*, vol. 141, no. 3–4, pp. 155–159, May 2009.
[47] J. L. Rossi, L. Gissmann, K. Jansen, and M. Müller, "Assembly of human papillomavirus type 16 pseudovirions in *Saccharomyces cerevisiae*.," *Hum. Gene Ther.*, vol. 11, no. 8, pp. 1165–1176, May 2000.
[48] S. J. Hale, S. C. Lovell, J. de Keyzer, and C. J. Stirling, "Interactions between Kar2p and its nucleotide exchange factors Sil1p and Lhs1p are mechanistically distinct.," *J. Biol. Chem.*, vol. 285, no. 28, pp. 21600–21606, Jul. 2010.
[49] P. Lajoie, R. D. Moir, I. M. Willis, and E. L. Snapp, "Kar2p availability defines distinct forms of endoplasmic reticulum stress in living cells.," *Mol. Biol. Cell*, vol. 23, no. 5, pp. 955–964, Mar. 2012.
[50] M. Delic, M. Valli, A. B. Graf, M. Pfeffer, D. Mattanovich, and B. Gasser, "The secretory pathway: exploring yeast diversity.," *FEMS Microbiol. Rev.*, vol. 37, no. 6, pp. 872–914, Nov. 2013.
[51] M. Delic, R. Göngrich, D. Mattanovich, and B. Gasser, "Engineering of protein folding and secretion-strategies to overcome bottlenecks for efficient production of recombinant proteins.," *Antioxid. Redox Signal.*, vol. 21, no. 3, pp. 414–437, Jul. 2014.
[52] A. Idiris, H. Tohda, H. Kumagai, and K. Takegawa, "Engineering of protein secretion in yeast: strategies and impact on protein production.," *Appl. Microbiol. Biotechnol.*, vol. 86, no. 2, pp. 403–417, Mar. 2010.
[53] M. C. Jonikas, S. R. Collins, V. Denic, E. Oh, E. M. Quan, V. Schmid, J. Weibezahn, B. Schwappach, P. Walter, J. S. Weissman, and M. Schuldiner, "Comprehensive characterization of genes required for protein folding in the endoplasmic reticulum.," *Science*, vol. 323, no.

5922, pp. 1693–1697, Mar. 2009.
[54] I. Fitzgerald and B. S. Glick, “Secretion of a foreign protein from budding yeasts is enhanced by cotranslational translocation and by suppression of vacuolar targeting.,” *Microb. Cell Fact.*, vol. 13, no. 1, p. 125, Jan. 2014.
[55] M. Tigges and M. Fussenegger, “Xbp1-based engineering of secretory capacity enhances the productivity of Chinese hamster ovary cells.,” *Metab. Eng.*, vol. 8, no. 3, pp. 264–272, May 2006.
[56] M. Vincent, M. Whidden, and S. Schnell, “Surveying the floodgates: estimating protein flux into the endoplasmic reticulum lumen in *Saccharomyces cerevisiae*.,” *Front. Physiol.*, vol. 5, p. 444, Jan. 2014.
[57] K.-U. Kalies, T. A. Rapoport, and E. Hartmann, “The β Subunit of the Sec61 Complex Facilitates Cotranslational Protein Transport and Interacts with the Signal Peptidase during Translocation,” *J. Cell Biol.*, vol. 141, no. 4, pp. 887–894, May 1998.
[58] R. Beckmann, “Alignment of Conduits for the Nascent Polypeptide Chain in the Ribosome-Sec61 Complex,” *Science (80-.).*, vol. 278, no. 5346, pp. 2123–2126, Dec. 1997.
[59] L. A. Kung, S.-C. Tao, J. Qian, M. G. Smith, M. Snyder, and H. Zhu, “Global analysis of the glycoproteome in Saccharomyces cerevisiae reveals new roles for protein glycosylation in eukaryotes.,” *Mol. Syst. Biol.*, vol. 5, no. 1, p. 308, 2009.
[60] M. Aebi, “N-linked protein glycosylation in the ER.,” *Biochim. Biophys. Acta*, vol. 1833, no. 11, pp. 2430–2437, Nov. 2013.
[61] J. Helenius, D. T. W. Ng, C. L. Marolda, P. Walter, M. A. Valvano, and M. Aebi, “Translocation of lipid-linked oligosaccharides across the ER membrane requires Rft1 protein.,” *Nature*, vol. 415, no. 6870, pp. 447–450, Jan. 2002.
[62] F. Schwarz and M. Aebi, “Mechanisms and principles of N-linked protein glycosylation.,” *Curr. Opin. Struct. Biol.*, vol. 21, no. 5, pp. 576–82, Oct. 2011.
[63] Q. Yan, G. D. Prestwich, and W. J. Lennarz, “The Ost1p subunit of yeast oligosaccharyl transferase recognizes the peptide glycosylation site sequence, -Asn-X-Ser/Thr-.,” *J. Biol. Chem.*, vol. 274, no. 8, pp. 5021–5025, Feb. 1999.
[64] E. Bause, R. Erkens, J. Schweden, and L. Jaenicke, “Purification and characterization of trimming glucosidase I from *Saccharomyces cerevisiae*,” *FEBS Lett.*, vol. 206, no. 2, pp. 208–212, Oct. 1986.
[65] A. Herscovics, “Processing glycosidases of Saccharomyces cerevisiae,” *Biochimica et Biophysica Acta - General Subjects*, vol. 1426, no. 2. pp. 275–285, 1999.
[66] A. Herscovics, “Structure and function of Class I alpha 1,2-mannosidases involved in glycoprotein synthesis and endoplasmic reticulum quality control.,” *Biochimie*, vol. 83, no. 8, pp. 757–762, Aug. 2001.
[67] A. Helenius, “How N-linked oligosaccharides affect glycoprotein folding in the endoplasmic reticulum.,” *Mol. Biol. Cell*, vol. 5, no. 3, pp. 253–265, Mar. 1994.
[68] A. Helenius and M. Aebi, “Roles of N-linked glycans in the endoplasmic reticulum.,” *Annu. Rev. Biochem.*, vol. 73, pp. 1019–1049, Jan. 2004.
[69] H. Hoshida, T. Fujita, K. Cha-aim, and R. Akada, “N-Glycosylation deficiency enhanced heterologous production of a *Bacillus licheniformis* thermostable α-amylase in *Saccharomyces cerevisiae*.,” *Appl. Microbiol. Biotechnol.*, vol. 97, no. 12, pp. 5473–5482, Jun. 2013.
[70] T.-Y. Wang, C.-J. Huang, H.-L. Chen, P.-C. Ho, H.-M. Ke, H.-Y. Cho, S.-K. Ruan, K.-Y. Hung, I.-L. Wang, Y.-W. Cai, H.-M. Sung, W.-H. Li, and M.-C. Shih, “Systematic screening of glycosylation- and trafficking-associated gene knockouts in *Saccharomyces cerevisiae* identifies mutants with improved heterologous exocellulase activity and host secretion.,” *BMC Biotechnol.*, vol. 13, p. 71, Jan. 2013.
[71] C. M. J. Sagt, B. Kleizen, R. Verwaal, M. D. M. De Jong, W. H. Muller, A. Smits, C. Visser, J. Boonstra, A. J. Verkleij, and C. T. Verrips, “Introduction of an N-glycosylation site increases secretion of heterologous proteins in yeasts,” *Appl. Environ. Microbiol.*, vol. 66, no. 11, pp. 4940–4944, Nov. 2000.
[72] J. F. Simons, S. Ferro-Novick, M. D. Rose, and A. Helenius, “BiP/Kar2p serves as a molecular chaperone during carboxypeptidase Y folding in yeast.,” *J. Cell Biol.*, vol. 130, no. 1, pp. 41–49, Jul. 1995.
[73] S. L. Sanders, K. M. Whitfield, J. P. Vogel, M. D. Rose, and R. W. Schekman, “Sec61p and BiP directly facilitate polypeptide translocation into the ER,” *Cell*, vol. 69, no. 2, pp. 353–365, Apr. 1992.

[74] G. C. Flynn, T. G. Chappell, and J. E. Rothman, “Peptide binding and release by proteins implicated as catalysts of protein assembly.,” *Science*, vol. 245, no. 4916, pp. 385–390, Jul. 1989.
[75] G. J. Steel, D. M. Fullerton, J. R. Tyson, and C. J. Stirling, “Coordinated activation of Hsp70 chaperones.,” *Science*, vol. 303, no. 5654, pp. 98–101, Jan. 2004.
[76] K. J. Kauffman, E. M. Pridgen, F. J. Doyle, P. S. Dhurjati, and A. S. Robinson, “Decreased protein expression and intermittent recoveries in BiP levels result from cellular stress during heterologous protein expression in *Saccharomyces cerevisiae*.,” *Biotechnol. Prog.*, vol. 18, no. 5, pp. 942–950, Jan. 2002.
[77] D. Pincus, M. W. Chevalier, T. Aragón, E. van Anken, S. E. Vidal, H. El-Samad, and P. Walter, “BiP binding to the ER-stress sensor Ire1 tunes the homeostatic behavior of the unfolded protein response.,” *PLoS Biol.*, vol. 8, no. 7, p. e1000415, Jan. 2010.
[78] A. Feizi, T. Österlund, D. Petranovic, S. Bordel, and J. Nielsen, “Genome-scale modeling of the protein secretory machinery in yeast.,” *PLoS One*, vol. 8, no. 5, p. e63284, Jan. 2013.
[79] R. Xiao, B. Wilkinson, A. Solovyov, J. R. Winther, A. Holmgren, J. Lundström-Ljung, and H. F. Gilbert, “The contributions of protein disulfide isomerase and its homologues to oxidative protein folding in the yeast endoplasmic reticulum.,” *J. Biol. Chem.*, vol. 279, no. 48, pp. 49780–49786, Nov. 2004.
[80] S. Grubb, L. Guo, E. A. Fisher, and J. L. Brodsky, “Protein disulfide isomerases contribute differentially to the endoplasmic reticulum-associated degradation of apolipoprotein B and other substrates.,” *Mol. Biol. Cell*, vol. 23, no. 4, pp. 520–532, Feb. 2012.
[81] A. R. Frand and C. A. Kaiser, “Ero1p Oxidizes Protein Disulfide Isomerase in a Pathway for Disulfide Bond Formation in the Endoplasmic Reticulum,” *Mol. Cell*, vol. 4, no. 4, pp. 469–477, Oct. 1999.
[82] S. Kim, D. P. Sideris, C. S. Sevier, and C. A. Kaiser, “Balanced Ero1 activation and inactivation establishes ER redox homeostasis.,” *J. Cell Biol.*, vol. 196, no. 6, pp. 713–725, Mar. 2012.
[83] M. G. Pollard, K. J. Travers, and J. S. Weissman, “Ero1p: A Novel and Ubiquitous Protein with an Essential Role in Oxidative Protein Folding in the Endoplasmic Reticulum,” *Mol. Cell*, vol. 1, no. 2, pp. 171–182, Jan. 1998.
[84] A. R. Frand and C. A. Kaiser, “The *ERO1* Gene of Yeast Is Required for Oxidation of Protein Dithiols in the Endoplasmic Reticulum,” *Mol. Cell*, vol. 1, no. 2, pp. 161–170, Jan. 1998.
[85] W. Wang, J. R. Winther, and C. Thorpe, “Erv2p: characterization of the redox behavior of a yeast sulfhydryl oxidase.,” *Biochemistry*, vol. 46, no. 11, pp. 3246–3254, Mar. 2007.
[86] Q. Wang and A. Chang, “Substrate recognition in ER-associated degradation mediated by Eps1, a member of the protein disulfide isomerase family.,” *EMBO J.*, vol. 22, no. 15, pp. 3792–3802, Aug. 2003.
[87] T. Kimura, Y. Hosoda, Y. Sato, Y. Kitamura, T. Ikeda, T. Horibe, and M. Kikuchi, “Interactions among yeast protein-disulfide isomerase proteins and endoplasmic reticulum chaperone proteins influence their activities.,” *J. Biol. Chem.*, vol. 280, no. 36, pp. 31438–31441, Sep. 2005.
[88] K. Dolinski, S. Muir, M. Cardenas, and J. Heitman, “All cyclophilins and FK506 binding proteins are, individually and collectively, dispensable for viability in *Saccharomyces cerevisiae*.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 94, no. 24, pp. 13093–13098, Nov. 1997.
[89] J. A. Partaledis and V. Berlin, “The FKB2 gene of *Saccharomyces cerevisiae*, encoding the immunosuppressant-binding protein FKBP-13, is regulated in response to accumulation of unfolded proteins in the endoplasmic reticulum.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 90, no. 12, pp. 5450–5454, Jun. 1993.
[90] V. S. Stronge, Y. Saito, Y. Ihara, and D. B. Williams, “Relationship between calnexin and BiP in suppressing aggregation and promoting refolding of protein and glycoprotein substrates.,” *J. Biol. Chem.*, vol. 276, no. 43, pp. 39779–39787, Oct. 2001.
[91] X. Xu, K. Kanbara, H. Azakami, and A. Kato, “Expression and characterization of *Saccharomyces cerevisiae* Cne1p, a calnexin homologue.,” *J. Biochem.*, vol. 135, no. 5, pp. 615–618, May 2004.
[92] D. N. Hebert, B. Foellmer, and A. Helenius, “Glucose trimming and reglucosylation determine glycoprotein association with calnexin in the endoplasmic reticulum,” *Cell*, vol. 81, no. 3. Cell Press, pp. 425–433, May-1995.
[93] W. A. Rodríguez-Limas, V. Tannenbaum, and K. E. J. Tyo, “Blocking endocytotic mechanisms to improve heterologous protein titers in *Saccharomyces cerevisiae*.,” *Biotechnol. Bioeng.*, vol. 112, no. 2, pp. 376–385, Feb. 2015.

[94] Y. R. Sevastsyanovich, S. N. Alfasi, and J. A. Cole, "Sense and nonsense from a systems biology approach to microbial recombinant protein production.," *Biotechnol. Appl. Biochem.*, vol. 55, no. 1, pp. 9–28, Jan. 2010.
[95] S. L. Powers and A. S. Robinson, "PDI improves secretion of redox-inactive beta-glucosidase.," *Biotechnol. Prog.*, vol. 23, no. 2, pp. 364–369, Jan. 2007.
[96] M. M. Harmsen, M. I. Bruyne, H. A. Raué, and J. Maat, "Overexpression of binding protein and disruption of the *PMR1* gene synergistically stimulate secretion of bovine prochymosin but not plant thaumatin in yeast.," *Appl. Microbiol. Biotechnol.*, vol. 46, no. 4, pp. 365–370, Nov. 1996.
[97] A. S. Robinson, J. A. Bockhaus, A. C. Voegler, and K. D. Wittrup, "Reduction of BiP levels decreases heterologous protein secretion in *Saccharomyces cerevisiae*.," *J. Biol. Chem.*, vol. 271, no. 17, pp. 10017–10022, Apr. 1996.
[98] E. V Shusta, R. T. Raines, A. Plückthun, and K. D. Wittrup, "Increasing the secretory capacity of *Saccharomyces cerevisiae* for production of single-chain antibody fragments.," *Nat. Biotechnol.*, vol. 16, no. 8, pp. 773–777, Aug. 1998.
[99] P. Xu, D. Raden, F. J. Doyle, and A. S. Robinson, "Analysis of unfolded protein response during single-chain antibody expression in *Saccaromyces cerevisiae* reveals different roles for BiP and PDI in folding," *Metab. Eng.*, vol. 7, no. 4, pp. 269–279, Jul. 2005.
[100] L. Xu, Y. Shen, J. Hou, B. Peng, H. Tang, and X. Bao, "Secretory pathway engineering enhances secretion of cellobiohydrolase I from *Trichoderma reesei* in *Saccharomyces cerevisiae*.," *J. Biosci. Bioeng.*, vol. 117, no. 1, pp. 45–52, Jan. 2014.
[101] A. S. Robinson, V. Hines, and K. D. Wittrup, "Protein disulfide isomerase overexpression increases secretion of foreign proteins in *Saccharomyces cerevisiae*.," *Biotechnology. (N. Y).*, vol. 12, no. 4, pp. 381–384, Apr. 1994.
[102] J. D. Smith, B. C. Tang, and A. S. Robinson, "Protein disulfide isomerase, but not binding protein, overexpression enhances secretion of a non-disulfide-bonded protein in yeast.," *Biotechnol. Bioeng.*, vol. 85, no. 3, pp. 340–350, Feb. 2004.
[103] T. Hayano, M. Hirose, and M. Kikuchi, "Protein disulfide isomerase mutant lacking its isomerase activity accelerates protein folding in the cell.," *FEBS Lett.*, vol. 377, no. 3, pp. 505–511, Dec. 1995.
[104] M. Mayer, U. Kies, R. Kammermeier, and J. Buchner, "BiP and PDI cooperate in the oxidative folding of antibodies in vitro.," *J. Biol. Chem.*, vol. 275, no. 38, pp. 29421–29425, Sep. 2000.
[105] A. E. Wentz and E. V Shusta, "A novel high-throughput screen reveals yeast genes that increase secretion of heterologous proteins.," *Appl. Environ. Microbiol.*, vol. 73, no. 4, pp. 1189–1198, Feb. 2007.
[106] S.-Y. Shin, Y.-H. Bae, S.-K. Kim, Y.-J. Seong, S.-H. Choi, K. H. Kim, Y.-C. Park, and J.-H. Seo, "Effects of signal sequences and folding accessory proteins on extracellular expression of carboxypeptidase Y in recombinant *Saccharomyces cerevisiae*.," *Bioprocess Biosyst. Eng.*, vol. 37, no. 6, pp. 1065–1071, Jun. 2014.
[107] Y. Song, J. Sata, A. Saito, M. Usui, H. Azakami, and A. Kato, "Effects of calnexin deletion in *Saccharomyces cerevisiae* on the secretion of glycosylated lysozymes.," *J. Biochem.*, vol. 130, no. 6, pp. 757–764, Dec. 2001.
[108] H. Arima, T. Kinoshita, H. R. Ibrahim, H. Azakami, and A. Kato, "Enhanced secretion of hydrophobic peptide fused lysozyme by the introduction of N-glycosylation signal and the disruption of calnexin gene in *Saccharomyces cerevisiae*.," *FEBS Lett.*, vol. 440, no. 1–2, pp. 89–92, Nov. 1998.
[109] F. Parlati, M. Dominguez, J. J. Bergeron, and D. Y. Thomas, "*Saccharomyces cerevisiae CNE1* encodes an endoplasmic reticulum (ER) membrane protein with sequence similarity to calnexin and calreticulin and functions as a constituent of the ER quality control apparatus.," *J. Biol. Chem.*, vol. 270, no. 1, pp. 244–253, Jan. 1995.
[110] E. Ciplys, K. Sasnauskas, and R. Slibinskas, "Overexpression of human calnexin in yeast improves measles surface glycoprotein solubility.," *FEMS Yeast Res.*, vol. 11, no. 6, pp. 514–523, Sep. 2011.
[111] B. M. Gardner and P. Walter, "Unfolded proteins are Ire1-activating ligands that directly induce the unfolded protein response.," *Science*, vol. 333, no. 6051, pp. 1891–1894, Sep. 2011.
[112] R. Mathuranyanon, T. Tsukamoto, A. Takeuchi, Y. Ishiwata-Kimata, Y. Tsuchiya, K. Kohno, and Y. Kimata, "Tight regulation of the unfolded protein sensor Ire1 by its intramolecularly antagonizing subdomain.," *J. Cell Sci.*, vol. 128, no. 9, pp. 1762–1772, May 2015.
[113] E. L. Snapp, "Unfolded protein responses with or without unfolded proteins?," *Cells*, vol. 1, no.

4, pp. 926–950, Jan. 2012.
[114] Y. Kimata, Y. I. Kimata, Y. Shimizu, H. Abe, I. C. Farcasanu, M. Takeuchi, M. D. Rose, and K. Kohno, "Genetic evidence for a role of BiP/Kar2 that regulates Ire1 in response to accumulation of unfolded proteins.," *Mol. Biol. Cell*, vol. 14, no. 6, pp. 2559–2569, Jun. 2003.
[115] L. Sathe, C. Bolinger, M. A. Mannan, T. E. Dever, and M. Dey, "Evidence That Base-pairing Interaction between Intron and mRNA Leader Sequences Inhibits Initiation of HAC1 mRNA Translation in Yeast.," *J. Biol. Chem.*, vol. 290, no. 36, pp. 21821–21832, Sep. 2015.
[116] T. Kawahara, H. Yanagi, T. Yura, and K. Mori, "Endoplasmic reticulum stress-induced mRNA splicing permits synthesis of transcription factor Hac1p/Ern4p that activates the unfolded protein response.," *Mol. Biol. Cell*, vol. 8, no. 10, pp. 1845–1862, Oct. 1997.
[117] K. J. Travers, C. K. Patil, L. Wodicka, D. J. Lockhart, J. S. Weissman, and P. Walter, "Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation.," *Cell*, vol. 101, no. 3, pp. 249–258, Apr. 2000.
[118] H. Higashio and K. Kohno, "A genetic link between the unfolded protein response and vesicle formation from the endoplasmic reticulum.," *Biochem. Biophys. Res. Commun.*, vol. 296, no. 3, pp. 568–574, Aug. 2002.
[119] D. T. Ng, E. D. Spear, and P. Walter, "The unfolded protein response regulates multiple aspects of secretory and membrane protein biogenesis and endoplasmic reticulum quality control.," *J. Cell Biol.*, vol. 150, no. 1, pp. 77–88, Jul. 2000.
[120] M. Valkonen, M. Ward, H. Wang, M. Penttilä, and M. Saloheimo, "Improvement of foreign-protein production in *Aspergillus niger* var. *awamori* by constitutive induction of the unfolded-protein response.," *Appl. Environ. Microbiol.*, vol. 69, no. 12, pp. 6979–6986, Dec. 2003.
[121] S. Schuck, W. A. Prinz, K. S. Thorn, C. Voss, and P. Walter, "Membrane expansion alleviates endoplasmic reticulum stress independently of the unfolded protein response.," *J. Cell Biol.*, vol. 187, no. 4, pp. 525–536, Nov. 2009.
[122] R. Friedlander, E. Jarosch, J. Urban, C. Volkwein, and T. Sommer, "A regulatory link between ER-associated protein degradation and the unfolded-protein response.," *Nat. Cell Biol.*, vol. 2, no. 7, pp. 379–384, Jul. 2000.
[123] G. Thibault, N. Ismail, and D. T. W. Ng, "The unfolded protein response supports cellular robustness as a broad-spectrum compensatory pathway.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 108, no. 51, pp. 20597–20602, Dec. 2011.
[124] T. Payne, C. Hanfrey, A. L. Bishop, A. J. Michael, S. V Avery, and D. B. Archer, "Transcript-specific translational regulation in the unfolded protein response of *Saccharomyces cerevisiae*.," *FEBS Lett.*, vol. 582, no. 4, pp. 503–509, Feb. 2008.
[125] N. Ogawa and K. Mori, "Autoregulation of the *HAC1* gene is required for sustained activation of the yeast unfolded protein response.," *Genes Cells*, vol. 9, no. 2, pp. 95–104, Feb. 2004.
[126] K. Mori, N. Ogawa, T. Kawahara, H. Yanagi, and T. Yura, "Palindrome with spacer of one nucleotide is characteristic of the cis-acting unfolded protein response element in *Saccharomyces cerevisiae*.," *J. Biol. Chem.*, vol. 273, no. 16, pp. 9912–9920, Apr. 1998.
[127] C. K. Patil, H. Li, and P. Walter, "Gcn4p and novel upstream activating sequences regulate targets of the unfolded protein response.," *PLoS Biol.*, vol. 2, no. 8, p. E246, Aug. 2004.
[128] P. M. Fordyce, D. Pincus, P. Kimmig, C. S. Nelson, H. El-Samad, P. Walter, and J. L. DeRisi, "Basic leucine zipper transcription factor Hac1 binds DNA in two distinct modes as revealed by microfluidic analyses.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 109, no. 45, pp. E3084-3093, Nov. 2012.
[129] P. Xu and A. S. Robinson, "Decreased secretion and unfolded protein response up-regulation are correlated with intracellular retention for single-chain antibody variants produced in yeast.," *Biotechnol. Bioeng.*, vol. 104, no. 1, pp. 20–29, Sep. 2009.
[130] D. Mattanovich, B. Gasser, H. Hohenblum, and M. Sauer, "Stress in recombinant protein producing yeasts.," *J. Biotechnol.*, vol. 113, no. 1–3, pp. 121–135, Sep. 2004.
[131] M. Valkonen, M. Penttilä, and M. Saloheimo, "Effects of inactivation and constitutive expression of the unfolded- protein response pathway on protein production in the yeast *Saccharomyces cerevisiae*.," *Appl. Environ. Microbiol.*, vol. 69, no. 4, pp. 2065–2072, Apr. 2003.
[132] K. E. J. Tyo, Z. Liu, D. Petranovic, and J. Nielsen, "Imbalance of heterologous protein folding and disulfide bond formation rates yields runaway oxidative stress.," *BMC Biol.*, vol. 10, no. 1, p. 16, Jan. 2012.

[133] B. Gasser, M. Maurer, J. Gach, R. Kunert, and D. Mattanovich, "Engineering of *Pichia pastoris* for improved production of antibody fragments.," *Biotechnol. Bioeng.*, vol. 94, no. 2, pp. 353–361, Jun. 2006.
[134] M. Guerfal, S. Ryckaert, P. P. Jacobs, P. Ameloot, K. Van Craenenbroeck, R. Derycke, and N. Callewaert, "The *HAC1* gene from *Pichia pastoris*: characterization and effect of its overexpression on the production of secreted, surface displayed and membrane proteins.," *Microb. Cell Fact.*, vol. 9, p. 49, Jan. 2010.
[135] A. Ruggiano, O. Foresti, and P. Carvalho, "Quality control: ER-associated degradation: protein quality control and beyond.," *J. Cell Biol.*, vol. 204, no. 6, pp. 869–879, Mar. 2014.
[136] C. A. Jakob, P. Burda, J. Roth, and M. Aebi, "Degradation of misfolded endoplasmic reticulum glycoproteins in *Saccharomyces cerevisiae* is determined by a specific oligosaccharide structure.," *J. Cell Biol.*, vol. 142, no. 5, pp. 1223–1233, Sep. 1998.
[137] R. Gauss, K. Kanehara, P. Carvalho, D. T. W. Ng, and M. Aebi, "A complex of Pdi1p and the mannosidase Htm1p initiates clearance of unfolded glycoproteins from the endoplasmic reticulum.," *Mol. Cell*, vol. 42, no. 6, pp. 782–793, Jun. 2011.
[138] A. Pfeiffer, H. Stephanowitz, E. Krause, C. Volkwein, C. Hirsch, E. Jarosch, and T. Sommer, "A Complex of Htm1 and the Oxidoreductase Pdi1 Accelerates Degradation of Misfolded Glycoproteins," *J. Biol. Chem.*, vol. 291, no. 23, pp. 12195–12207, Jun. 2016.
[139] S. Clerc, C. Hirsch, D. M. Oggier, P. Deprez, C. Jakob, T. Sommer, and M. Aebi, "Htm1 protein generates the N-glycan signal for glycoprotein degradation in the endoplasmic reticulum.," *J. Cell Biol.*, vol. 184, no. 1, pp. 159–172, Jan. 2009.
[140] E. M. Quan, Y. Kamiya, D. Kamiya, V. Denic, J. Weibezahn, K. Kato, and J. S. Weissman, "Defining the glycan destruction signal for endoplasmic reticulum-associated degradation.," *Mol. Cell*, vol. 32, no. 6, pp. 870–877, Dec. 2008.
[141] R. Szathmary, R. Bielmann, M. Nita-Lazar, P. Burda, and C. A. Jakob, "Yos9 protein is essential for degradation of misfolded glycoproteins and may function as lectin in ERAD.," *Mol. Cell*, vol. 19, no. 6, pp. 765–775, Sep. 2005.
[142] E. M. Benitez, A. Stolz, and D. H. Wolf, "Yos9, a control protein for misfolded glycosylated and non-glycosylated proteins in ERAD.," *FEBS Lett.*, vol. 585, no. 19, pp. 3015–3019, Oct. 2011.
[143] R. Gauss, E. Jarosch, T. Sommer, and C. Hirsch, "A complex of Yos9p and the HRD ligase integrates endoplasmic reticulum quality control into the degradation machinery.," *Nat. Cell Biol.*, vol. 8, no. 8, pp. 849–854, Aug. 2006.
[144] M. Mehnert, F. Sommermeyer, M. Berger, S. Kumar Lakshmipathy, R. Gauss, M. Aebi, E. Jarosch, and T. Sommer, "The interplay of Hrd3 and the molecular chaperone system ensures efficient degradation of malfolded secretory proteins.," *Mol. Biol. Cell*, vol. 26, no. 2, pp. 185–194, Jan. 2015.
[145] M. Mehnert, T. Sommer, and E. Jarosch, "Der1 promotes movement of misfolded proteins through the endoplasmic reticulum membrane.," *Nat. Cell Biol.*, vol. 16, no. 1, pp. 77–86, Jan. 2014.
[146] R. G. Gardner, G. M. Swarbrick, N. W. Bays, S. R. Cronin, S. Wilhovsky, L. Seelig, C. Kim, and R. Y. Hampton, "Endoplasmic Reticulum Degradation Requires Lumen to Cytosol Signaling: Transmembrane Control of Hrd1p by Hrd3p," *J. Cell Biol.*, vol. 151, no. 1, pp. 69–82, Oct. 2000.
[147] N. W. Bays, R. G. Gardner, L. P. Seelig, C. A. Joazeiro, and R. Y. Hampton, "Hrd1p/Der3p is a membrane-anchored ubiquitin ligase required for ER-associated degradation.," *Nat. Cell Biol.*, vol. 3, no. 1, pp. 24–29, Jan. 2001.
[148] M. M. Hiller, A. Finger, M. Schweiger, and D. H. Wolf, "ER degradation of a misfolded luminal protein by the cytosolic ubiquitin-proteasome pathway.," *Science*, vol. 273, no. 5282, pp. 1725–1728, Sep. 1996.
[149] M. B. Metzger, Y.-H. Liang, R. Das, J. Mariano, S. Li, J. Li, Z. Kostova, R. A. Byrd, X. Ji, and A. M. Weissman, "A structurally unique E2-binding domain activates ubiquitination by the ERAD E2, Ubc7p, through multiple mechanisms.," *Mol. Cell*, vol. 50, no. 4, pp. 516–527, May 2013.
[150] A. Stein, A. Ruggiano, P. Carvalho, and T. A. Rapoport, "Key steps in ERAD of luminal ER proteins reconstituted with purified components.," *Cell*, vol. 158, no. 6, pp. 1375–1388, Sep. 2014.
[151] K. Nakatsukasa, J. L. Brodsky, and T. Kamura, "A stalled retrotranslocation complex reveals physical linkage between substrate recognition and proteasomal degradation during ER-associated degradation.," *Mol. Biol. Cell*, vol. 24, no. 11, pp. 1765–1775, S1-8, Jun. 2013.

[152] T. Zhang and Y. Ye, "The final moments of misfolded proteins en route to the proteasome.," *DNA Cell Biol.*, vol. 33, no. 8, pp. 477–483, Aug. 2014.
[153] G. Thibault and D. T. W. Ng, "The endoplasmic reticulum-associated degradation pathways of budding yeast.," *Cold Spring Harb. Perspect. Biol.*, vol. 4, no. 12, Dec. 2012.
[154] T. Suzuki, H. Park, N. M. Hollingsworth, R. Sternglanz, and W. J. Lennarz, "*PNG1*, a yeast gene encoding a highly conserved peptide:N-glycanase.," *J. Cell Biol.*, vol. 149, no. 5, pp. 1039–1052, May 2000.
[155] I. Chantret, V. P. Kodali, C. Lahmouich, D. J. Harvey, and S. E. H. Moore, "Endoplasmic reticulum-associated degradation (ERAD) and free oligosaccharide generation in *Saccharomyces cerevisiae*.," *J. Biol. Chem.*, vol. 286, no. 48, pp. 41786–41800, Dec. 2011.
[156] C. Schauber, L. Chen, P. Tongaonkar, I. Vega, D. Lambertson, W. Potts, and K. Madura, "Rad23 links DNA repair to the ubiquitin/proteasome pathway.," *Nature*, vol. 391, no. 6668, pp. 715–718, Feb. 1998.
[157] M. C. S. Lee, L. Orci, S. Hamamoto, E. Futai, M. Ravazzola, and R. Schekman, "Sar1p N-terminal helix initiates membrane curvature and completes the fission of a COPII vesicle.," *Cell*, vol. 122, no. 4, pp. 605–617, Aug. 2005.
[158] K. Matsuoka, L. Orci, M. Amherdt, S. Y. Bednarek, S. Hamamoto, R. Schekman, and T. Yeung, "COPII-coated vesicle formation reconstituted with purified coat proteins and chemically defined liposomes.," *Cell*, vol. 93, no. 2, pp. 263–275, Apr. 1998.
[159] E. A. Miller, T. H. Beilharz, P. N. Malkus, M. C. S. Lee, S. Hamamoto, L. Orci, and R. Schekman, "Multiple cargo binding sites on the COPII subunit Sec24p ensure capture of diverse membrane proteins into transport vesicles.," *Cell*, vol. 114, no. 4, pp. 497–509, Aug. 2003.
[160] E. Miller, B. Antonny, S. Hamamoto, and R. Schekman, "Cargo selection into COPII vesicles is driven by the Sec24p subunit.," *EMBO J.*, vol. 21, no. 22, pp. 6105–6113, Nov. 2002.
[161] R. Peng, A. De Antoni, and D. Gallwitz, "Evidence for overlapping and distinct functions in protein transport of coat protein Sec24p family members.," *J. Biol. Chem.*, vol. 275, no. 15, pp. 11521–11528, Apr. 2000.
[162] M. Sato, K. Sato, and A. Nakano, "Evidence for the intimate relationship between vesicle budding from the ER and the unfolded protein response.," *Biochem. Biophys. Res. Commun.*, vol. 296, no. 3, pp. 560–567, Aug. 2002.
[163] N. G. Tsvetanova, D. P. Riordan, and P. O. Brown, "The yeast Rab GTPase Ypt1 modulates unfolded protein response dynamics by regulating the stability of HAC1 RNA.," *PLoS Genet.*, vol. 8, no. 7, p. e1002862, Jan. 2012.
[164] N. G. Tsvetanova, "The secretory pathway in control of endoplasmic reticulum homeostasis.," *Small GTPases*, vol. 4, no. 1, pp. 28–33, Jan. 2013.
[165] H. Cai, S. Yu, S. Menon, Y. Cai, D. Lazarova, C. Fu, K. Reinisch, J. C. Hay, and S. Ferro-Novick, "TRAPPI tethers COPII vesicles by binding the coat subunit Sec23.," *Nature*, vol. 445, no. 7130, pp. 941–944, Feb. 2007.
[166] X. Cao, N. Ballew, and C. Barlowe, "Initial docking of ER-derived vesicles requires Uso1p and Ypt1p but is independent of SNARE proteins.," *EMBO J.*, vol. 17, no. 8, pp. 2156–2165, Apr. 1998.
[167] T. Hayashi, H. McMahon, S. Yamasaki, T. Binz, Y. Hata, T. C. Südhof, and H. Niemann, "Synaptic vesicle membrane fusion complex: action of clostridial neurotoxins on assembly.," *EMBO J.*, vol. 13, no. 21, pp. 5051–5061, Nov. 1994.
[168] J. Malsam, S. Kreye, and T. H. Söllner, "Membrane fusion: SNAREs and regulation.," *Cell. Mol. Life Sci.*, vol. 65, no. 18, pp. 2814–2832, Sep. 2008.
[169] J. Stolz and S. Munro, "The components of the *Saccharomyces cerevisiae* mannosyltransferase complex M-Pol I have distinct functions in mannan synthesis.," *J. Biol. Chem.*, vol. 277, no. 47, pp. 44801–44808, Nov. 2002.
[170] K. Nakayama, Y. Nakanishi-Shindo, A. Tanaka, Y. Haga-Toda, and Y. Jigami, "Substrate specificity of alpha-1,6-mannosyltransferase that initiates N-linked mannose outer chain elongation in *Saccharomyces cerevisiae*.," *FEBS Lett.*, vol. 412, no. 3, pp. 547–550, Aug. 1997.
[171] J. C. Rayner and S. Munro, "Identification of the MNN2 and MNN5Mannosyltransferases Required for Forming and Extending the Mannose Branches of the Outer Chain Mannans of *Saccharomyces cerevisiae*," *J. Biol. Chem.*, vol. 273, no. 41, pp. 26836–26843, Oct. 1998.
[172] E. W. Jones, "Three proteolytic systems in the yeast *saccharomyces cerevisiae*.," *J. Biol. Chem.*, vol. 266, no. 13, pp. 7963–7966, May 1991.
[173] H. Abe, Y. Takaoka, Y. Chiba, N. Sato, S. Ohgiya, A. Itadani, M. Hirashima, C. Shimoda, Y.

Jigami, and K. Nakayama, “Development of valuable yeast strains using a novel mutagenesis technique for the effective production of therapeutic glycoproteins.,” *Glycobiology*, vol. 19, no. 4, pp. 428–436, Apr. 2009.

[174] H. Tang, S. Wang, J. Wang, M. Song, M. Xu, M. Zhang, Y. Shen, J. Hou, and X. Bao, “N-hypermannose glycosylation disruption enhances recombinant protein production by regulating secretory pathway and cell wall integrity in *Saccharomyces cerevisiae*.,” *Sci. Rep.*, vol. 6, p. 25654, 2016.

[175] R. S. Fuller, A. Brake, and J. Thorner, “Yeast prohormone processing enzyme (*KEX2* gene product) is a Ca2+-dependent serine protease.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 86, no. 5, pp. 1434–1438, Mar. 1989.

[176] R. Parekh, K. Forrester, and D. Wittrup, “Multicopy overexpression of bovine pancreatic trypsin inhibitor saturates the protein folding and secretory capacity of *Saccharomyces cerevisiae*.,” *Protein Expr. Purif.*, vol. 6, no. 4, pp. 537–545, Aug. 1995.

[177] Y.-K. Park, S.-M. Jung, H.-K. Lim, Y.-J. Son, Y.-C. Park, and J.-H. Seo, “Effects of Trx2p and Sec23p expression on stable production of hepatitis B surface antigen S domain in recombinant *Saccharomyces cerevisiae*.,” *J. Biotechnol.*, vol. 160, no. 3–4, pp. 151–60, Aug. 2012.

[178] E. Harsay and A. Bretscher, “Parallel secretory pathways to the cell surface in yeast.,” *J. Cell Biol.*, vol. 131, no. 2, pp. 297–310, Oct. 1995.

[179] M. R. Heider and M. Munson, “Exorcising the exocyst complex.,” *Traffic*, vol. 13, no. 7, pp. 898–907, Jul. 2012.

[180] R. Pleskot, L. Cwiklik, P. Jungwirth, V. Žárský, and M. Potocký, “Membrane targeting of the yeast exocyst complex.,” *Biochim. Biophys. Acta*, vol. 1848, no. 7, pp. 1481–1489, Jul. 2015.

[181] M. L. Dubuke, S. Maniatis, S. A. Shaffer, and M. Munson, “The Exocyst Subunit Sec6 Interacts with Assembled Exocytic SNARE Complexes.,” *J. Biol. Chem.*, vol. 290, no. 47, pp. 28245–28256, Nov. 2015.

[182] K. L. Nicholson, M. Munson, R. B. Miller, T. J. Filip, R. Fairman, and F. M. Hughson, “Regulation of SNARE complex assembly by an N-terminal domain of the t-SNARE Sso1p.,” *Nat. Struct. Biol.*, vol. 5, no. 9, pp. 793–802, Sep. 1998.

[183] L. Ruohonen, J. Toikkanen, V. Tieaho, M. Outola, H. Soderlund, and S. Keranen, “Enhancement of protein secretion in *Saccharomyces cerevisiae* by overproduction of Sso protein, a late-acting component of the secretory machinery.,” *Yeast*, vol. 13, no. 4, pp. 337–351, Mar. 1997.

[184] J. H. D. Van Zyl, R. Den Haan, and W. H. Van Zyl, “Overexpression of native *Saccharomyces cerevisiae* ER-to-Golgi SNARE genes increased heterologous cellulase secretion.,” *Appl. Microbiol. Biotechnol.*, vol. 100, no. 1, pp. 505–518, Jan. 2016.

[185] H. Holkeri and M. Makarow, “Different degradation pathways for heterologous glycoproteins in yeast.,” *FEBS Lett.*, vol. 429, no. 2, pp. 162–166, Jun. 1998.

[186] E. Hong, A. R. Davidson, and C. A. Kaiser, “A pathway for targeting soluble misfolded proteins to the yeast vacuole.,” *J. Cell Biol.*, vol. 135, no. 3, pp. 623–633, Nov. 1996.

[187] J. A. Rakestraw, S. L. Sazinsky, A. Piatesi, E. Antipov, and K. D. Wittrup, “Directed evolution of a secretory leader for the improved expression of heterologous proteins and full-length antibodies in Saccharomyces cerevisiae.,” *Biotechnol. Bioeng.*, vol. 103, no. 6, pp. 1192–1201, Aug. 2009.

[188] B. Zhang, A. Chang, T. B. Kjeldsen, and P. Arvan, “Intracellular retention of newly synthesized insulin in yeast is caused by endoproteolytic processing in the Golgi complex.,” *J. Cell Biol.*, vol. 153, no. 6, pp. 1187–1198, Jun. 2001.

[189] T. Kitagawa, K. Kohda, K. Tokuhiro, H. Hoshida, R. Akada, H. Takahashi, and T. Imaeda, “Identification of genes that enhance cellulase protein production in yeast.,” *J. Biotechnol.*, vol. 151, no. 2, pp. 194–203, Jan. 2011.

[190] M. A. Gleeson, C. E. White, D. P. Meininger, and E. A. Komives, “Generation of protease-deficient strains and their use in heterologous protein expression.,” *Methods Mol. Biol.*, vol. 103, pp. 81–94, 1998.

[191] K. Tomimoto, Y. Fujita, T. Iwaki, Y. Chiba, Y. Jigami, K. Nakayama, Y. Nakajima, and H. Abe, “Protease-deficient Saccharomyces cerevisiae strains for the synthesis of human-compatible glycoproteins.,” *Biosci. Biotechnol. Biochem.*, vol. 77, no. 12, pp. 2461–2466, Jan. 2013.

[192] H. A. Kang, E. S. Choi, W. K. Hong, J. Y. Kim, S. M. Ko, J. H. Sohn, and S. K. Rhee, “Proteolytic stability of recombinant human serum albumin secreted in the yeast *Saccharomyces cerevisiae*.,” *Appl. Microbiol. Biotechnol.*, vol. 53, no. 5, pp. 575–582, May 2000.

[193] N. A. P. S. Buss, S. J. Henderson, M. McFarlane, J. M. Shenton, and L. de Haan, "Monoclonal antibody therapeutics: history and future.," *Curr. Opin. Pharmacol.*, vol. 12, no. 5, pp. 615–622, Oct. 2012.
[194] A. de Marco, "Biotechnological applications of recombinant single-domain antibody fragments.," *Microb. Cell Fact.*, vol. 10, p. 44, Jan. 2011.
[195] G. Vidarsson, G. Dekkers, and T. Rispens, "IgG Subclasses and Allotypes: From Structure to Effector Functions," *Front. Immunol.*, vol. 5, p. 520, Oct. 2014.
[196] P. Bork, L. Holm, and C. Sander, "The Immunoglobulin Fold," *J. Mol. Biol.*, vol. 242, no. 4, pp. 309–320, 1994.
[197] D. Hristodorov, R. Fischer, H. Joerissen, B. Müller-Tiemann, H. Apeler, and L. Linden, "Generation and comparative characterization of glycosylated and aglycosylated human IgG1 antibodies.," *Mol. Biotechnol.*, vol. 53, no. 3, pp. 326–335, Mar. 2013.
[198] M.-S. Ju and S. T. Jung, "Aglycosylated full-length IgG antibodies: steps toward next-generation immunotherapeutics.," *Curr. Opin. Biotechnol.*, vol. 30, pp. 128–139, Dec. 2014.
[199] N. Lenny and M. Green, "Regulation of endoplasmic reticulum stress proteins in COS cells transfected with immunoglobulin mu heavy chain cDNA.," *J. Biol. Chem.*, vol. 266, no. 30, pp. 20532–20537, Oct. 1991.
[200] M. Vanhove, Y.-K. Usherwood, and L. M. Hendershot, "Unassembled Ig Heavy Chains Do Not Cycle from BiP In Vivo but Require Light Chains to Trigger Their Release," *Immunity*, vol. 15, no. 1, pp. 105–114, Jul. 2001.
[201] M. J. Feige, L. M. Hendershot, and J. Buchner, "How antibodies fold.," *Trends Biochem. Sci.*, vol. 35, no. 4, pp. 189–198, Apr. 2010.
[202] H. Lilie, R. Rudolph, and J. Buchner, "Association of antibody chains at different stages of folding: prolyl isomerization occurs after formation of quaternary structure.," *J. Mol. Biol.*, vol. 248, no. 1, pp. 190–201, Apr. 1995.
[203] M. H. Ryan, D. Petrone, J. F. Nemeth, E. Barnathan, L. Björck, and R. E. Jordan, "Proteolysis of purified IgGs by human and bacterial enzymes in vitro and the detection of specific proteolytic fragments of endogenous IgG in rheumatoid synovial fluid.," *Mol. Immunol.*, vol. 45, no. 7, pp. 1837–1846, Apr. 2008.
[204] J. B. Adlersberg, "The immunoglobulin hinge (Interdomain) region," *La Ricerca in Clinica e in Laboratorio*, vol. 6, no. 3, Springer-Verlag, pp. 191–205, 1976.
[205] P. Holliger and P. J. Hudson, "Engineered antibody fragments and the rise of single domains.," *Nat. Biotechnol.*, vol. 23, no. 9, pp. 1126–1136, Sep. 2005.
[206] R. J. Flanagan and A. L. Jones, "Fab Antibody Fragments," *Drug Saf.*, vol. 27, no. 14, pp. 1115–1133, 2004.
[207] Z. A. Ahmad, S. K. Yeap, A. M. Ali, W. Y. Ho, N. B. M. Alitheen, and M. Hamid, "scFv antibody: principles and clinical application.," *Clin. Dev. Immunol.*, vol. 2012, p. 980250, Jan. 2012.
[208] T. Yokota, D. E. Milenic, M. Whitlow, and J. Schlom, "Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms.," *Cancer Res.*, vol. 52, no. 12, pp. 3402–3408, Jun. 1992.
[209] D. E. Milenic, T. Yokota, D. R. Filpula, M. A. Finkelman, S. W. Dodd, J. F. Wood, M. Whitlow, P. Snoy, and J. Schlom, "Construction, binding properties, metabolism, and tumor targeting of a single-chain Fv derived from the pancarcinoma monoclonal antibody CC49.," *Cancer Res.*, vol. 51, no. 23 Pt 1, pp. 6363–6371, Dec. 1991.
[210] F. Unverdorben, F. Richter, M. Hutt, O. Seifert, P. Malinge, N. Fischer, and R. E. Kontermann, "Pharmacokinetic properties of IgG and various Fc fusion proteins in mice.," *MAbs*, vol. 8, no. 1, pp. 120–128, Jan. 2016.
[211] Y. Ye, S. Nian, W. Xu, T. Wu, X. Wang, Y. Gao, and Q. Yuan, "Construction and expression of human scFv-Fc against interleukin-33.," *Protein Expr. Purif.*, vol. 114, pp. 58–63, Oct. 2015.
[212] X. Xiao, Y. Chen, S. Mugabe, C. Gao, C. Tkaczyk, Y. Mazor, P. Pavlik, H. Wu, W. Dall'Acqua, and P. S. Chowdhury, "A Novel Dual Expression Platform for High Throughput Functional Screening of Phage Libraries in Product like Format.," *PLoS One*, vol. 10, no. 10, p. e0140691, Jan. 2015.
[213] G. Walsh, "Biopharmaceutical benchmarks 2014.," *Nat. Biotechnol.*, vol. 32, no. 10, pp. 992–1000, Oct. 2014.
[214] G. Walsh, "Biopharmaceutical benchmarks 2010.," *Nat. Biotechnol.*, vol. 28, no. 9, pp. 917–924, Sep. 2010.
[215] R. Kunert and D. Reinhart, "Advances in recombinant antibody manufacturing.," *Appl. Microbiol. Biotechnol.*, vol. 100, no. 8, pp. 3451–3461, Apr. 2016.

[216] Y.-M. Huang, W. Hu, E. Rustandi, K. Chang, H. Yusuf-Makagiansar, and T. Ryll, "Maximizing productivity of CHO cell-based fed-batch culture using chemically defined media conditions and typical manufacturing equipment.," *Biotechnol. Prog.*, vol. 26, no. 5, pp. 1400–1410, 2010.
[217] A. Berlec and B. Strukelj, "Current state and recent advances in biopharmaceutical production in *Escherichia coli*, yeasts and mammalian cells.," *J. Ind. Microbiol. Biotechnol.*, vol. 40, no. 3–4, pp. 257–274, Apr. 2013.
[218] A. Frenzel, M. Hust, and T. Schirrmann, "Expression of recombinant antibodies.," *Front. Immunol.*, vol. 4, p. 217, Jan. 2013.
[219] O. Spadiut, S. Capone, F. Krainer, A. Glieder, and C. Herwig, "Microbials for the production of monoclonal antibodies and antibody fragments," *Trends in Biotechnology*, vol. 32, no. 1. pp. 54–60, 2014.
[220] V. Jäger, K. Büssow, A. Wagner, S. Weber, M. Hust, A. Frenzel, and T. Schirrmann, "High level transient production of recombinant antibodies and antibody fusion proteins in HEK293 cells.," *BMC Biotechnol.*, vol. 13, p. 52, Jan. 2013.
[221] S. Carroll and M. Al-Rubeai, "The selection of high-producing cell lines using flow cytometry and cell sorting.," *Expert Opin. Biol. Ther.*, vol. 4, no. 11, pp. 1821–1829, Nov. 2004.
[222] D. Nishimiya, "Proteins improving recombinant antibody production in mammalian cells.," *Appl. Microbiol. Biotechnol.*, vol. 98, no. 3, pp. 1031–1042, Feb. 2014.
[223] S. C. Y. Ku, D. T. W. Ng, M. G. S. Yap, and S.-H. Chao, "Effects of overexpression of X-box binding protein 1 on recombinant protein production in Chinese hamster ovary and NS0 myeloma cells.," *Biotechnol. Bioeng.*, vol. 99, no. 1, pp. 155–164, Jan. 2008.
[224] S. Weikert, D. Papac, J. Briggs, D. Cowfer, S. Tom, M. Gawlitzek, J. Lofgren, S. Mehta, V. Chisholm, N. Modi, S. Eppler, K. Carroll, S. Chamow, D. Peers, P. Berman, and L. Krummen, "Engineering Chinese hamster ovary cells to maximize sialic acid content of recombinant glycoproteins.," *Nat. Biotechnol.*, vol. 17, no. 11, pp. 1116–1121, Nov. 1999.
[225] P. Hossler, S. F. Khattak, and Z. J. Li, "Optimal and consistent protein glycosylation in mammalian cell culture.," *Glycobiology*, vol. 19, no. 9, pp. 936–949, Sep. 2009.
[226] S. M. Singh and A. K. Panda, "Solubilization and refolding of bacterial inclusion body proteins.," *J. Biosci. Bioeng.*, vol. 99, no. 4, pp. 303–310, Apr. 2005.
[227] M. Venturi, C. Seifert, and C. Hunte, "High level production of functional antibody Fab fragments in an oxidizing bacterial cytoplasm.," *J. Mol. Biol.*, vol. 315, no. 1, pp. 1–8, Jan. 2002.
[228] M. Dueñas, J. Vázquez, M. Ayala, E. Söderlind, M. Ohlin, L. Pérez, C. A. Borrebaeck, and J. V Gavilondo, "Intra- and extracellular expression of an scFv antibody fragment in *E. coli*: effect of bacterial strains and pathway engineering using GroES/L chaperonins.," *Biotechniques*, vol. 16, no. 3, pp. 476–7, 480–483, Mar. 1994.
[229] R. Levy, K. Ahluwalia, D. J. Bohmann, H. M. Giang, L. J. Schwimmer, H. Issafras, N. B. Reddy, C. Chan, A. H. Horwitz, and T. Takeuchi, "Enhancement of antibody fragment secretion into the Escherichia coli periplasm by co-expression with the peptidyl prolyl isomerase, FkpA, in the cytoplasm.," *J. Immunol. Methods*, vol. 394, no. 1–2, pp. 10–21, Aug. 2013.
[230] F. Hatahet, V. D. Nguyen, K. E. H. Salo, and L. W. Ruddock, "Disruption of reducing pathways is not essential for efficient disulfide bond formation in the cytoplasm of *E. coli*.," *Microb. Cell Fact.*, vol. 9, p. 67, Jan. 2010.
[231] V. D. Nguyen, F. Hatahet, K. E. H. Salo, E. Enlund, C. Zhang, and L. W. Ruddock, "Pre-expression of a sulfhydryl oxidase significantly increases the yields of eukaryotic disulfide bond containing proteins expressed in the cytoplasm of *E.coli*.," *Microb. Cell Fact.*, vol. 10, no. 1, p. 1, 2011.
[232] A. Gaciarz, J. Veijola, Y. Uchida, M. J. Saaranen, C. Wang, S. Hörkkö, and L. W. Ruddock, "Systematic screening of soluble expression of antibody fragments in the cytoplasm of *E. coli*," *Microb. Cell Fact.*, vol. 15, no. 1, p. 22, Dec. 2016.
[233] T. Makino, G. Skretas, T.-H. Kang, and G. Georgiou, "Comprehensive engineering of *Escherichia coli* for enhanced expression of IgG antibodies.," *Metab. Eng.*, vol. 13, no. 2, pp. 241–251, Mar. 2011.
[234] Q. Cai, J. A. Hanson, A. R. Steiner, C. Tran, M. R. Masikat, R. Chen, J. F. Zawada, A. K. Sato, T. J. Hallam, and G. Yin, "A simplified and robust protocol for immunoglobulin expression in *Escherichia coli* cell-free protein synthesis systems.," *Biotechnol. Prog.*, vol. 31, no. 3, pp. 823–831, Jan. 2015.
[235] D.-M. Kim and J. R. Swartz, "Efficient production of a bioactive, multiple disulfide-bonded

protein using modified extracts of *Escherichia coli*.," *Biotechnol. Bioeng.*, vol. 85, no. 2, pp. 122–129, Jan. 2004.

[236] D. Groff, S. Armstrong, P. J. Rivers, J. Zhang, J. Yang, E. Green, J. Rozzelle, S. Liang, J. D. Kittle, A. R. Steiner, R. Baliga, C. D. Thanos, T. J. Hallam, A. K. Sato, and A. Y. Yam, "Engineering toward a bacterial 'endoplasmic reticulum' for the rapid expression of immunoglobulin proteins.," *MAbs*, vol. 6, no. 3, pp. 671–678, Jan. 2014.

[237] G. H. Wegner, "Emerging applications of the methylotrophic yeasts.," *FEMS Microbiol. Rev.*, vol. 7, no. 3–4, pp. 279–283, Dec. 1990.

[238] M. Pfeffer, M. Maurer, G. Köllensperger, S. Hann, A. B. Graf, and D. Mattanovich, "Modeling and measuring intracellular fluxes of secreted recombinant protein in *Pichia pastoris* with a novel 34S labeling procedure," *Microb. Cell Fact.*, vol. 10, no. 1, p. 47, 2011.

[239] M. Pfeffer, M. Maurer, J. Stadlmann, J. Grass, M. Delic, F. Altmann, and D. Mattanovich, "Intracellular interactome of secreted antibody Fab fragment in *Pichia pastoris* reveals its routes of secretion and degradation.," *Appl. Microbiol. Biotechnol.*, vol. 93, no. 6, pp. 2503–2512, Mar. 2012.

[240] M. Van Roy, C. Ververken, E. Beirnaert, S. Hoefman, J. Kolkman, M. Vierboom, E. Breedveld, B. 't Hart, S. Poelmans, L. Bontinck, A. Hemeryck, S. Jacobs, J. Baumeister, and H. Ulrichts, "The preclinical pharmacology of the high affinity anti-IL-6R Nanobody® ALX-0061 supports its clinical development in rheumatoid arthritis.," *Arthritis Res. Ther.*, vol. 17, p. 135, Jan. 2015.

[241] L. Detalle, T. Stohr, C. Palomo, P. A. Piedra, B. E. Gilbert, V. Mas, A. Millar, U. F. Power, C. Stortelers, K. Allosery, J. A. Melero, and E. Depla, "Generation and Characterization of ALX-0171, a Potent Novel Therapeutic Nanobody for the Treatment of Respiratory Syncytial Virus Infection.," *Antimicrob. Agents Chemother.*, vol. 60, no. 1, pp. 6–13, Jan. 2016.

[242] L. M. Damasceno, K. A. Anderson, G. Ritter, J. M. Cregg, L. J. Old, and C. A. Batt, "Cooverexpression of chaperones for enhanced secretion of a single-chain antibody fragment in *Pichia pastoris*.," *Appl. Microbiol. Biotechnol.*, vol. 74, no. 2, pp. 381–389, Feb. 2007.

[243] B. Gasser and D. Mattanovich, "Antibody production with yeasts and filamentous fungi: on the road to large scale?," *Biotechnol. Lett.*, vol. 29, no. 2, pp. 201–212, Feb. 2007.

[244] K. Takahashi, T. Yuuki, T. Takai, C. Ra, K. Okumura, T. Yokota, and Y. Okumura, "Production of humanized Fab fragment against human high affinity IgE receptor in Pichia pastoris.," *Biosci. Biotechnol. Biochem.*, vol. 64, no. 10, pp. 2138–2144, Oct. 2000.

[245] D. Ning, X. Junjian, Z. Qing, X. Sheng, C. Wenyin, R. Guirong, and W. Xunzhang, "Production of recombinant humanized anti-HBsAg Fab fragment from *Pichia pastoris* by fermentation.," *J. Biochem. Mol. Biol.*, vol. 38, no. 3, pp. 294–299, May 2005.

[246] A. Maccani, N. Landes, G. Stadlmayr, D. Maresch, C. Leitner, M. Maurer, B. Gasser, W. Ernst, R. Kunert, and D. Mattanovich, "*Pichia pastoris* secretes recombinant proteins less efficiently than Chinese hamster ovary cells but allows higher space-time yields for less complex proteins.," *Biotechnol. J.*, vol. 9, no. 4, pp. 526–537, Apr. 2014.

[247] A. A. Ogunjimi, J. M. Chandler, C. M. Gooding, A. R. III, and P. V. Choudary, "High-level secretory expression of immunologically active intact antibody from the yeast *Pichia pastoris*," *Biotechnol. Lett.*, vol. 21, no. 6, pp. 561–567, 1999.

[248] T. I. Potgieter, S. D. Kersey, M. R. Mallem, A. C. Nylen, and M. d'Anjou, "Antibody expression kinetics in glycoengineered *Pichia pastoris*.," *Biotechnol. Bioeng.*, vol. 106, no. 6, pp. 918–927, Aug. 2010.

[249] A. H. Horwitz, C. P. Chang, M. Better, K. E. Hellstrom, and R. R. Robinson, "Secretion of functional antibody and Fab fragment from yeast cells.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 85, no. 22, pp. 8678–8682, Nov. 1988.

[250] L. G. J. Frenken, E. van Tuijl, W. J. Bos, W. H. Mueller, A. J. Verkleij, and C. T. Verrips, "ScFv Antibody Fragments Produced in *Saccharomyces cerevisiae* Accumulate in the Endoplasmic Reticulum and the Vacuole," in *Biological Membranes: Structure, Biogenesis and Dynamics*, 1994, pp. 223–236.

[251] B. J. Hackel, D. Huang, J. C. Bubolz, X. X. Wang, and E. V Shusta, "Production of soluble and active transferrin receptor-targeting single-chain antibody using *Saccharomyces cerevisiae*.," *Pharm. Res.*, vol. 23, no. 4, pp. 790–797, Apr. 2006.

[252] F. Parsaie Nasab, M. Aebi, G. Bernhard, and A. D. Frey, "A combined system for engineering glycosylation efficiency and glycan structure in *Saccharomyces cerevisiae*.," *Appl. Environ. Microbiol.*, vol. 79, no. 3, pp. 997–1007, Feb. 2013.

[253] M. A. Piirainen, H. Boer, J. C. de Ruijter, and A. D. Frey, "A dual approach for improving

homogeneity of a human-type N-glycan structure in *Saccharomyces cerevisiae*," *Glycoconj. J.*, vol. 33, no. 2, pp. 189–199, 2016.

[254] P. P. Jacobs, S. Geysens, W. Vervecken, R. Contreras, and N. Callewaert, "Engineering complex-type N-glycosylation in *Pichia pastoris* using GlycoSwitch technology.," *Nat. Protoc.*, vol. 4, no. 1, pp. 58–70, 2009.

[255] W. Vervecken, N. Callewaert, V. Kaigorodov, S. Geysens, and R. Contreras, "Modification of the N-glycosylation pathway to produce homogeneous, human-like glycans using GlycoSwitch plasmids.," *Methods Mol Biol*, vol. 389, pp. 119–138, 2007.

[256] P. Bobrowicz, R. C. Davidson, H. Li, T. I. Potgieter, J. H. Nett, S. R. Hamilton, T. A. Stadheim, R. G. Miele, B. Bobrowicz, T. Mitchell, S. Rausch, E. Renfer, and S. Wildt, "Engineering of an artificial glycosylation pathway blocked in core oligosaccharide assembly in the yeast *Pichia pastoris*: Production of complex humanized glycoproteins with terminal galactose," *Glycobiology*, vol. 14, no. 9, pp. 757–766, May 2004.

[257] H. Li, N. Sethuraman, T. A. Stadheim, D. Zha, B. Prinz, N. Ballew, P. Bobrowicz, B.-K. Choi, W. J. Cook, M. Cukan, N. R. Houston-Cummings, R. Davidson, B. Gong, S. R. Hamilton, J. P. Hoopes, Y. Jiang, N. Kim, R. Mansfield, J. H. Nett, S. Rios, R. Strawbridge, S. Wildt, and T. U. Gerngross, "Optimization of humanized IgGs in glycoengineered *Pichia pastoris*.," *Nat. Biotechnol.*, vol. 24, no. 2, pp. 210–215, Feb. 2006.

[258] S. R. Hamilton, P. Bobrowicz, B. Bobrowicz, R. C. Davidson, H. Li, T. Mitchell, J. H. Nett, S. Rausch, T. A. Stadheim, H. Wischnewski, S. Wildt, and T. U. Gerngross, "Production of complex human glycoproteins in yeast.," *Science*, vol. 301, no. 5637, pp. 1244–1246, 2003.

[259] N. Zhang, L. Liu, C. Dan Dumitru, N. R. H. Cummings, M. Cukan, Y. Jiang, Y. Li, F. Li, T. Mitchell, M. R. Mallem, Y. Ou, R. N. Patel, K. Vo, H. Wang, I. Burnina, B.-K. Choi, H. E. Huber, T. A. Stadheim, and D. Zha, "Glycoengineered *Pichia* produced anti-HER2 is comparable to trastuzumab in preclinical study," *MAbs*, vol. 3, no. 3, pp. 289–298, Oct. 2014.

[260] L. Liu, A. Stadheim, L. Hamuro, T. Pittman, W. Wang, D. Zha, J. Hochman, and T. Prueksaritanont, "Pharmacokinetics of IgG1 monoclonal antibodies produced in humanized *Pichia pastoris* with specific glycoforms: a comparative study with CHO produced materials.," *Biologicals*, vol. 39, no. 4, pp. 205–210, Jul. 2011.

[261] P. Mease, V. Strand, L. Shalamberidze, A. Dimic, T. Raskina, L.-A. Xu, Y. Liu, and J. Smith, "A phase II, double-blind, randomised, placebo-controlled study of BMS945429 (ALD518) in patients with rheumatoid arthritis with an inadequate response to methotrexate.," *Ann. Rheum. Dis.*, vol. 71, no. 7, pp. 1183–1189, Jul. 2012.

[262] T. J. Bayliss, J. T. Smith, M. Schuster, K. H. Dragnev, and J. R. Rigas, "A humanized anti-IL-6 antibody (ALD518) in non-small cell lung cancer.," *Expert Opin. Biol. Ther.*, vol. 11, no. 12, pp. 1663–1668, Dec. 2011.

[263] Y. Chiba, M. Suzuki, S. Yoshida, A. Yoshida, H. Ikenaga, M. Takeuchi, Y. Jigami, and E. Ichishima, "Production of human compatible high mannose-type (Man5GlcNAc2) sugar chains in *Saccharomyces cerevisiae*," *J. Biol. Chem.*, vol. 273, no. 41, pp. 26298–26304, Oct. 1998.

[264] E. Nyyssönen, M. Penttilä, A. Harkki, A. Saloheimo, J. K. Knowles, and S. Keränen, "Efficient production of antibody fragments by the filamentous fungus *Trichoderma reesei*.," *Biotechnology. (N. Y).*, vol. 11, no. 5, pp. 591–595, May 1993.

[265] E. Nyyssönen and S. Keränen, "Multiple roles of the cellulase CBHI in enhancing production of fusion antibodies by the filamentous fungus *Trichoderma reesei*.," *Curr. Genet.*, vol. 28, no. 1, pp. 71–79, Jun. 1995.

[266] C. P. Landowski, A. Huuskonen, R. Wahl, A. Westerholm-Parvinen, A. Kanerva, A.-L. Hänninen, N. Salovuori, M. Penttilä, J. Natunen, C. Ostermeier, B. Helk, J. Saarinen, and M. Saloheimo, "Enabling Low Cost Biopharmaceuticals: A Systematic Approach to Delete Proteases from a Well-Known Protein Production Host *Trichoderma reesei*.," *PLoS One*, vol. 10, no. 8, p. e0134723, Jan. 2015.

[267] C. Landowski, A. Huuskonen, J. Saarinen, A. Westerholm-Parvinen, A. Kanerva, J. Natunen, A.-L. Hänninen, N. Salovuori, M. Penttilä, and M. Saloheimo, "Protease Deficient Filamentous Fungal Cells And Methods of Use Thereof.," International #WO/2013/102674, 2013.

[268] C. Landowski, A. Huuskonen, A. Westerholm-Parvinen, M. Saloheimo, A. Kanerva, and J. Hiltunen, "Multiple proteases deficient filamentous fungal cells and methods of use thereof.," International #WO/2015/004241, 2015.

[269] A. Sotiriadis, T. Keshavarz, and E. Keshavarz-Moore, "Factors Affecting the Production of a Single-Chain Antibody Fragment by Aspergillus awamori in a Stirred Tank Reactor,"

Biotechnol. Prog., vol. 17, no. 4, pp. 618–623, Aug. 2001.
[270] R. J. Gouka, P. J. Punt, and C. A. M. J. J. van den Hondel, "Efficient production of secreted proteins by *Aspergillus* : progress, limitations and prospects," *Appl. Microbiol. Biotechnol.*, vol. 47, no. 1, pp. 1–11, Jan. 1997.
[271] L. G. J. Frenken, J. G. M. Hessing, C. A. M. J. J. Van den Hondel, and C. T. Verrips, "Recent advances in the large-scale production of antibody fragments using lower eukaryotic microorganisms," *Res. Immunol.*, vol. 149, no. 6, pp. 589–599, Jul. 1998.
[272] M. Ward, C. Lin, D. C. Victoria, B. P. Fox, J. A. Fox, D. L. Wong, H. J. Meerman, J. P. Pucci, R. B. Fong, M. H. Heng, N. Tsurushita, C. Gieswein, M. Park, and H. Wang, "Characterization of humanized antibodies secreted by *Aspergillus niger*.," *Appl. Environ. Microbiol.*, vol. 70, no. 5, pp. 2567–76, May 2004.
[273] R. Strasser, F. Altmann, and H. Steinkellner, "Controlled glycosylation of plant-produced recombinant proteins," *Current Opinion in Biotechnology*, vol. 30. pp. 95–100, 2014.
[274] K. M. Cox, J. D. Sterling, J. T. Regan, J. R. Gasdaska, K. K. Frantz, C. G. Peele, A. Black, D. Passmore, C. Moldovan-Loomis, M. Srinivasan, S. Cuison, P. M. Cardarelli, and L. F. Dickey, "Glycan optimization of a human monoclonal antibody in the aquatic plant *Lemna minor*.," *Nat. Biotechnol.*, vol. 24, no. 12, pp. 1591–1597, Dec. 2006.
[275] V. Gomord and L. Faye, "Posttranslational modification of therapeutic proteins in plants.," *Curr. Opin. Plant Biol.*, vol. 7, no. 2, pp. 171–181, Apr. 2004.
[276] R. Piron, F. Santens, A. De Paepe, A. Depicker, and N. Callewaert, "Using GlycoDelete to produce proteins lacking plant-specific N-glycan modification in seeds.," *Nat. Biotechnol.*, vol. 33, no. 11, pp. 1135–1137, Nov. 2015.
[277] G. Moussavou, K. Ko, J.-H. Lee, and Y.-K. Choo, "Production of monoclonal antibodies in plants for cancer immunotherapy.," *Biomed Res. Int.*, vol. 2015, p. 306164, Jan. 2015.
[278] V. Yusibov, N. Kushnir, and S. J. Streatfield, "Antibody Production in Plants and Green Algae.," *Annu. Rev. Plant Biol.*, vol. 67, pp. 669–701, Apr. 2016.
[279] J. R. Gasdaska, S. Sherwood, J. T. Regan, and L. F. Dickey, "An afucosylated anti-CD20 monoclonal antibody with greater antibody-dependent cellular cytotoxicity and B-cell depletion and lower complement-dependent cytotoxicity than rituximab.," *Mol. Immunol.*, vol. 50, no. 3, pp. 134–141, Mar. 2012.
[280] R. Valdés, L. Gómez, S. Padilla, J. Brito, B. Reyes, T. Alvarez, O. Mendoza, O. Herrera, W. Ferro, M. Pujol, V. Leal, M. Linares, Y. Hevia, C. García, L. Milá, O. García, R. Sánchez, A. Acosta, D. Geada, R. Paez, J. Luis Vega, and C. Borroto, "Large-scale purification of an antibody directed against hepatitis B surface antigen from transgenic tobacco plants.," *Biochem. Biophys. Res. Commun.*, vol. 308, no. 1, pp. 94–100, Aug. 2003.
[281] R. Valdés, B. Reyes, T. Alvarez, J. García, J. A. Montero, A. Figueroa, L. Gómez, S. Padilla, D. Geada, M. C. Abrahantes, L. Dorta, D. Fernández, O. Mendoza, N. Ramirez, M. Rodriguez, M. Pujol, C. Borroto, and J. Brito, "Hepatitis B surface antigen immunopurification using a plant-derived specific antibody produced in large scale.," *Biochem. Biophys. Res. Commun.*, vol. 310, no. 3, pp. 742–747, Oct. 2003.
[282] M. Pujol, N. I. Ramírez, M. Ayala, J. V Gavilondo, R. Valdés, M. Rodríguez, J. Brito, S. Padilla, L. Gómez, B. Reyes, R. Peral, M. Pérez, J. L. Marcelo, L. Milá, R. F. Sánchez, R. Páez, J. A. Cremata, G. Enríquez, O. Mendoza, M. Ortega, and C. Borroto, "An integral approach towards a practical application for a plant-made monoclonal antibody in vaccine purification.," *Vaccine*, vol. 23, no. 15, pp. 1833–1837, Mar. 2005.
[283] R. B. Santos, R. Abranches, R. Fischer, M. Sack, and T. Holland, "Putting the Spotlight Back on Plant Suspension Cultures," *Front. Plant Sci.*, vol. 7, p. 297, 2016.
[284] T. Holland, M. Sack, T. Rademacher, K. Schmale, F. Altmann, J. Stadlmann, R. Fischer, and S. Hellwig, "Optimal nitrogen supply as a key to increased and sustained production of a monoclonal full-size antibody in BY-2 suspension culture," *Biotechnol. Bioeng.*, vol. 107, no. 2, pp. 278–289, Oct. 2010.
[285] A. Yano, F. Maeda, and M. Takekoshi, "Transgenic Tobacco Cells Producing the Human Monoclonal Antibody to Hepatitis B Virus Surface Antigen," *J. Med. Virol.*, vol. 73, no. 2, pp. 208–215, Jun. 2004.
[286] N. Raven, S. Rasche, C. Kuehn, T. Anderlei, W. Klöckner, F. Schuster, M. Henquet, D. Bosch, J. Büchs, R. Fischer, and S. Schillberg, "Scaled-up manufacturing of recombinant antibodies produced by plant cells in a 200-L orbitally-shaken disposable bioreactor," *Biotechnol. Bioeng.*, vol. 112, no. 2, pp. 308–321, Feb. 2015.
[287] S. Kim, D. Y. Lee, G. Wohlgemuth, H. S. Park, O. Fiehn, and K. H. Kim, "Evaluation and

optimization of metabolome sample preparation methods for *Saccharomyces cerevisiae*.," *Anal. Chem.*, vol. 85, no. 4, pp. 2169–76, Feb. 2013.
[288] F. E. Demircioglu, P. Burkhardt, and D. Fasshauer, "The SM protein Sly1 accelerates assembly of the ER-Golgi SNARE complex.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 111, no. 38, pp. 13828–13833, Sep. 2014.
[289] J. H. D. Van Zyl, R. Den Haan, and W. H. Van Zyl, "Over-expression of native *Saccharomyces cerevisiae* exocytic SNARE genes increased heterologous cellulase secretion.," *Appl. Microbiol. Biotechnol.*, vol. 98, no. 12, pp. 5567–5578, Mar. 2014.
[290] J. Hou, K. E. J. Tyo, Z. Liu, D. Petranovic, and J. Nielsen, "Metabolic engineering of recombinant protein secretion by *Saccharomyces cerevisiae*.," *FEMS Yeast Res.*, vol. 12, no. 5, pp. 491–510, Aug. 2012.
[291] N. Alikhani, A.-K. Berglund, T. Engmann, E. Spånning, F.-N. Vögtle, P. Pavlov, C. Meisinger, T. Langer, and E. Glaser, "Targeting capacity and conservation of PreP homologues localization in mitochondria of different species.," *J. Mol. Biol.*, vol. 410, no. 3, pp. 400–410, Jul. 2011.
[292] L. Jønson, J. F. Rehfeld, and A. H. Johnsen, "Enhanced peptide secretion by gene disruption of *CYM1*, a novel protease in *Saccharomyces cerevisiae*.," *Eur. J. Biochem.*, vol. 271, no. 23–24, pp. 4788–97, Dec. 2004.
[293] J. S. Cox, R. E. Chapman, and P. Walter, "The unfolded protein response coordinates the production of endoplasmic reticulum protein and endoplasmic reticulum membrane.," *Mol. Biol. Cell*, vol. 8, no. 9, pp. 1805–1814, Sep. 1997.
[294] P. Chumnanpuen, I. Nookaew, and J. Nielsen, "Integrated analysis, transcriptome-lipidome, reveals the effects of INO-level (*INO2* and *INO4*) on lipid metabolism in yeast.," *BMC Syst. Biol.*, vol. 7 Suppl 3, p. S7, Jan. 2013.
[295] G. M. Carman and G.-S. Han, "Regulation of phospholipid synthesis in yeast," *J. Lipid Res.*, vol. 50, no. Supplement, pp. S69–S73, Dec. 2008.
[296] U. Güldener, S. Heck, T. Fielder, J. Beinhauer, and J. H. Hegemann, "A new efficient gene disruption cassette for repeated use in budding yeast.," *Nucleic Acids Res.*, vol. 24, no. 13, pp. 2519–2524, Jul. 1996.
[297] M. Guerfal, K. Claes, O. Knittelfelder, R. De Rycke, S. D. Kohlwein, and N. Callewaert, "Enhanced membrane protein expression by engineering increased intracellular membrane production.," *Microb. Cell Fact.*, vol. 12, no. 1, p. 122, Jan. 2013.
[298] Z. Zhang, L. P. Song, M. Fang, F. Wang, D. He, R. Zhao, J. Liu, Z. Y. Zhou, C. C. Yin, Q. Lin, and H. L. Huang, "Production of soluble and functional engineered antibodies in *Escherichia coli* improved by FkpA," *Biotechniques*, vol. 35, no. 5, pp. 1032–1042, Nov. 2003.
[299] A. Bertolotti, Y. Zhang, L. M. Hendershot, H. P. Harding, and D. Ron, "Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response.," *Nat. Cell Biol.*, vol. 2, no. 6, pp. 326–332, Jun. 2000.
[300] J. Chillarón and I. G. Haas, "Dissociation from BiP and retrotranslocation of unassembled immunoglobulin light chains are tightly coupled to proteasome activity.," *Mol. Biol. Cell*, vol. 11, no. 1, pp. 217–226, Jan. 2000.
[301] M. Valaviciute, M. Norkiene, K. Goda, R. Slibinskas, and A. Gedvilaite, "Survey of molecular chaperone requirement for the biosynthesis of hamster polyomavirus VP1 protein in *Saccharomyces cerevisiae*.," *Arch. Virol.*, vol. 161, no. 7, pp. 1807–1819, Jul. 2016.
[302] R. Gonzalez, B. A. Andrews, J. Molitor, and J. A. Asenjo, "Metabolic analysis of the synthesis of high levels of intracellular human SOD in *Saccharomyces cerevisiae* rhSOD 2060 411 SGA122.," *Biotechnol. Bioeng.*, vol. 82, no. 2, pp. 152–169, Apr. 2003.
[303] A. Kazemi Seresht, A. L. Cruz, E. de Hulster, M. Hebly, E. A. Palmqvist, W. van Gulik, J.-M. Daran, J. Pronk, and L. Olsson, "Long-term adaptation of *Saccharomyces cerevisiae* to the burden of recombinant insulin production.," *Biotechnol. Bioeng.*, vol. 110, no. 10, pp. 2749–2763, Apr. 2013.
[304] J. F. Görgens, W. H. van Zyl, J. H. Knoetze, and B. Hahn-Hägerdal, "The metabolic burden of the PGK1 and ADH2 promoter systems for heterologous xylanase production by *Saccharomyces cerevisiae* in defined medium.," *Biotechnol. Bioeng.*, vol. 73, no. 3, pp. 238–245, May 2001.
[305] J. Apodaca, I. Kim, and H. Rao, "Cellular tolerance of prion protein PrP in yeast involves proteolysis and the unfolded protein response.," *Biochem. Biophys. Res. Commun.*, vol. 347, no. 1, pp. 319–326, Aug. 2006.
[306] S. Li, R. A. Spooner, R. Y. Hampton, J. M. Lord, and L. M. Roberts, "Cytosolic entry of Shiga-

like toxin a chain from the yeast endoplasmic reticulum requires catalytically active Hrd1p.," *PLoS One*, vol. 7, no. 7, p. e41119, Jan. 2012.

[307] N. D. S. P. Carvalho, M. Arentshorst, R. Kooistra, H. Stam, C. M. Sagt, C. A. M. J. J. van den Hondel, and A. F. J. Ram, "Effects of a defective ERAD pathway on growth and heterologous protein production in *Aspergillus niger*.," *Appl. Microbiol. Biotechnol.*, vol. 89, no. 2, pp. 357–373, Jan. 2011.

[308] U.-M. Bailey and B. L. Schulz, "Deglycosylation systematically improves N-glycoprotein identification in liquid chromatography-tandem mass spectrometry proteomics for analysis of cell wall stress responses in *Saccharomyces cerevisiae* lacking Alg3p.," *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.*, vol. 923–924, pp. 16–21, Apr. 2013.

[309] T. Izawa, H. Nagai, T. Endo, and S. Nishikawa, "Yos9p and Hrd1p mediate ER retention of misfolded proteins for ER-associated degradation.," *Mol. Biol. Cell*, vol. 23, no. 7, pp. 1283–1293, Apr. 2012.

[310] R. Z. Edros, S. McDonnell, and M. Al-Rubeai, "Using molecular markers to characterize productivity in Chinese hamster ovary cell lines.," *PLoS One*, vol. 8, no. 10, p. e75935, Jan. 2013.

[311] N. Borth, K. Strutzenberger, R. Kunert, W. Steinfellner, and H. Katinger, "Analysis of changes during subclone development and ageing of human antibody-producing heterohybridoma cells by northern blot and flow cytometry.," *J. Biotechnol.*, vol. 67, no. 1, pp. 57–66, Jan. 1999.

[312] M. Vanhove, Y. K. Usherwood, and L. M. Hendershot, "Unassembled Ig heavy chains do not cycle from BiP in vivo but require light chains to trigger their release.," *Immunity*, vol. 15, no. 1, pp. 105–114, Jul. 2001.

[313] N. Kanjou, A. Nagao, Y. Ohmiya, and S. Ohgiya, "Yeast mutant with efficient secretion identified by a novel secretory reporter, Cluc.," *Biochem. Biophys. Res. Commun.*, vol. 358, no. 2, pp. 429–434, Jul. 2007.

[314] J. M. Wingfield and J. R. Dickinson, "The activity of a model heterologous protein in pep4-3 mutants of *Saccharomyces cerevisiae*.," *Appl. Microbiol. Biotechnol.*, vol. 36, no. 6, pp. 754–758, Mar. 1992.

[315] Z. X. Sun H, Wang T, Zhang J, Liu Q, Wang L, Chen P, Wang F, Li H, Xiao Y, "Display of heterologous proteins on the *Saccharomyces cerevisiae* surface display system using a single constitutive expression vector.," *Biotechnol. Prog.*, vol. 30, no. 2, pp. 443–450, Jan. 2013.

[316] A. Rakestraw and K. D. Wittrup, "Contrasting secretory processing of simultaneously expressed heterologous proteins in *Saccharomyces cerevisiae*.," *Biotechnol. Bioeng.*, vol. 93, no. 5, pp. 896–905, Apr. 2006.

[317] A. Kumar, M. Seringhaus, M. C. Biery, R. J. Sarnovsky, L. Umansky, S. Piccirillo, M. Heidtman, K.-H. Cheung, C. J. Dobry, M. B. Gerstein, N. L. Craig, and M. Snyder, "Large-scale mutagenesis of the yeast genome using a Tn7-derived multipurpose transposon.," *Genome Res.*, vol. 14, no. 10A, pp. 1975–1986, Oct. 2004.

[318] G. Giaever, A. M. Chu, L. Ni, C. Connelly, L. Riles, S. Véronneau, S. Dow, A. Lucau-Danila, K. Anderson, B. André, A. P. Arkin, A. Astromoff, M. El-Bakkoury, R. Bangham, R. Benito, S. Brachat, S. Campanaro, M. Curtiss, K. Davis, A. Deutschbauer, K.-D. Entian, P. Flaherty, F. Foury, D. J. Garfinkel, M. Gerstein, D. Gotte, U. Güldener, J. H. Hegemann, S. Hempel, Z. Herman, D. F. Jaramillo, D. E. Kelly, S. L. Kelly, P. Kötter, D. LaBonte, D. C. Lamb, N. Lan, H. Liang, H. Liao, L. Liu, C. Luo, M. Lussier, R. Mao, P. Menard, S. L. Ooi, J. L. Revuelta, C. J. Roberts, M. Rose, P. Ross-Macdonald, B. Scherens, G. Schimmack, B. Shafer, D. D. Shoemaker, S. Sookhai-Mahadeo, R. K. Storms, J. N. Strathern, G. Valle, M. Voet, G. Volckaert, C. Wang, T. R. Ward, J. Wilhelmy, E. A. Winzeler, Y. Yang, G. Yen, E. Youngman, K. Yu, H. Bussey, J. D. Boeke, M. Snyder, P. Philippsen, R. W. Davis, and M. Johnston, "Functional profiling of the *Saccharomyces cerevisiae* genome.," *Nature*, vol. 418, no. 6896, pp. 387–391, Jul. 2002.

[319] S. Ben-Aroya, C. Coombes, T. Kwok, K. A. O'Donnell, J. D. Boeke, and P. Hieter, "Toward a comprehensive temperature-sensitive mutant repository of the essential genes of *Saccharomyces cerevisiae*.," *Mol. Cell*, vol. 30, no. 2, pp. 248–258, Apr. 2008.

[320] M. Zagorec, J. M. Buhler, I. Treich, T. Keng, L. Guarente, and R. Labbe-Bois, "Isolation, sequence, and regulation by oxygen of the yeast *HEM13* gene coding for coproporphyrinogen oxidase.," *J. Biol. Chem.*, vol. 263, no. 20, pp. 9718–9724, Jul. 1988.

[321] P. S. R. Coelho, A. C. Bryan, A. Kumar, G. S. Shadel, and M. Snyder, "A novel mitochondrial protein, Tar1p, is encoded on the antisense strand of the nuclear 25S rDNA.," *Genes Dev.*, vol. 16, no. 21, pp. 2755–2760, Nov. 2002.

[322] A. Galopier and S. Hermann-Le Denmat, “Mitochondria of the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis* contain nuclear rDNA-encoded proteins.,” *PLoS One*, vol. 6, no. 1, p. e16325, Jan. 2011.
[323] N. D. Bonawitz, M. Chatenay-Lapointe, C. M. Wearn, and G. S. Shadel, “Expression of the rDNA-encoded mitochondrial protein Tar1p is stringently controlled and responds differentially to mitochondrial respiratory demand and dysfunction.,” *Curr. Genet.*, vol. 54, no. 2, pp. 83–94, Aug. 2008.
[324] M. N. Seaman, E. G. Marcusson, J. L. Cereghino, and S. D. Emr, “Endosome to Golgi retrieval of the vacuolar protein sorting receptor, Vps10p, requires the function of the VPS29, VPS30, and VPS35 gene products.,” *J. Cell Biol.*, vol. 137, no. 1, pp. 79–92, Apr. 1997.
[325] Z. Liu, L. Liu, T. Österlund, J. Hou, M. Huang, L. Fagerberg, D. Petranovic, M. Uhlén, and J. Nielsen, “Improved production of a heterologous amylase in *Saccharomyces cerevisiae* by inverse metabolic engineering.,” *Appl. Environ. Microbiol.*, vol. 80, no. 17, pp. 5542–5550, Sep. 2014.
[326] K. B. Kruse, J. L. Brodsky, and A. A. McCracken, “Characterization of an ERAD gene as *VPS30/ATG6* reveals two alternative and functionally distinct protein quality control pathways: one for soluble Z variant of human alpha-1 proteinase inhibitor (A1PiZ) and another for aggregates of A1PiZ.,” *Mol. Biol. Cell*, vol. 17, no. 1, pp. 203–212, Jan. 2006.
[327] B. R. Glick, “Metabolic load and heterologous gene expression,” *Biotechnology Advances*, vol. 13, no. 2. Elsevier, pp. 247–261, Jan-1995.
[328] J. F. Görgens, W. H. Van Zyl, J. H. Knoetze, and B. Hahn-Hägerdal, “The metabolic burden of the *PGK1* and *ADH2* promoter systems for heterologous xylanase production by *Saccharomyces cerevisiae* in defined medium,” *Biotechnol. Bioeng.*, vol. 73, no. 3, pp. 238–245, May 2001.
[329] A. S. Karim, K. A. Curran, and H. S. Alper, “Characterization of plasmid burden and copy number in *Saccharomyces cerevisiae* for optimization of metabolic engineering applications.,” *FEMS Yeast Res.*, vol. 13, no. 1, pp. 107–116, Feb. 2013.
[330] E. Van Rensburg, R. Den Haan, J. Smith, W. H. Van Zyl, and J. F. Görgens, “The metabolic burden of cellulase expression by recombinant *Saccharomyces cerevisiae* Y294 in aerobic batch culture,” *Appl. Microbiol. Biotechnol.*, vol. 96, no. 1, pp. 197–209, Oct. 2012.
[331] S. Jin, K. Ye, and K. Shimizu, “Metabolic flux distributions in recombinant *Saccharomyces cerevisiae* during foreign protein production,” *J. Biotechnol.*, vol. 54, no. 3, pp. 161–174, May 1997.
[332] S. Karlin, L. Brocchieri, J. Trent, B. E. Blaisdell, and J. Mrázek, “Heterogeneity of Genome and Proteome Content in Bacteria, Archaea, and Eukaryotes,” *Theor. Popul. Biol.*, vol. 61, no. 4, pp. 367–390, Jun. 2002.
[333] J. F. Görgens, W. H. Van Zyl, J. H. Knoetze, and B. Hahn-Hägerdal, “Amino acid supplementation improves heterologous protein production by *Saccharomyces cerevisiae* in defined medium,” *Appl. Microbiol. Biotechnol.*, vol. 67, no. 5, pp. 684–691, Jun. 2005.
[334] J. W. Cuozzo and C. A. Kaiser, “Competition between glutathione and protein thiols for disulphide-bond formation.,” *Nat. Cell Biol.*, vol. 1, no. 3, pp. 130–135, Jul. 1999.
[335] S. T. Coleman, T. K. Fang, S. A. Rovinsky, F. J. Turano, and W. S. Moye-Rowley, “Expression of a glutamate decarboxylase homologue is required for normal oxidative stress tolerance in *Saccharomyces cerevisiae*.,” *J. Biol. Chem.*, vol. 276, no. 1, pp. 244–250, Jan. 2001.
[336] C. Panozzo, M. Nawara, C. Suski, R. Kucharczyka, M. Skoneczny, A. M. Bécam, J. Rytka, and C. J. Herbert, “Aerobic and anaerobic NAD+ metabolism in Saccharomyces cerevisiae,” *FEBS Lett.*, vol. 517, no. 1–3, pp. 97–102, Apr. 2002.
[337] A. Kazemi Seresht, E. A. Palmqvist, G. Schluckebier, I. Pettersson, and L. Olsson, “The Challenge of Improved Secretory Production of Active Pharmaceutical Ingredients in *Saccharomyces cerevi*siae: A Case Study on Human Insulin Analogs.,” *Biotechnol. Bioeng.*, vol. 110, no. 10, pp. 2764–2774 Apr. 2013.
[338] P. I. Merksamer, A. Trusina, and F. R. Papa, “Real-time redox measurements during endoplasmic reticulum stress reveal interlinked protein folding functions.,” *Cell*, vol. 135, no. 5, pp. 933–947, Nov. 2008.
[339] B. Hahn-Hägerdal, K. Karhumaa, C. U. Larsson, M. Gorwa-Grauslund, J. Görgens, W. H. van Zyl, “Role of cultivation media in the development of yeast strains for large scale industrial use,” *Microb. Cell Fact.*, vol. 4, no. 1, p. 31, 2005.
[340] O. Mendoza-Vega, J. Sabatié, S. W. Brown, “Industrial production of heterologous proteins by fed-batch cultures of the yeast *Saccharomyces cerevisiae*.,” *FEMS Microbiol. Rev.*, vol. 15, no.

4, pp. 369–410, Dec. 1994.
[341] F. M. Wurm, “Production of recombinant protein therapeutics in cultivated mammalian cells,” *Nat Biotechnol*, vol. 22, no. 11, pp. 1393–1398, Nov. 2004.
[342] W. Zhang, H.-L. Zhao, C. Xue, X. Xiong, X. Yao, X. Li, H. Chen, and Z. Liu, “Enhanced secretion of heterologous proteins in Pichia pastoris following overexpression of *Saccharomyces cerevisiae* chaperone proteins.,” *Biotechnol. Prog.*, vol. 22, no. 4, pp. 1090–1095, 2006.

The yeast *Saccharomyces cerevisiae* is a well known organism in biotechnology, and it has been widely used as an expression host for the manufacturing of products like biofuels, small molecules, and of recombinant proteins. To increase the yields of economically interesting proteins, the secretory pathway has been engineered extensively, however, secretion titers have often remained low. One example for these shortcomings are full-length IgG antibodies, which play an important role in human diagnostics, therapeutical applications, and are used in a wide range of research methods. The recombinant antibodies are currently mostly produced in CHO cells, although microbial and plant based production platforms are emerging. In this thesis, using targeted and random screening approaches, we aim to identify genetic factors that can be used to create yeast strains with an increased secretion efficiency of a full-length IgG molecule.

Aalto-DD 277/2016

ISBN 978-952-60-7223-4 (printed)
ISBN 978-952-60-7222-7 (pdf)
ISSN-L 1799-4934
ISSN 1799-4934 (printed)
ISSN 1799-4942 (pdf)

Aalto University
School of Chemical Technology
Department of Biotechnology and Chemical Technology
www.aalto.fi

BUSINESS + ECONOMY

ART + DESIGN + ARCHITECTURE

SCIENCE + TECHNOLOGY

CROSSOVER

DOCTORAL DISSERTATIONS