THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

# Engineering the Secretory Pathway for Recombinant Protein Secretion in *Saccharomyces cerevisiae*

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## Abstract

Over the past few decades, there has been an increasing demand for biopharmaceutical proteins. Several types of cell factories are used to produce different pharmaceutical proteins. However, manufacturers prefer to use a few favourable biological platforms with low costs, high productivity and proper post-translational modifications to undertake production tasks. The yeast *Saccharomyces cerevisiae* is a preferred cell factory because it has many advantages. There are several reports on the improvement of recombinant protein production by *S. cerevisiae* through the rational engineering of different stages of the protein secretion pathway.

Here we engineered protein anterograde trafficking by over-expressing *SEC16* to increase the secretory capacity of yeast. We performed bioreactor fermentation to further characterize the engineered strains, and we analysed the reactive oxygen species accumulation, endoplasmic reticulum exit sites, amount of endoplasmic reticulum membranes in different strains, etc. Next, we engineered retrograde trafficking by over-expressing *GLO3* and *GCS1* to further increase the secretory capacity of yeast based on the strain over-expressing *SEC16*. Physiological changes in the engineered strains were analysed. We also performed additional experiments to investigate the changes in the amount of endoplasmic reticulum membranes and reactive oxygen species accumulation. We also performed a systems-level analysis of strains with high α-amylase production, which were screened for UV mutations in an earlier study. We identified common regulation patterns and consequently could specify some general rules for efficient protein secretion. Lastly, we report on an efficient yeast secretion platform for biomedical and biotechnological applications. This platform is responsive to secretory disturbances from both chemicals and proteins and is potentially applicable for drug screening and for selecting cell engineering targets for protein production.

**Keywords:** *Saccharomyces cerevisiae*, protein secretion, *SEC16*, *GLO3*, transcriptomics, oxidative stress, endoplasmic reticulum, drug screening

## LIST OF PUBLICATIONS

The thesis is based on the following publications, referred to as Paper I to IV in the text:

I. **Bao J**.*, Huang M.*, Petranovic D., & Nielsen J. (2017) Moderate expression of SEC16 increases protein secretion by Saccharomyces cerevisiae. *Applied and Environmental Microbiology*, doi:10.1128/AEM.03400-16

II. **Bao J.**, Huang M., Petranovic D., & Nielsen J. Balanced trafficking between the ER and the Golgi apparatus increases protein secretion in yeast (Submitted to *Applied Microbiology and Biotechnology*)

III. Huang M., **Bao J.**, Hallström B M., Petranovic D., & Nielsen J., Efficient protein production by yeast requires global tuning of metabolism (Accepted by *Nature Communications*)

IV. Huang M., **Bao J.**, & Petranovic D., Yeast secretion assay platform for biomedical and biotechnological applications (Submitted to *FEMS Yeast research)*

Additional publications during doctoral research not included in this thesis:

V. Huang M., **Bao J.**, & Nielsen J. (2014) Biopharmaceutical protein production by *Saccharomyces cerevisiae*: current state and future prospects. *Pharmaceutical Bioprocessing*, 2, 167-182 (Review)

VI. Chen Y., **Bao J.**, Kim I., Siewers V., & Nielsen J. (2014) Coupled incremental precursor and co-factor supply improves 3-hydroxypropionic acid production in *Saccharomyces cerevisiae*. *Metabolic engineering*, 22, 104-109.

VII. Hu Y., Zhou Y., **Bao J.**, Huang L., Nielsen J. & Krivoruchko A. (2017) Metabolic engineering of *Saccharomyces cerevisiae* for production of germacrene A, a precursor of beta-elemene. *The Journal of Industrial Microbiology & Biotechnology*, doi:10.1007/s10295-017-1934-z

* Authors contributed equally to this work.

## CONTRIBUTION SUMMARY

I. Designed the study, performed the experiments, analysed the data, wrote the manuscript
II. Designed the study, performed the experiments, analysed the data, wrote the manuscript
III. Performed the experiments, assisted in manuscript preparation
IV. Performed the experiments, assisted in manuscript preparation
V. Assisted in manuscript preparation
VI. Performed the experiments, analysed the data, assisted in manuscript preparation
VII. Performed the experiments, analysed the data, assisted in manuscript preparation

## Preface

This dissertation is submitted for the partial fulfillment of the degree of doctor of philosophy. It is based on work carried out between December 2012 and February 2017 in the Systems and Synthetic Biology group, Department of Biology and Biological Engineering, Chalmers University of Technology, under the supervision of Professor Jens Nielsen. The research was funded by the Knut and Alice Wallenberg Foundation, the Chalmers Foundation, the European Research Council and the Novo Nordisk Foundation.

# Content

# Introduction

## *Saccharomyces cerevisiae*

*Saccharomyces cerevisiae* has been widely used by our ancestors to brew alcohol and bake bread. With the development of modern biotechnology and bioinformatics, extensive genome data have been generated, detailed metabolic networks have been deciphered leading to new insight into many physiological processes of *S. cerevisiae* [1-5]. These progresses have made *S. cerevisiae* one of the most studied microorganisms. Some properties of *S. cerevisiae* favour industrial use, such as fast growth and robustness. Owing to these benefits, *S. cerevisiae* is widely used as a cell factory for chemical and pharmaceutical production.

## Recombinant protein production

Over the past few years, the use of pharmaceutical proteins in disease therapy has increased [6]. The development of molecular biotechnology has paved the way for the production of pharmaceutical proteins by microbial cell factories [7]. Hundreds of pharmaceutical proteins have been approved for the market since the first recombinant therapy protein, human insulin, was launched in 1982 [7]. Recombinant biopharmaceuticals account for a quarter of commercial pharmaceuticals. In addition, biopharmaceutical sales account for 40% of total sales [8]. Insulin and insulin analogues, anti-TNF antibodies and cancer antibodies, as the top three categories, account for over 50% of sales and have a high growth rate of approximately 10-20% [9].

Recombinant proteins can be produced by different cell factories, for example, *Escherichia coli*, *S. cerevisiae*, insect cells, and mammalian cells [10, 11]. Each recombinant producer host has different advantages and disadvantages (Fig. 1). *E. coli* grows very fast; it is preferably used to produce simple and small recombinant proteins. However, because of the inefficient secretory capacity of *E. coli*, it might cost more in the downstream purification. In addition, as a prokaryotic organism, it is difficult for *E. coli* to perform proper posttranslational modifications (PTMs) on recombinant proteins. By contrast, Chinese hamster ovary (CHO) cells have an analogical glycosylation pattern similar to that of human cells; thus, CHO cells could perform complicated PTMs on recombinant proteins for human use. However, CHO cells have obvious drawbacks, including slow growth rate, sensitive culture

conditions, expensive medium and limited scale-up capacity. *S. cerevisiae* as a single-cell microorganism combines the advantages of both *E. coli* and CHO cells. *S. cerevisiae* has a high growth rate, low contamination risk, and complete protein secretory pathway. In addition, because *S. cerevisiae* is a eukaryotic microorganism, it can perform disulfide bond formation and proper glycosylation. Since it has high mannose type N-glycosylation, which is different from human cells, *S. cerevisiae* is limited in the production of complex recombinant proteins [12].

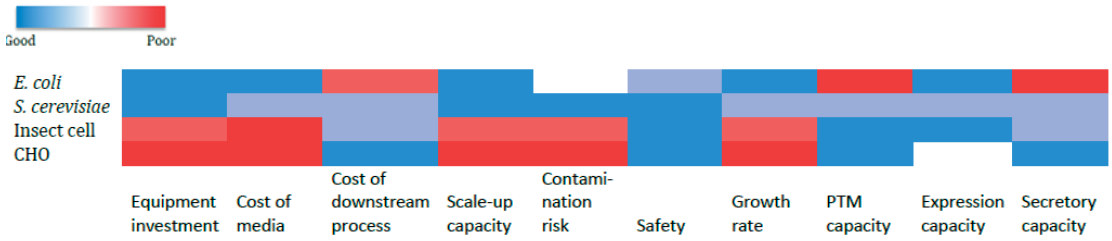


**Fig. 1 The criteria of the different expression systems.**

## Recombinant protein production in *S. cerevisiae*

*S. cerevisiae* has a long history of being widely used for recombinant biopharmaceutical production. In 1987, Novo (now Novo Nordisk) began industrial production of insulin using genetically engineered *S. cerevisiae*, which started a new era of insulin production instead of extraction of porcine insulin [7]. Currently, approximately 20% of recombinant pharmaceutical proteins are produced by *S. cerevisiae* [13], including insulin, vaccines, and blood factors. [14]. By June 2012, 23 recombinant therapeutic proteins produced by *S. cerevisiae* had been approved by the European Medicines Agency (Table 1). The advantages of *S. cerevisiae* are as follows: 1) it is a eukaryote, which makes it capable of folding recombinant proteins correctly; 2) *S. cerevisiae* grows faster than mammalian cells, and the cost of recombinant protein production by *S. cerevisiae* is normally cheaper than that by mammalian cells; 3) it has a complete protein secretory pathway so that the recombinant protein can be secreted into the medium, which lowers the cost of downstream processes; and 4) its complete genome information, available systems biology tools for studying *S. cerevisiae* and convenient genetic engineering operations make *S. cerevisiae* the cell factory of choice for industry. While many targets for improving recombinant protein secretion have been reported, many of these attempts did not show general effects on a range of protein secretion [15-18].

**Table 1 European Medicines Agency-approved biopharmaceuticals produced by *S. cerevisiae*, authorized year, company.**

| Biopharmaceutical | Authorized year | Company | Uses |
|---|---|---|---|
| Victoza® | 2009 | NVO | Diabetes |
| Silgard® | 2006 | MSD | Human Papillomavirus vaccination |
| Valtropin® | 2006 | BPG* | Somatropin for growth failure therapy |
| Fendrix® | 2005 | GSK | Hepatitis B vaccination |
| Levemir® | 2004 | NVO | Diabetes |
| Protaphane® | 2002 | NVO | Diabetes |
| Mixtard® | 2002 | NVO | Diabetes |
| Insulatard® | 2002 | NVO | Diabetes |
| Actrapid® | 2002 | NVO | Diabetes |
| Actraphane® | 2002 | NVO | Diabetes |
| Ambirix® | 2002 | GSK | Hepatitis B vaccination |
| HBVaxPro® | 2001 | SPM | Hepatitis B vaccination |
| Fasturtec® | 2001 | SAG | Hyperuricaemia |
| Infanrix® Penta | 2000 | GSK | Diphtheria, tetanus, and acellular pertussis vaccination |
| Infanrix® Hexa | 2000 | GSK | Diphtheria, tetanus, and acellular pertussis vaccination |
| NovoMix® | 2000 | NVO | Diabetes |
| NovoRapid® | 1999 | NVO | Diabetes |
| Regranex® | 1999 | JCI | Human growth factor for wound healing |
| Revasc® | 1997 | CPL | Venous thrombosis |
| Twinrix® Paediatric | 1997 | GSK | Hepatitis A and hepatitis B vaccination |
| Twinrix® Adult | 1996 | GSK | Hepatitis A and hepatitis B vaccination |
| Tritanrix® HepB | 1996 | GSK | Hepatitis B vaccination |

## Secretory pathway

### Translocation into the ER

The secretory pathway is highly conserved among eukaryotes (Fig. 2). The first step is called co-translational translocation. The mRNA of the recombinant protein is collected by a ribosome in the cytoplasm. Then, the ribosome starts translating the mRNA into protein. The first translated part of the polypeptide is called the signal peptide, which consists of pre- and pro-signal sequences and is recognized and bound by a signal recognition particle (SRP). In addition, the SRP hampers the continuous translation of the mRNA. When the complex is caught by an SRP receptor on the endoplasmic reticulum (ER) membrane, the mRNA is co-translationally translocated into the ER with the help of the Sec63p complex and translocon Sec61p. Once the translation is done, the pre-signal sequence is cleaved by signal peptidase. Next, the nascent polypeptide can fold freely with the assistance of chaperones, e.g., protein disulfide isomerase (Pdi1p) and protein binding protein (Kar2p). Before leaving the ER, the nascent polypeptide undergoes primary glycosylation by calnexin.

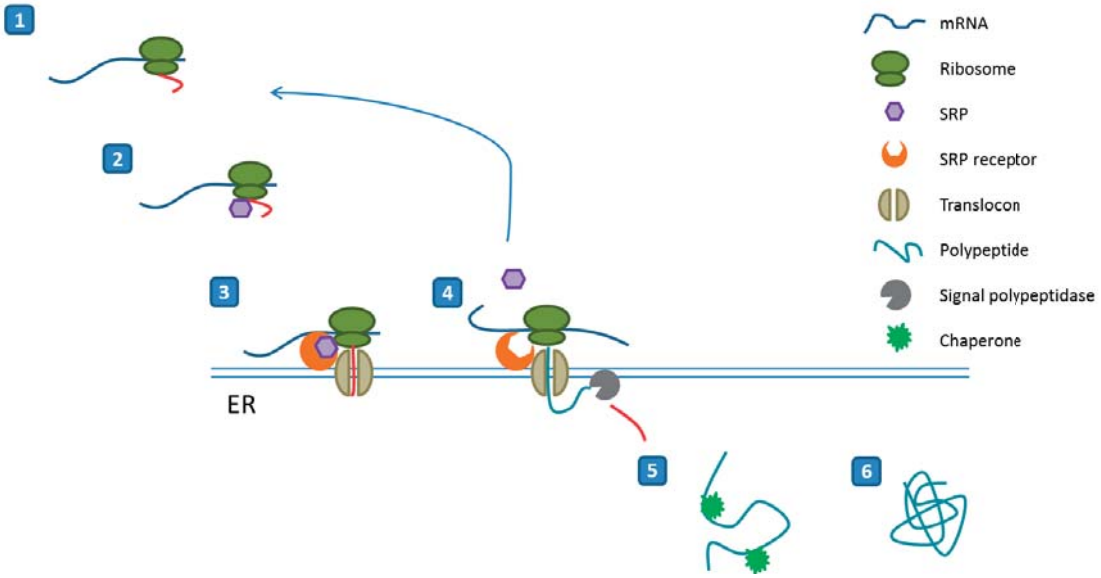


**Fig. 2 The process of co-translational translocation.**

### Ready for trafficking (COPII vesicle formation)

Folded proteins have to been transported into the Golgi for further PTMs.

COPII-coated vesicles mediate trafficking from the ER to the Golgi (Fig. 3). The formation of a COPII vesicle is triggered by Sar1p-GDP binding to the ER membrane. A guanine exchange factor (GEF), Sec12p, activates Sar1p by replacing GDP with GTP. Then, the activated Sar1p-GTP recruits the inner coat Sec23p-Sec24p complex. Sec24p catches the cargo proteins for the next transportation [19]. Sar1p-GTP, Sec23p-Sec24p and the cargo are called the pre-budding complex. The pre-budding complex accumulates at ER exit sites (ERES). Subsequently, the outer coat complex Sec13p-Sec31p is recruited by the pre-budding complex. The assembly of an intact COPII vesicle results in detachment from the ER membrane [20]. The COPII-coated vesicle is matured by the disassembly of coatomers from the liposome. This process is driven by the hydrolysis of GTP.

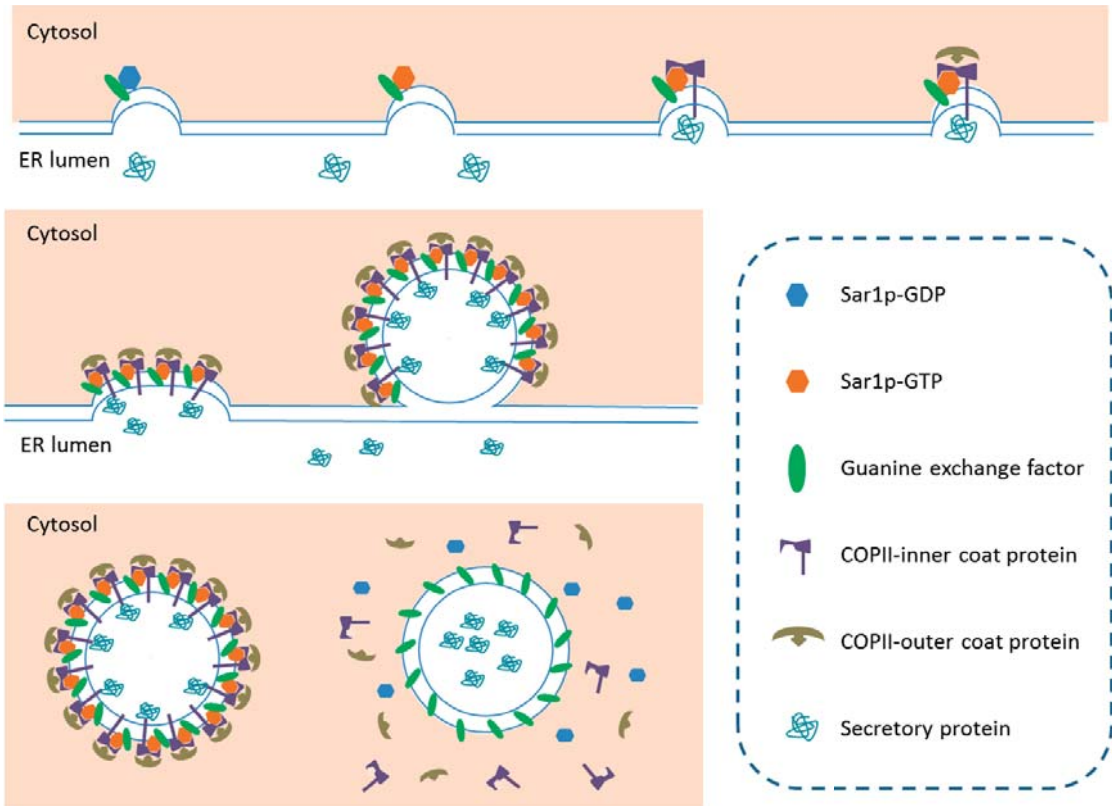


**Fig. 3 The process of COPII-coated vesicle formation.**

## Travel to the Golgi

Vesicle trafficking is carried out by the cooperation of N-ethylmaleimide-sensitive fusion protein (NSF), soluble NSF attachment protein (SNAP) and soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE). First, the Rab protein on the vesicle is activated by Rab-GEF by exchanging GDP with GTP. Then,

Rab-GTP directs the vesicle to the specific spots on the target membrane and is tethered with Rab effector. Then, v-SNAREs and t-SNAREs twine together to facilitate the fusion of the lipid bilayers (Fig. 4).


**Fig. 4 The process of vesicle docking and fusion.**

## What occurs in the Golgi?

After the fusion of lipid bilayers, the cargo protein is translocated into the Golgi apparatus. The cargo protein will undergo a series of further glycosylations (Fig. 5). The recombinant protein is initially glycosylated in the ER, resulting in $Man_8GlcNAc_2$ precursor glycoprotein [21]. In the Golgi of *S. cerevisiae*, mannosyltransferase (Och1p) first adds α-1,6-mannose to α-1,3-mannose of the trimannosyl core [22]. Then, with the help of other mannosyltransferases, such as Mnn1p, Mnn5, and Mnn9, the hypermannose glycan is formed. Before the glycosylated protein can be secreted, the pro-leader has to be removed by Kex2p in the trans-Golgi network (TGN).

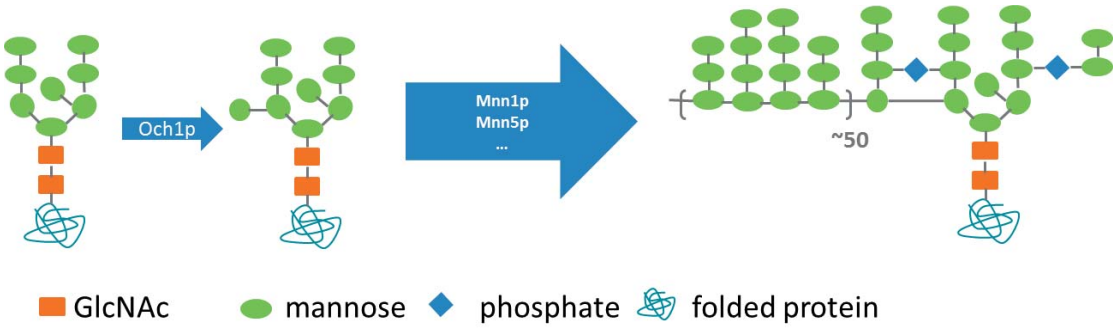


**Fig. 5 Glycosylation in the Golgi of *S. cerevisiae***

## Ready to be transported out of the cell

The process of trafficking from the TGN to the plasma membrane (PM) was elucidated previously [5]. Sec2p, Sec4p, Sec15p and Ypt32p are involved in this process. Sec2p is a GEF and converges at the Golgi membrane through binding to Ypt32p-GTP and phosphatidyl inositol 4 phosphate (PI(4)P). Then, the Golgi-derived vesicle detaches from the Golgi, followed by activation of the Rab GTPase Sec4p through Sec2p. Next, the effector Sec15p is recruited to the complex. During the delivery, the conformation of Sec2p is changed with the decreased level of PI(4)P, which enables the effector Sec15p to substitute Ypt32p-GTP on Sec2p. Then, Sec2p is phosphorylated, which strengthens the interaction between Sec2p and Sec15p and prevents binding to Ypt32p-GTP and PI(4)P to drive the reaction forward [5] (Fig. 6).

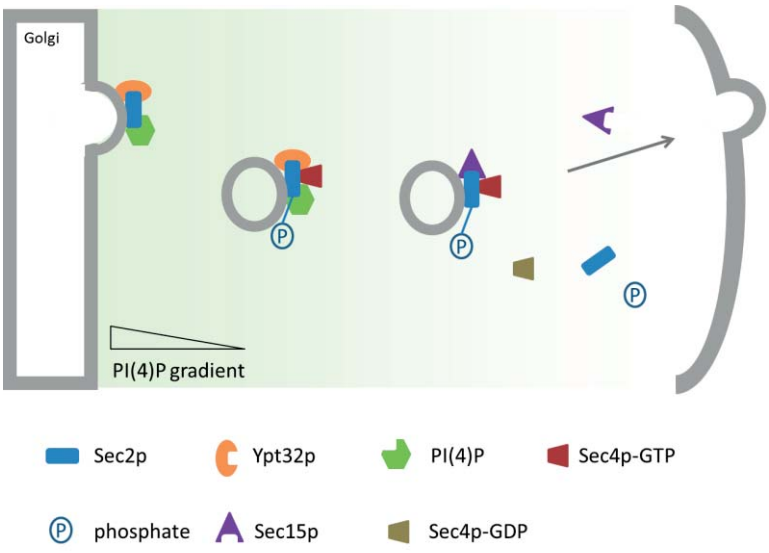


**Fig. 6 The process of the vesicle trafficking from the Golgi to plasma membrane**

## Examples of engineering protein secretion

### Signal peptide

The signal peptide is also sometimes referred to as the leader sequence and can affect recombinant protein secretion. The leader sequence can be a native signal peptide, a heterologous one from another organism or a synthetic one [23-25]. The leader sequence is first translated, after which translation is halted. The translated leader sequence will be recognized by the SRP. This process is endowed mainly by the hydrophobic core of the signal peptide [26]. The leader sequence is composed of pre- and pro-leaders, which define the flow of the target proteins. The pre- and pro-leaders are cleaved in the ER and Golgi, respectively. The signal peptide has been successfully engineered to improve protein secretion by directed evolution [23]. It was recently found that the alpha factor leader sequence is better than a synthetic leader sequence for α-amylase secretion in *S. cerevisiae*. By contrast, the secretion of insulin precursor is higher with a synthetic leader sequence than with the alpha factor leader sequence [27]. Thus, the effect of signal peptides on recombinant protein secretion is protein-specific. Eiden-Plach et al. found the viral K28 prep-pro-toxin signal peptide can endow four different yeast species with the ability to secrete green fluorescent protein (GFP) [28]. This suggested that viral signal peptides are novel strategies for recombinant protein secretion.

### Folding and quality control

After nascent polypeptides are translocated into the ER, they are folded with the help of ER chaperones. Kar2p is an essential protein belonging to the Hsp70 family, which is responsible for folding nascent polypeptides to native structures. Over-expression of *KAR2* enhanced antithrombotic hirudin and bovine prochymosin secretion in *S. cerevisiae* [17, 18]. However, this benefit of over-expressing *KAR2* does not apply to all cases. A negative effect of over-expressing *KAR2* on the production of glucose oxidase in *Hansenula polymorpha* was reported by van der Heide et al [15]. In addition, the level of the heterologous protein β-glucosidase in *S. cerevisiae* decreased with the increased level of *KAR2* [16]. Thus, the effect of over-expressing *KAR2* varies among different proteins and hosts; by contrast, another ER chaperone, Pdi1p, seems to be versatile in improving recombinant protein secretion. Pdi1p is protein disulfide isomerase, which is an essential protein that processes disulfide bond formation in the ER lumen. The introduction of *S. cerevisiae PDI1* into *Pichia pastoris* enhances rhG-CSF secretion around four times [29]. Over-expression of PDI1 from a single chromosomally integrated copy in *S. cerevisiae* increases the secretion

of human-derived growth factor B homodimer and *Schizosaccharomyces pombe* acid phosphatase by ten- and four- fold, respectively [30]. Unexpectedly, over-expression of *PDI1* also improves the secretion of the non-disulfide-bonded protein β-glucosidase from *Pyrococcus furiosus* [16]. This result indicates that Pdi1p may play a role in protein folding but not in disulfide bond formation. After the recombinant protein is processed by Pdi1p, it will undergo primary glycosylation in the ER; this process is completed by calnexin (Cne1p), an ER membrane protein. Over-expression of human calnexin in *S. cerevisiae* noticeably improves measles surface glycoprotein (MeH) solubility, whereas over-expression of yeast endogenous CNE1 does not help [31]. This result is due to the measles virus adopting the ER glycosylation system of human cells [32, 33]. These findings provide novel insight that when producing heterologous proteins, co-expressing heterologous protein-sourced chaperones can be a good strategy to improve secretion.

## Vesicle trafficking

Recombinant proteins are translocated between sub-organelles via vesicle trafficking. As described above, cargo protein transport includes three processes: 1) vesicle formation, 2) vesicle tethering to the target membrane, and 3) membrane fusion. Engineering the second process successfully increases heterologous protein secretion. Hou et al found that over-expression of Sly1p, an SM (Sec1/Munc-18) family protein that regulates trafficking from the ER to the Golgi, enhances α-amylase secretion but not for human insulin precursor or invertase [34]. Additionally, over-expression of Sec1p, which also belongs to the SM family, improves the secretion of all three proteins [34]. These results indicate that the process limiting recombinant proteins occurs at different stages depending on the properties of the recombinant proteins. Over-expression of SNARE proteins individually, such as Snc1p, Snc2p, Sso1p, Sso2p, and Sec9p involved in membrane fusion between Golgi-derived vesicles and the PM, increases cellobiohydrolase secretion in *S. cerevisiae*. This result means that engineering the fusion process indeed improves the secretion of cellobiohydrolase [35]. Meanwhile, over-expression of Sso1p enhances the titre of β-glucosidase by approximately 43.8%. Thus, the published results reveal that engineering the vesicle trafficking process is important

## Post-translational modification

Glycosylation is a common PTM. However, it has negative effects when expressing bacterially sourced enzymes in yeast, which is not glycosylated in nature. The heterologous protein α-amylase from *Bacillus licheniformis* has six N-linked glycosylations in *S. cerevisiae*, whereas the native α-amylase is not glycosylated in *B.*

*licheniformis* [36]. The titre (enzyme activity) of non-N-linked glycosylation α-amylase is approximately three-fold higher than the wild-type α-amylase in *S. cerevisiae*, whilst the amount of non-glycosylated α-amylase is also three-fold higher than the wild type. Tang et al. found that blocking the formation of hypermannose glycan with deletion of *MNN1* and *OCH1* remarkably improves fungal sourced cellulases secretion but not enzyme activities in *S. cerevisiae* [37]. These findings suggest that over-glycosylation on the recombinant protein can lower the secretory capacity in *S. cerevisiae*.

## Unfolded protein response

While protein transportation is a landmark event in protein secretion, unfolded protein response (UPR) is also a very important issue. When proteins are misfolded or aggregated in the ER, the UPR will be triggered with large-scale transcriptional alterations for approximately 400 genes in order to recover the secretory pathway to homeostasis [38]. A sensing system and an activation system constitute the UPR. The ER transmembrane protein Ire1p directly binds to chaperone Kar2p under non-stress conditions. When a protein is misfolded, Kar2p binds to the misfolded protein instead of Ire1p, which causes dimerization of Ire1p. The dimerized Ire1p has mRNA endonuclease activity, which is specific to the mRNA of *HAC1*. The spliced *HAC1* mRNA can be translated, after which Hac1p is translocated to the nucleus to activate the transcription of corresponding genes.

The over-expression of endogenous truncated *HAC1* in *S. cerevisiae* improves the secretion of the native proteins invertase and endoglucanase I from *Trichoderma reesei* but not that of α-amylase from *Bacillus amyloliquefaciens* [39]. Interestingly, heterologous over-expression of active *HAC1* from *T. reesei* in *S. cerevisiae* also showed 2.4-fold enhancement in *B. amyloliquefaciens* α-amylase secretion [39]. Payne et al found that over-expression of spliced HAC1 induces chaperone expression of *SIL1, JEM1* and *LHS1* and improves the production of recombinant human albumin [40]. In addition, over-expression of *HAC1* or its homologs is beneficial for recombinant protein secretion by CHO cells and *Aspergillus niger* [41, 42].

## Systems biology tools

Over the past few decades, systems biology has emerged, and its approaches have been developed as highly useful tools for strain engineering to produce valuable compounds and chemicals in *S. cerevisiae* [43-45]. Systems biology provides a global view of experimental design. As mentioned before, the effect of target engineering on recombinant protein secretion could vary among different proteins. Combining genome scale metabolic models (GEMs) with other omics data might provide a good solution for this problem. There are two methodologies for systems biology: a top-down approach and a bottom-up approach. The former relies on numerous sources of omics data and results from high-throughput analyses; the latter requires researchers to have a deep level of knowledge of specific cellular parts to generate or reconstruct the models. The two approaches are usually combined together in an effort to gather global information and design strategies for chemical production by cell factories [43].

### Top-down approach:

Genome analysis makes it possible to construct an entire cellular network. Transcriptome analysis provides scientists with a global view of the dynamic change in the expression of all genes upon cellular response [46]. The transcriptome profile of *S. cerevisiae* revealed that the production of membrane and soluble protein could be improved by increasing the transcript level of *BMS1*, which encodes the GTPase required for ribosomal subunit synthesis and rRNA processing [47]. The functions of several stress response cofactors, Yap6p, Cin5p, Phd1p, and Skn7p, were discovered from the results of a transcriptome analysis [48]. Gasser et al. performed a transcriptome analysis in *Pichia pastoris* and found effective targets to increase the yield of the Fab antibody approximately 2.5-fold [49]. Over-expression of heat shock factor (*HSF1*), a transcription factor (TF) that regulates the expression of hundreds of genes in response to heat shock response (HSR), increases insulin and α-amylase secretion in *S. cerevisiae*. Transcriptome data showed that ER stress was relieved in an *HSF1* over-expression strain [50].

Flux balance analysis quantitates the metabolic networks, mimicking the phenotype *in vitro* [51]. Fluxome analysis of *S. cerevisiae* demonstrated weakened flux to the pentose phosphate pathway and tricarboxylic acid cycle in the strain with human superoxide dismutase [52]. Fürch et al. found that using pyruvate as the sole carbon source is better for recombinant protein hydrolase from *Thermobifida fusca* in *Bacillus megaterium* according to metabolic flux analysis [53, 54].

## Bottom-up approach:

The development of mathematical models provides new scope for scientists to understand cell metabolism [55]. Bottom-up models usually concentrate on the specific cellular process, for example, glycosylation [56, 57] and UPR [58]. The construction of bottom-up models requires a deep understanding of the specific cellular process.

Feizi et al. constructed the first yeast genome-scale model for secretory machinery [59]. PTM features, transport steps and protein abundances were integrated in this model. This model provides the systems biology framework for recombinant protein production strategies. The mathematical model constructed by Raden et al. simulated the early steps of UPR in *S. cerevisiae* and revealed that over-expression of only the ER chaperone binding protein is not sufficient to activate the UPR [58]. Umana et al. constructed a mathematical model for N-linked glycoform biosynthesis in CHO cells. The qualitative trends of glycoprotein secretion could be monitored using this model [56].

# Results

## Paper I: Moderate over-expression of *SEC16* increases α-amylase secretion in *S. cerevisiae*

As described in the introduction, the yeast *S. cerevisiae* is a widely used cell factory for the production of recombinant proteins [12] owing to its advantages of fast growth, defined cultivation, and ability to perform PTMs and secrete proteins into the extracellular medium, which facilities purification [60]. However, limitations of the secretory pathway may reduce the yield of recombinant proteins. Therefore, it is meaningful to study and engineer the yeast secretory pathway to improve recombinant protein secretion. In this study, we chose α-amylase from *A. oryzae* as a reporter to evaluate the secretory capacities of yeast strains. A-amylase is a three-domain protein with 478 amino acids, four disulfide bonds and one N-linked glycosylation site [61].

Over-expression of several genes coding for proteins involved in ER-to-Golgi and Golgi-to-PM transport by the low-copy-number plasmid p416TEF were tested. We found that over-expression of *SEC16* improved protein secretion approximately 2-fold in the yield of α-amylase compared with the control strain (Fig. 7a). However, over-expression of *SAR1*, which is also involved in COPII vesicle formation, did not increase the secretion of yeast in our study. The results indicate that the limitation might be in the recruitment step. In addition, over-expression of Golgi-to-PM transport proteins did not significantly increase the yield of α-amylase, which indicates that the limitation might not be in this step for the control strain (Fig. 7a). To investigate whether elevating the dosage of *SEC16* would further increase the secretory capacity of *S. cerevisiae*, a high-copy-number plasmid was tested. Unexpectedly, when *SEC16* was over-expressed by a high-copy-number plasmid, the yield was at the same level as in the control strain. This result is consistent with the previous finding that over-expression of *SEC16* by a high-copy-number plasmid and deletion of *SEC16* will block the secretory pathway or be lethal to the cell [62]. Thus, moderate over-expression of *SEC16* is beneficial to recombinant protein secretion. To obtain a stable strain for further analysis, the native promoter of *SEC16* was replaced by the constitutive promoter $P_{GPD}$, resulting in YIGS16. The yield of α-amylase in YIGS16 was approximately 2-fold as the control strain AACK (Fig. 7b).

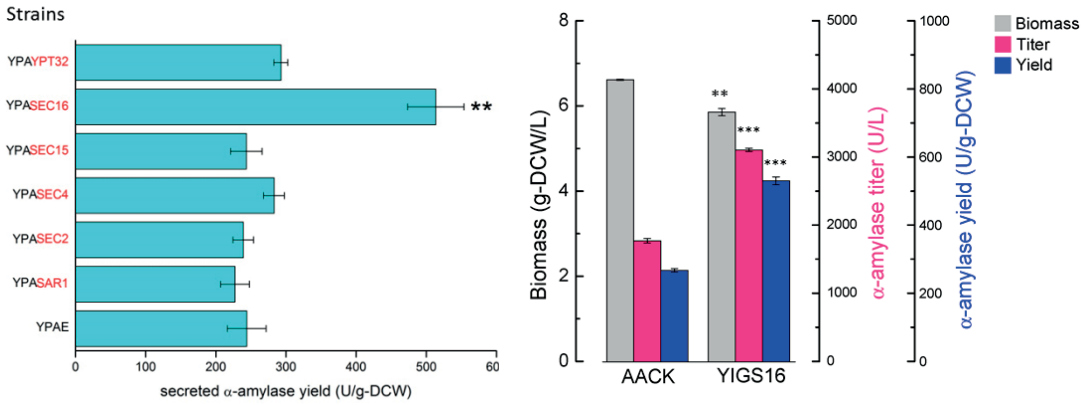


**Fig. 7 a) The secreted α-amylase yield of strains that overexpress genes that are involved in ER to Golgi or Golgi to membrane transport.** The overexpressed genes are marked in red. **b) The final biomass, α-amylase titer and α-amylase yield of strains YIGS16 and AACK in tube fermentation**.**P-value<0.01; ***P-value<0.001.

Batch fermentation was performed to further study the physiological change between YIGS16 and AACK. As shown in Fig. 8a & 8b, higher production of α-amylase was gained by batch fermentation. This result is probably due to the better control of conditions such as pH and aeration rather than tube fermentation. The biomass yield of YIGS16 was slightly lower than that of AACK. The glucose consumption rate was almost the same in both strains (Table 2). The ethanol titre for YIGS16 was 1.6 g/L, whereas it was 2.1 g/L for AACK at the end of the glucose phase; however, glycerol production was higher in YIGS16 than in AACK (Fig. 8c). The changed physiological behaviour implied a difference in redox balance between the two strains. Table 2 summarizes the physiological parameters for AACK and YIGS16. Over-expression of *SEC16* influenced the final biomass but resulted in a slower maximum specific growth rate. Moderate over-expression of *SEC16* led to a higher specific α-amylase production rate and a yield of α-amylase production in batch fermentation, at approximately 54% and 65%, respectively.

To investigate the intracellular accumulation of α-amylase between the two strains, cell samples taken from six different time points were analysed (Fig. 8d). The retained α-amylase in AACK increased from 60 U/g-DCW in the exponential phase to 140 U/g-DCW at the end of fermentation. The percentage of intracellular α-amylase in AACK increased sharply during the glucose phase and then stabilized (Fig. 8d). In comparison to AACK, the retained α-amylase in YIGS16 increased smoothly during the whole process from 110 U/g-DCW to 140 U/g-DCW. Moreover, the percentage of intracellular α-amylase in YIGS16 increased slightly during the glucose phase and was lower than AACK throughout fermentation (Fig. 8d). This suggests that moderate expression of *SEC16* allows for a better secretory capacity, and the relatively stable intracellular α-amylase percentage indicates that the secretory capacity matches

protein synthesis though the whole fermentation process. By contrast, the secretory capacity of AACK may lag behind its protein synthesis, as the intracellular α-amylase amount was increasing throughout fermentation.

**Table 2 Physiological parameters of AACK and YIGS16**

| Strain | $\mu_{max}$ | $r_S$ | $r_P$ | $Y_{s\alpha}$ | $r_E$ | $r_G$ |
|---|---|---|---|---|---|---|
| **AACK** | 0.249±0.004 | 1.35±0.04 | 103.6±14.5 | 80.6±9.4 | 0.163±0.004 | 0.205±0.008 |
| **YIGS16** | 0.233±0.008 | 1.26±0.04 | 159.9±12.0 | 132.2±6.4 | 0.122±0.011 | 0.200±0.006 |

$\mu_{max}$: Maximum specific growth rate ($h^{-1}$) on glucose; $r_S$: Specific glucose uptake rate (g/(g-DCW)/h); $r_P$: Specific secreted α-amylase production rate (U/(g-DCW)/h) on glucose; $Y_{s\alpha}$: Yield of secreted α-amylase from glucose (U/g); $r_E$: Specific ethanol production rate (g/(g-DCW)/h); and $r_G$: Specific glycerol production rate (g/(g-DCW)/h). Measurements are reported as the average value ± standard deviation from independent quadruplicates.

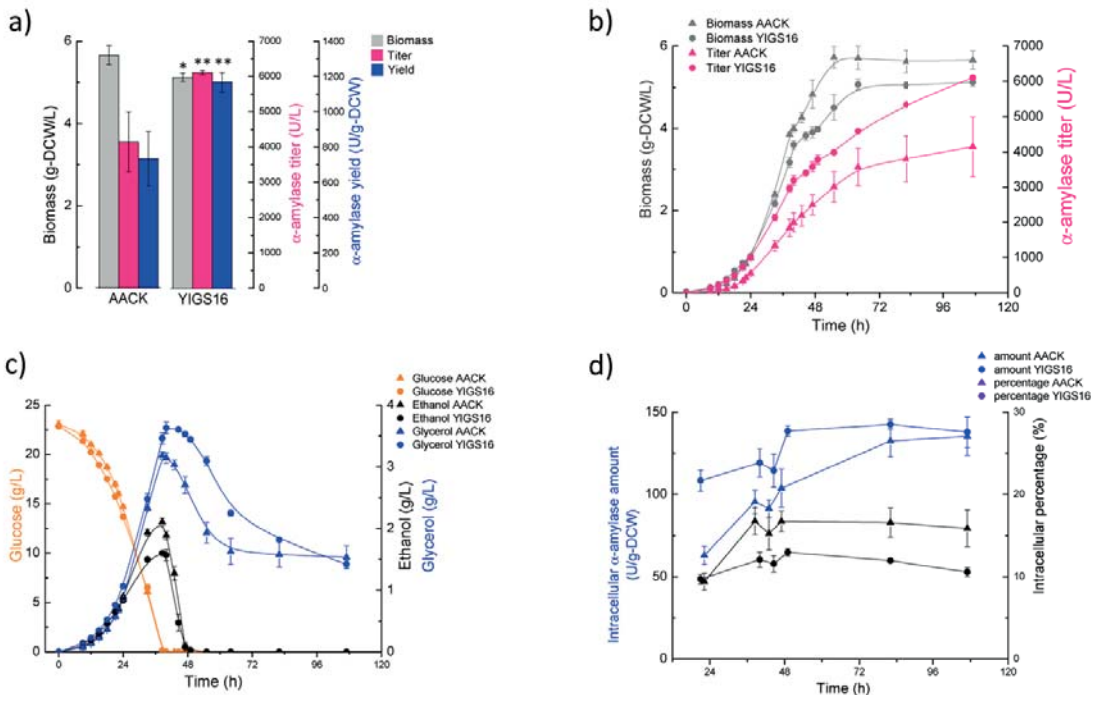


**Fig. 8 Batch fermentation of the strain YIGS16 and the control strain AACK.** a) The final biomass, secreted amylase titer and secreted amylase yield in batch fermentation. b) The time course of the biomass and secreted amylase titer. c) The time course of the glucose consumption and ethanol and glycerol production. d) The intracellular α-amylase amount and the intracellular percentages (calculated by dividing the intracellular α-amylase amount by the total amount of α-amylase) at six different time points. *P-value<0.05; **P-value<0.01.

The secretion and degradation of retained intracellular α-amylase in AACK and YIGS16 were measured to further quantify the protein secretory capacity of both strains. The cells were incubated in SD-2xSCAA medium until the $OD_{600}$ reached 1 and then were transferred to fresh carbon -free S-2xSCAA medium for 48 hours. During this time, the cells could not synthesize α-amylase because of the lack of a carbon source; however, retained intracellular α-amylase would continue to be secreted out of the cells. The secretion of retained intracellular α-amylase with YIGS16 was more than 2-fold higher than with AACK. Furthermore, there was no significant change in the degradation of retained intracellular α-amylase between AACK and YIGS16 (Table 3).

**Table 3 Details of intracellular α-amylase secretion test for AACK and YIGS16.**

| | AACK | YIGS16 | P. value (YIGS16 vs. AACK) |
|---|---|---|---|
| **Secreted (U/g-DCW)** | 14.75±1.69 | 36.14±3.88 | 0.0022 |
| **Retained (U/g-DCW)** | 37.70±1.78 | 42.50±2.08 | 0.0198 |
| **Degraded (U/g-DCW)** | 34.66±2.49 | 30.33±3.46 | 0.0801 |
| **Total (U/g-DCW)** | 87.11 | 108.96 | - |

Secreted: the amount of extracellular α-amylase after the test; Residual: the amount of intracellular α-amylase after the test; Degraded: the amount of degraded α-amylase; Total: the amount of intracellular α-amylase at the beginning of the 48-h incubation.

Recombinant protein production normally introduces extra ROS into cells because of protein folding in the ER [63]. ROS in the wild-type background strain 530-1CK with the empty plasmid Y1EK was set as 1 RFU (Fig. 9). When producing α-amylase in the wild-type background 530-1CK, ROS increased by 0.41 RFU (AACK vs. Y1EK). ROS from the moderate over-expression of *SEC16* was huge, with an approximately 0.69 RFU increase (Y6EK vs. Y1EK). However, ROS from producing α-amylase in YIGS16 was 0.27 RFU, which was less than that of AACK ((YIGS16-Y6EK) vs. (AACK-Y1EK)), even though the α-amylase yield was 2-fold higher than that of AACK.

Additionally, from the transcriptome data of AACK and YIGS16 in the glucose phase, approximately 50% of genes regulated by Hac1p and Gcn4p, which are TFs that activate the expression of hundreds of genes in response to UPR, were significantly down-regulated. These results indicate that the relative amount of ROS formed owing to the production of α-amylase was lower in the *SEC16* moderate expression strain compared with the wild-type strain. Interestingly, moderate expression of *SEC16* itself also increased intracellular ROS accumulation, which might come from elsewhere instead of ER-related stress. This result may partly explain why over-expression of *SEC16* with a high-copy-number plasmid did not increase α-amylase production as more ROS from the high expression of *SEC16* could be harmful for recombinant protein production.

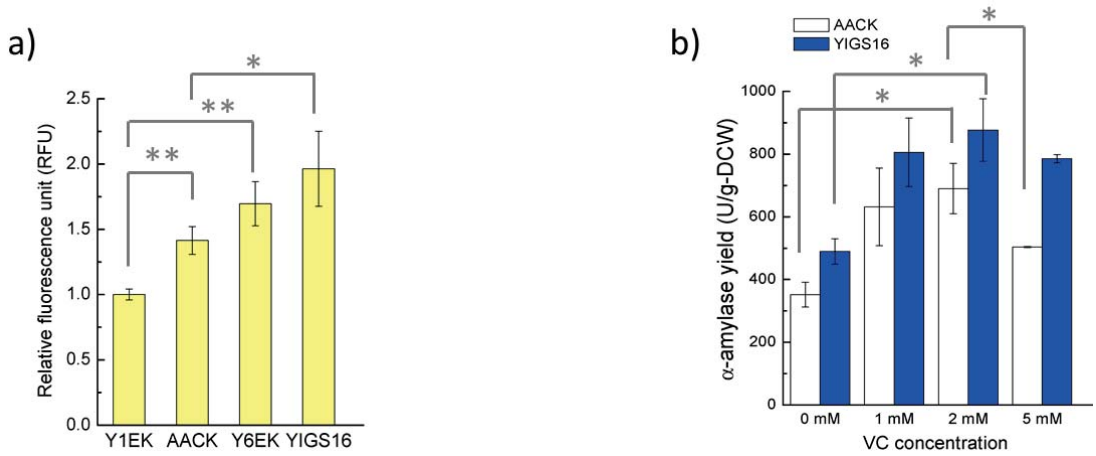


**Fig. 9 Oxidative stress study.** a) Intracellular ROS level of strains Y1EK (wt), AACK (production of α-amylase in wt strain), Y6EK (*SEC16* overexpression) and YIGS16 (production of α-amylase in *SEC16* overexpression) in shake flask cultivation. b) The final yield of α-amylase with different concentration of vitamin C supplied to medium in tube fermentation.

Vitamin C is an antioxidant that can protect cells against the harmful effects of ROS [64]. Different concentrations of vitamin C were added to the medium in order to investigate whether a reduction in ROS level could increase α-amylase production. The pH of the medium was hardly affected by vitamin C since the medium was buffered. When a low concentration (2 mM) of vitamin C was added to the medium, the α-amylase yield of both strains increased (Fig. 9b). However, when the concentration of vitamin C increased, the α-amylase yield of both strains decreased (Fig. 9b). When the concentration increased to 10 mM, the growth of both strains was strongly inhibited, and the titre of α-amylase was consequently very low (data not shown). Thus, a proper level of antioxidants could help cells to resist the damage caused by ROS accumulation and could be beneficial for protein production. The α-amylase yield decreased significantly by 27% when vitamin C increased from 2 mM to 5 mM in the AACK strain and decreased slightly, by 10%, in the YIGS16 strain.

The function of *SEC16* is to mediate COPII vesicle formation. *SEC16* co-localizes with COPII coat proteins at ERESs and is related to ERES organization [65-67]. The level of Sec16p in *S. cerevisiae* is lower than that of other COPII proteins [59]. In an effort to prove that moderate over-expression of *SEC16* indeed increases the number of ERESs in yeast, we fused GFP with Sec16p and RFP with Sec13p, an outer protein of COPII vesicles. The fluorescence signal intensity of SEC16-GFP in the *SEC16* moderate over-expression strain was approximately 5-fold higher in comparison to AACK (Fig. 10a). The RFP signal in the *SEC16* moderate over-expression strain had an approximately 50% higher intensity than that in AACK, although the mRNA level of *SEC13* was almost the same in both strains. These results suggest that the amount of ERESs increases with increased Sec16p abundance. The enhancement in the number

of ERESs provides more places for COPII formation, which facilitates the translocation of α-amylase from the ER to the Golgi.

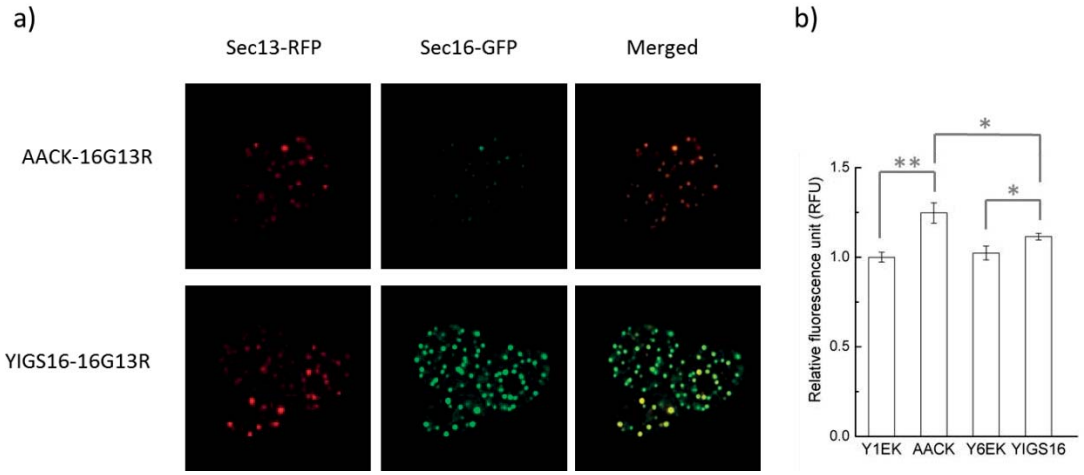


**Fig. 10 The strain YIGS16 has more ERESs and a reduced amount of ER membrane compared to strain AACK.** a) Airyscan images showing ERESs by the colocalization of Sec16-GFP and Sec13-RFP. *SEC16* was fused with *GFP*, and *SEC13* was fused with *RFP* in strains AACK and YIGS16. b) ER staining was performed, and the ER membrane changes were quantified by their fluorescence intensity. *P-value<0.05; **P-value<0.01.

As moderate over-expression of *SEC16* increased the number of ERESs, which improved the translocation from the ER to the Golgi, we wondered if the modification affected ER morphology. The signal of the ER membrane in Y1EK was set as 1 RFU (Fig. 10b). Production of α-amylase introduced extra ER stress to the cell, which was accompanied by ER expansion (Y1EK vs. AACK). ER expansion helps to alleviate ER stress in the cell [68]. Schuck et al found that the *hac1⊿* yeast strain is unable to properly expand the ER membrane and increase the chaperone levels and shows hypersensitivity to tunicamycin, which introduces ER stress to cells [68]. However, deletion of *OPI1*, a transcriptional regulator that negatively mediates phospholipid biosynthesis by binding to the Ino2p/Ino4p complex, or over-expression of *INO2*, a transcriptional activator for lipid biosynthesis, and its Opi1p-insensitive version *ino2(L119A)* enhanced the ER stress tolerance of the *hac1⊿* mutant through ER expansion [68]. There was no significant change in the signal of the ER membrane between Y1EK and Y6EK, although the ROS in Y6EK was higher than in Y1EK. We also noticed that the ER membrane expanded less in YIGS16 than in AACK ((YIGS16-Y6EK)-(AACK-Y1EK)), which agreed with the lower accumulation of ROS in YIGS16 caused by recombinant protein production compared with AACK (Fig. 9a). These results provide more evidence that the ROS caused by *SEC16* over-expression might come from other sources instead of ER stress.

Additionally, we detected a lower amount of Kar2p of YIGS16 in the extracellular medium. Kar2p is an ER resident chaperone. The secretion of Kar2p implies an impairment of the secretory pathway [69]. Thus, the production of recombinant protein had a negative effect on the secretory pathway, while moderate over-expression of *SEC16* could recover this damage.

Transcriptome analysis was performed in order to understand the change in global gene expression between AACK and YIGS16. The samples were taken from the glucose phase and ethanol phase. Principal component analysis (PCA) showed that YIGS16 has significantly different expression levels compared to AACK at both growth phases. The expression levels of 612 genes and 579 genes were significantly up- and down-regulated more than two-fold in the glucose phase and ethanol phase, respectively. There were 203 genes whose expression significantly changed more than two-fold under both conditions. Among these 203 genes, many belonged to three processes: cell wall organization, trafficking and cell cycle. The number of genes whose transcript levels changed significantly between the two growth phases was similar for both strains, and these genes are associated with the diauxic shift when cells change their metabolism from fermentative growth on glucose to respiratory growth on ethanol.

To gain more molecular insight, reporter GO term analysis and reporter TF analysis were performed for transcriptome profiling in the glucose phase. We noticed that the processes regulation of cell cycle, DNA replication, cytoskeleton organization, regulation of organelle organization, chromatin organization, regulation of DNA metabolic process, DNA repair, cellular response to DNA damage stimulus, chromosome segregation, mitotic cell cycle, organelle fission and meiotic cell cycle were up-regulated (Fig. 11). According to the results of the TF analysis, the genes regulated by glycolysis activators Tye7p and Gcr1p were significantly down-regulated (Fig. 12). This result suggests that carbohydrate metabolism was altered in YIGS16. We also noticed that the level two TFs Tye7p and Gcr1p themselves were down-regulated approximately 2.6-fold and 2-fold, respectively.

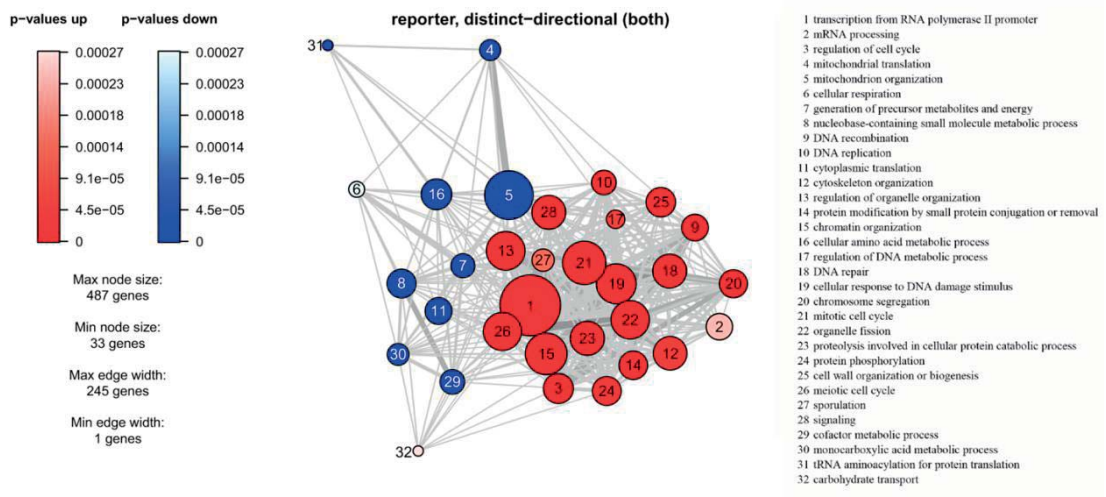


**Fig. 11 Reporter GO terms analysis of transcriptome profiling in the glucose phase.** The nodes represent GO terms, and the thickness of the edges correlate to the number of shared genes. The size of the nodes represents the number of genes within the GO terms.

According to the results from ER staining, ROS accumulation showed lower ER stress in YIGS16. In the reporter TF analysis, we found that the genes regulated by Hsf1p, which induces gene expression in response to HSR, were down-regulated (Fig. 12). The activation of HSR is a way to relieve ER stress [70]. Thus, a down-regulated HSR in YIGS16 strengthened the finding of lower ER stress in the *SEC16* moderate over-expression strain at the transcriptome level.

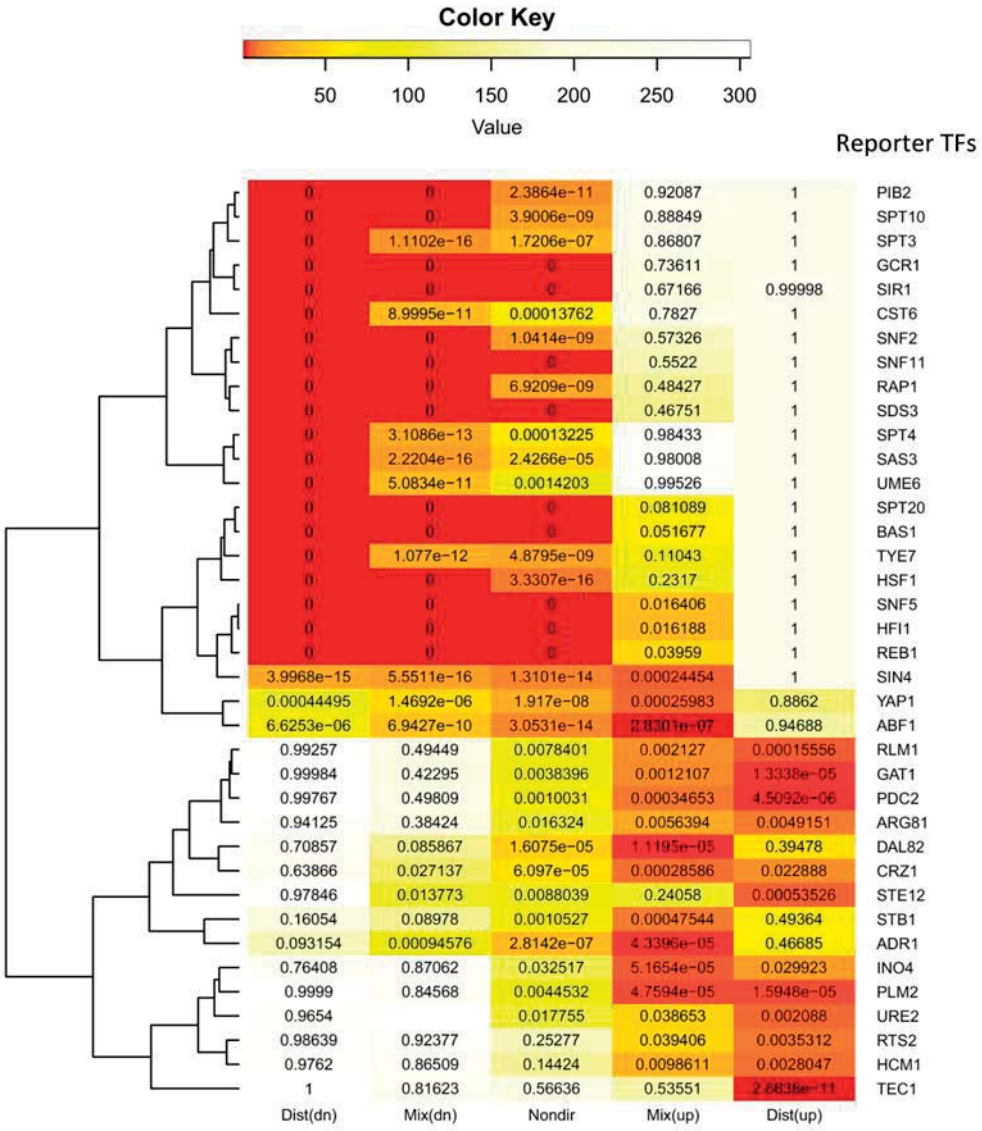


**Fig. 12 Reporter TF analysis for transcriptome profiling in the glucose phase.** This reporter TF analysis calculates the significance for expression change (up-regulation or down-regulation) of a gene set (controlled by a TF). Gene sets (TFs) received a consensus rank <10 in five groups (distinct-directional down, mixed-directional down, Non-directional change, Mix-directional up and distinct-directional up), were included in the heatmap. The ranking of TFs was shown by colors. The p-values for reporter TFs are present inside each cell of the heatmap. The TFs clustered at the upper part (from *PIB2* to *ABF1*) are showing patterns of mostly down-regulation whereas the TFs in the lower part (from *RLM1* to *TEC1*) are showing patterns of mainly up-regulation.

The UPR is activated by increasing ER stress when proteins are overloaded in ER. The TFs Hac1p and Gcn4p co-up-regulate the expression of hundreds of genes in response to the UPR [71]. Around half of the genes that are co-regulated by the two TFs were significantly down-regulated. Furthermore, the genes involved in ER folding

were down-regulated as well, such as *CNE1, STI1, SSE2, SSA4*, and *SIS1* [50]. These results also support the conclusion that moderate expression of *SEC16* leads to lower ER stress.

GO term analysis showed that the transcript levels of the genes involved in mitochondrial organization formation and mitochondrial translation were significantly down-regulated (Fig. 11). According to the results of mitochondrial staining, we found YIGS16 has fewer mitochondria than AACK (Fig. 13). This was probably caused by the high accumulation of ROS in YIGS16. Since mitochondrial biogenesis is tightly related to lipid metabolism [72], a higher level of ROS in YIGS16 may stimulate lipid peroxidation, which can affect mitochondrial organization and function [73]. Lipid metabolism was also affected by *SEC16* moderate expression according to the transcriptome analysis. Therefore, a combined effect of high levels of ROS and disturbed lipid metabolism resulted in limited mitochondrial function in the *SEC16* moderate expression strain. In addition, reduced respiration of YIGS16 was observed, which agreed with fewer mitochondria being organized in YIGS16.

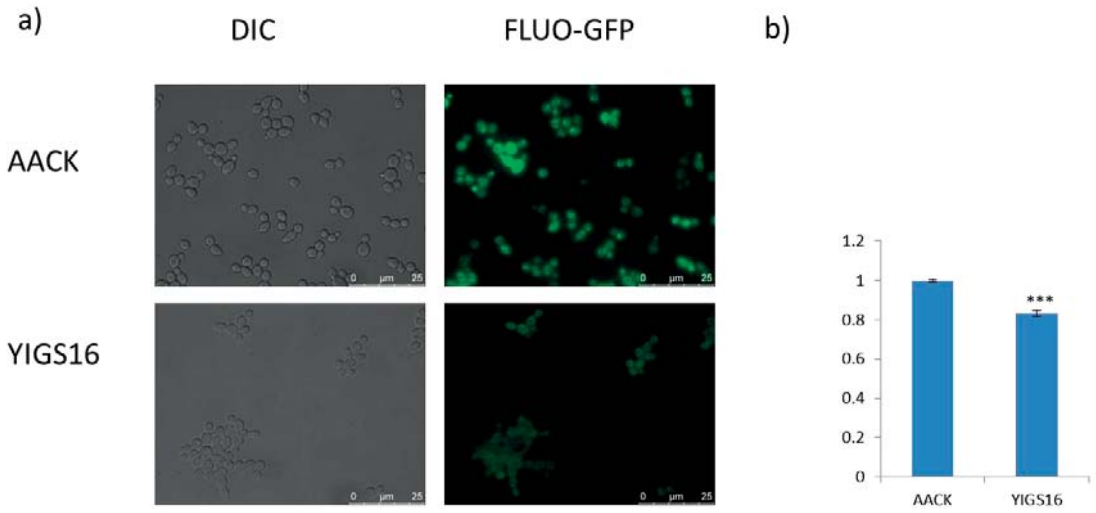


**Fig. 13 Mitochondria staining for AACK and YIGS16.** a) Fluorescence microscopy images of cells after mitochondria staining. b) Quantification of fluorescent intensity of mitochondria staining by plate reader. ***P-value<0.001.

In an effort to determine whether moderate expression of *SEC16* could increase secretion of other recombinant proteins, two fungal recombinant proteins, endoglucanase I from *Trichoderma reesei* and glucan-1,4-α-glucosidase from *Rhizopus oryzae,* were expressed. The recombinant protein yield of endoglucanase I in the *SEC16* moderate expression strain was approximately 50% higher than the reference, whereas the yield of glucan-1,4-α-glucosidase in the *SEC16* moderate over-expression strain was more than 3-fold higher than the reference (Fig. 14).

These results demonstrate that moderate over-expression of *SEC16* could increase a range of recombinant protein secretion and it may be a general strategy for the enhancement of recombinant protein secretion.

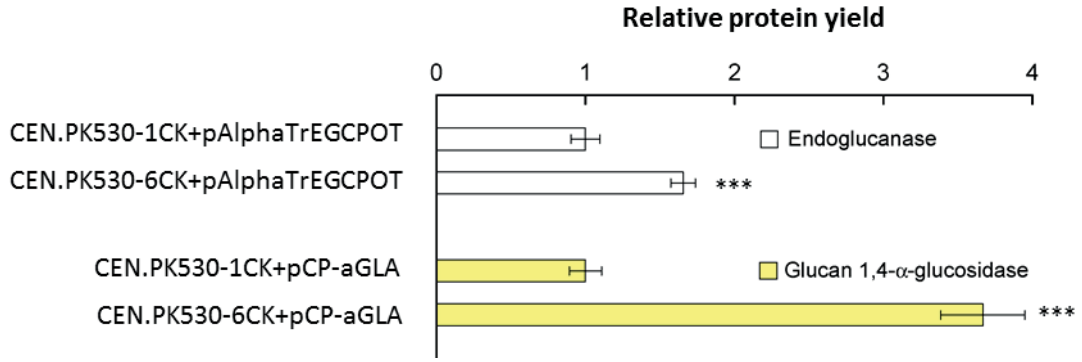


**Fig. 14 Secretion of two recombinant proteins, endoglucanase and glucan 1,4-α-glucosidase, in the wild-type and *SEC16* overexpression strains.** ***P-value<0.001.

In conclusion, moderate over-expression of *SEC16* could increase the secretion of a range of recombinant proteins in *S. cerevisiae*. Moderate over-expression of *SEC16* resulted in a higher level of ROS accumulation but lower ER stress compared with the wild-type strain. The high secretory capacity in the *SEC16* moderate over-expression strain might be due to more ERES and lower ER stress. Hence, moderate over-expression of *SEC16* could serve as a general strategy for improving recombinant protein secretion.

## Paper II: Balanced trafficking between the ER and the Golgi apparatus increases protein secretion in yeast

As described in paper I, we found that moderate expression of *SEC16* could increase recombinant protein secretion in *S. cerevisiae*. *SEC16* encodes an ER peripheral membrane protein and mediates COPII vesicle formation. According to the results of paper I, the number of ERESs increased with moderate over-expression of *SEC16*. However, the enhanced flux of ER-to-Golgi transportation brings excess ER-associated proteins to the Golgi via COPII vesicles, for example, v-SNARE proteins [74]. In eukaryotes, COPI vesicles transport the necessary component from the Golgi to the ER for continued anterograde trafficking [75, 76]. We amplified the retrograde trafficking in the *SEC16* moderate over-expression strain in order to further increase

the secretory capacity for recombinant proteins. Two GTP-activating proteins (GAP), Gcs1p and Glo3p, which mediate COPI vesicle formation, were over-expressed separately. In this study, YIGS16 was used as the starting strain. The native promoters of *GCS1* and *GLO3* were replaced with the constitutive strong promoter $P_{TEF}$ by integration.

The results of tube fermentation for strains YIGS16, YIGCS1 and YIGLO3 show that over-expression of *GCS1* and *GLO3* individually could further increase the titre of α-amylase by 19.7% and 25.1% (Fig. 15a). Interestingly, we found that overexpression of *GCS1* and *GLO3* in the wild-type strain (yeast strain without overexpression of *SEC16*) significantly decreased α-amylase secretion (Fig. 15b).

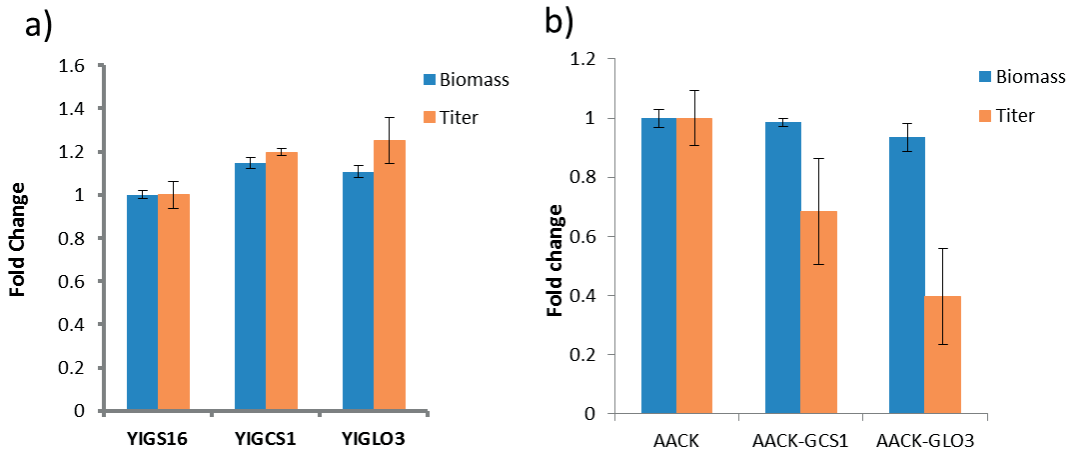


**Fig. 15** a) Overexpression of *GCS1* or *GLO3* by promoter replacement in *SEC16*-overexpression strain has a positive effect on α-amylase secretion. b) Overexpression of *GCS1* or *GLO3* in wildtype strain AACK (without *SEC16* overexpression) decrease the protein secretion.

ROS accumulation was measured in order to investigate whether the enhanced α-amylase secretion would cause an increase in intracellular ROS accumulation. The results of ROS accumulation indicated that there was no significant difference among the three strains, although the titres of α-amylase in YIGCS1 and YIGLO3 were higher than YIGS16 (Fig. 16a).

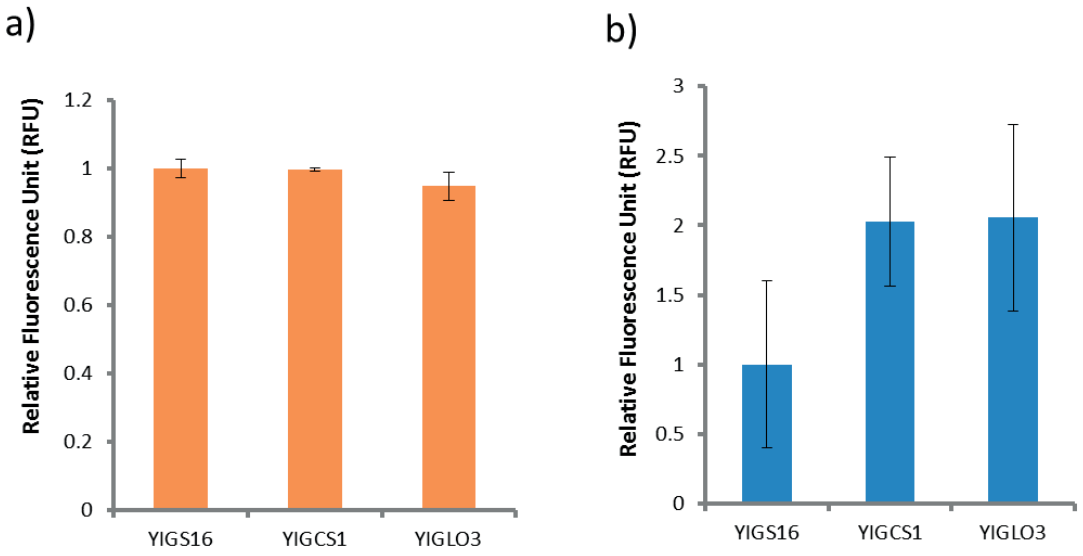


**Fig. 16 ROS and ER membrane of YIGS16, YIGCS1 and YIGLO3.** a) Intracellular ROS levels of strains YIGS16, YIGCS1 and YIGLO3. b) The amounts of ER membrane of strains YIGS16, YIGCS1 and YIGLO3.

Gcs1p and Glo3p mediate COPI vesicle formation. In an effort to determine whether amplification of retrograde trafficking might return more ER membrane, we stained the ER membrane for each strain. We detected a larger ER membrane in YIGCS1 and YIGLO3 in comparison to YIGS16 (Fig. 16b).

To elucidate the physiological change in *GCS1* and *GLO3* over-expression strains, batch fermentation for the three strains was performed. Over-expression of *GCS1* and *GLO3* resulted in a higher maximum specific growth rate than the reference strain (Table 4), although these strains grow slowly at the beginning (Fig. 17a). The final titres of α-amylase in YIGCS1 and YIGLO3 increased by 18.8% and 33.4%, respectively (Fig. 17b). The final biomass of both strains was undoubtedly higher than that of the reference strain (Fig. 17a), which was consistent with the results from tube fermentation. There was no significant difference in glucose consumption (data not shown) or the specific glucose uptake rate among the three strains (Table 4). However, YIGLO3 had a significantly higher specific α-amylase production rate and yield of α-amylase from glucose in comparison to the reference strain. Meanwhile, YIGLO3 had a higher specific ethanol production rate and higher ethanol production and a lower specific glycerol production rate and lower glycerol production than those in YIGS16. These results suggest that over-expression of YIGS16 might affect the central carbon metabolism. In addition, the difference in the level of remaining glycerol in these three strains at the end of fermentation reflected a different redox balance in the cell.

**Table 4 Physiological parameters of YIGS16, YIGCS1 and YIGLO3.**

| Strain | $\mu_{max}$ | $r_S$ | $r_A$ | $y_{SA}$ | $r_E$ | $r_G$ |
|---|---|---|---|---|---|---|
| **YIGS16** | 0.194±0.003 | 1.21±0.01 | 158.12±7.80 | 130.47±4.98 | 0.147±0.002 | 0.253±0.005 |
| **YIGCS1** | 0.226±0.004 | 1.16±0.01 | 154.36±1.74 | 128.93±2.48 | 0.147±0.005 | 0.261±0.007 |
| **YIGLO3** | 0.217±0.002 | 1.25±0.03 | 182.90±7.31 | 145.92±5.76 | 0.165±0.001 | 0.231±0.006 |

$\mu_{max}$: Maximum specific growth rate ($h^{-1}$) on glucose; $r_S$: Specific glucose uptake rate (g/(g-DCW)/h); $r_P$: Specific α-amylase production rate (U/(g-DCW)/h) on glucose; $Y_{s\alpha}$: Yield of α-amylase from glucose (U/g); $r_E$: Specific ethanol production rate (g/(g-DCW)/h); and $r_G$: Specific glycerol production rate (g/(g-DCW)/h).

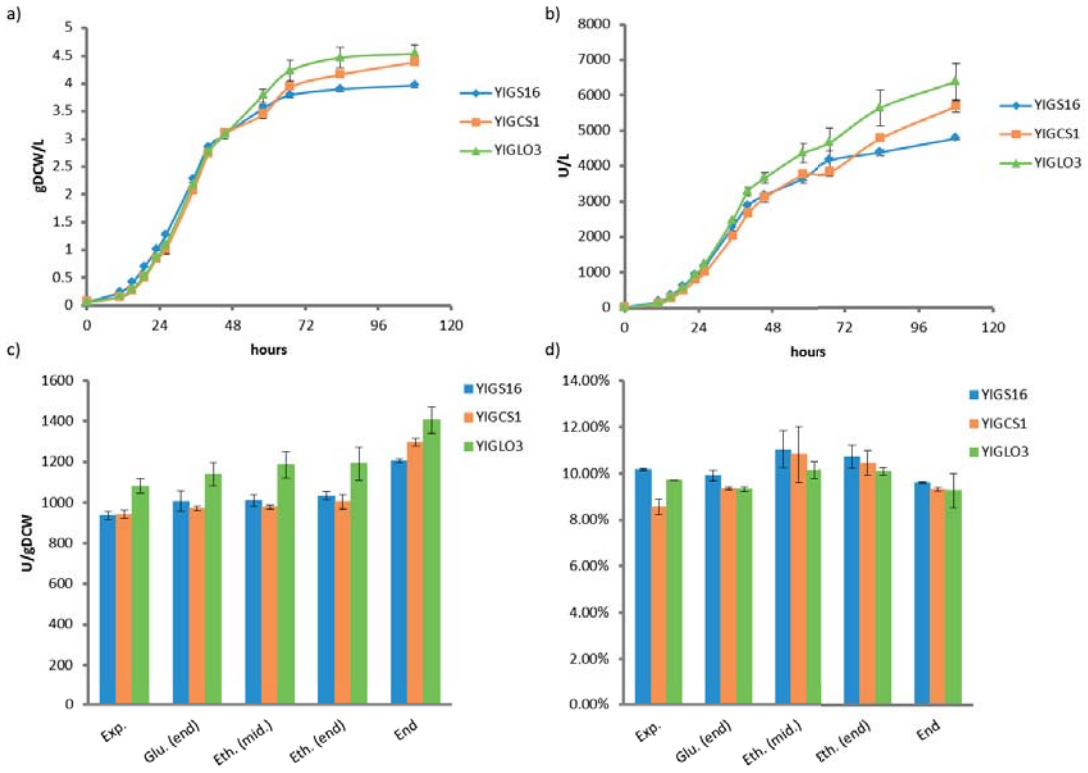


**Fig. 17 Batch fermentation of the strain YIGS16, YIGCS1 and YIGLO3.** The time course of a) the biomass and b) the α-amylase titer. c) The titer/biomass ratio and d) the percentage of intracellular α-amylase of the three strains at five different time points.

To evaluate the secretory capacity of each strain throughout fermentation, we calculated the titre-to-biomass ratio (titre/biomass) in five different stages (Fig. 17c). It is obvious that over-expression of *GLO3* endowed the *SEC16* moderate expression strain with a better secretory capacity as the titre/biomass was always higher than in

YIGS16. However, titre/biomass in YIGCS1 showed a similar pattern as in YIGS16. These results suggest that over-expression of *GLO3* in the *SEC16* moderate expression strain could enhance α-amylase secretion throughout fermentation, whereas the effect of *GCS1* over-expression on α-amylase secretion was not that significant in the bioreactor.

We analysed the intracellular α-amylase accumulation among the three strains. The samples were taken from five different time points, exponential phase (OD≈1), the end of glucose phase, the end of glucose phase, the middle of ethanol phase, the end of ethanol phase and the end of fermentation (Fig. 17d). All the three strains showed a similar profile of intracellular accumulation.

To investigate whether the effect of over-expression of *GLO3* in *SEC16* moderate over-expression strain on protein secretion is general, we tested another two heterologous proteins, endoglucanase I from *T. reesei* and glucan-1,4-α-glucosidase from *R. oryzae,* in a *GLO3* over-expression strain. The yields of the two recombinant proteins increased approximately 25% compared with the reference strain (Fig. 18). The final biomass of the *GLO3* over-expression strain was slightly but significantly higher than the reference.

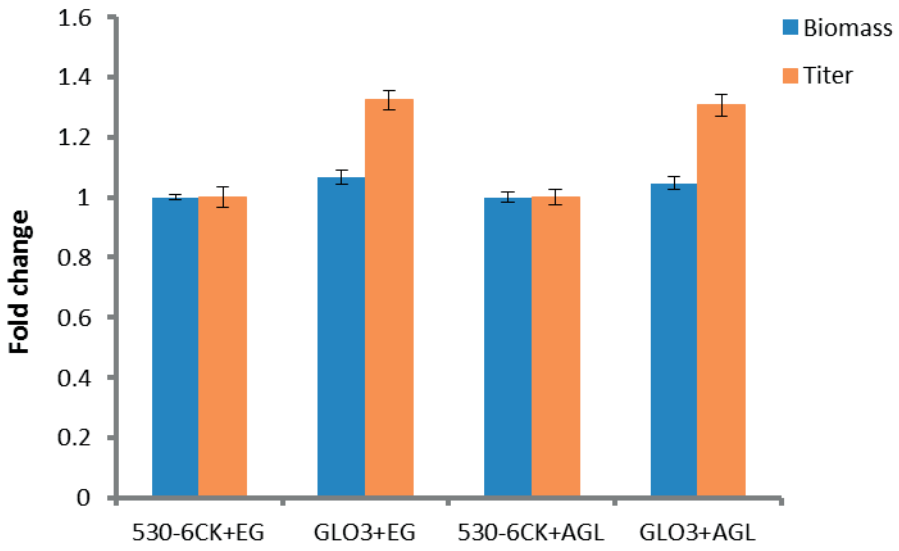


**Fig. 18 Secretion of two recombinant proteins, endoglucanase and glucan 1,4-α-glucosidase, in the reference strain and *GLO3*-overexpression strain.**

Moderate expression of *SEC16* in *S. cerevisiae* improves the secretion of a range of recombinant proteins by amplifying the trafficking flow from the ER to the Golgi. However, the enhanced flux of ER-to-Golgi transportation brings excess ER-associated proteins to the Golgi via COPII vesicles, for example, v-SNARE proteins

[74]. COPI vesicles return the necessary components from the Golgi to the ER for continued anterograde trafficking [75, 76]. Over-expression of Gcs1p and Glo3p, which are involved in COPI vesicle formation, could further increase α-amylase secretion in the *SEC16* moderate over-expression strain. However, the ROS accumulation in the *GCS1* and *GLO3* over-expression strain was at the same level as the reference, although their titres were higher. These results indicated that over-expression of GCS1 and *GLO3* in the SEC16 moderate expression strain enhanced α-amylase secretion without burdening the yeast cells. We also observed an increase in the size of the ER, which could be a clue to demonstrate the increased flux from the Golgi to the ER by over-expression of *GCS1* and *GLO3*. In the bioreactor batch fermentation, YIGCS1 and YIGLO3 grew slower than YIGS16 in the very beginning, but the two strains showed a higher specific growth rate than YIGS16, and their final biomasses were higher than YIGS16 (Fig. 17a). We also observed a higher biomass in the tube fermentation (Fig. 15a). These results suggest that over-expression of *GLO3* and *GCS1* would not negatively affect cell growth. To some extent, they improved the cell growth. The increase in the α-amylase titre in YIGCS1 in the bioreactor was not as high as that in tube fermentation, indicating that the effect of over-expressing *GCS1* on α-amylase production was more significant under poorly controlled conditions; when the conditions were well controlled, the advantage of *GCS1* over-expression was less. However, there was a significant effect of over-expression of *GLO3* on α-amylase production under both poorly controlled conditions and well-controlled conditions. The difference between over-expression of *GCS1* and *GLO3* might be due to that *GLO3* plays a dominant role in COPI vesicle formation, although *GCS1* and *GLO3* have an overlap in function [77]. We also noticed that when *GLO3* and *GCS1* were co-over-expressed in the *SEC16* moderate expression strain, the titre of α-amylase hardly increased compared with YIGLO3 in the bioreactor (data not shown). The retained intracellular α-amylase and the percentage of intracellular α-amylase accumulation indicate that YIGCS1 and YIGLO3 had a better secretory capacity in the glucose phase. In this study, another two recombinant proteins, endoglucanase I and glucan-1,4-α-glucosidase, were tested in the *GLO3* over-expression strain. The increase in the yield of both recombinant proteins was approximately 25%, implying that over-expression of *GLO3* in the *SEC16* moderate expression strain could serve as a general strategy to increase the secretion of a range of recombinant proteins.

In summary, over-expression of *GCS1* and *GLO3* in the *SEC16* moderate expression strain could enhance recombinant protein secretion. Since Glo3p plays a greater role than Gcs1p in COPI vesicle formation, the effect of over-expressing Arf-GAP on α-amylase secretion was not inconspicuous in YIGCS1 but was in YIGLO3 in bioreactor batch fermentation. Over-expression of *GLO3* could further improve the protein secretory capacity in the *SEC16* moderate expression strain.

## Paper III: Efficient protein production by yeast requires global tuning of metabolism

In a previous study, the high-protein-secretion strain B184 with a 5-fold increase in α-amylase secretion was attained through three rounds of UV mutagenesis. In paper III, we studied the genome-wide transcriptional response to protein secretion using transcriptome data from the starting strain, AAC; the high-protein-secretion strain, B184; and another six strains with higher secretory capacity than AAC, which were identified during the three rounds of UV mutagenesis.

The physiological profile of the 8 strains was obtained by batch fermentation (Fig. 19). All seven mutant strains could secrete more α-amylase than the reference strain AACK throughout fermentation (Fig. 19a), while the biomass yield of the seven mutant strains was slightly lower than that in AACK (except for F83, which had a 27% lower biomass yield) (Fig. 19b). Notably, the specific α-amylase production rate in B184 was highest among all strains; M715 had the lowest specific glycerol production rate and specific acetate production rate. However, the rates increased again in the descendants (Table 5). Intracellular α-amylase accumulation was also investigated, and B184 had the lowest α-amylase accumulation among all strains. As shown in Table 5, we observed an increase in the specific growth rate and specific ethanol production rate of all mutant strains.

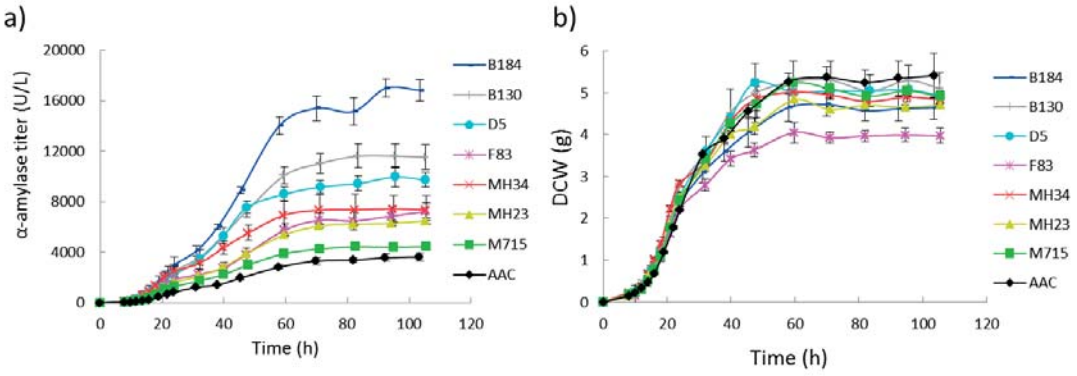


**Fig. 19 α-amylase production of mutant strains.** a) α-amylase titer of strains in batch fermentation. b) cell growth of the mutant strains in batch fermentation.

**Table 5 Physiological parameters of the mutant strains.**

| Strain | $\mu_{max}$ | $r_S$ | $r_E$ | $r_G$ | $r_A$ | $r_P$ |
|---|---|---|---|---|---|---|
| AAC | 0.276 ± 0.010 | 1.351±0.024 | 0.347±0.006 | 0.099±0.002 | 0.040±0.001 | 101.37±4.90 |
| M715 | 0.309 ± 0.017 | 1.329±0.249 | 0.360±0.036 | 0.083±0.011 | 0.033±0.002 | 164.01±12.61 |
| MH23 | 0.304 ± 0.007 | 1.408±0.147 | 0.439±0.024 | 0.091±0.005 | 0.039±0.003 | 223.45±10.18 |
| F83 | 0.296 ± 0.005 | 1.588±0.158 | 0.495±0.051 | 0.117±0.005 | 0.040±0.005 | 264.55±69.41 |
| MH34 | 0.329 ± 0.016 | 1.649±0.141 | 0.460±0.023 | 0.087±0.006 | 0.034±0.002 | 262.96±35.56 |
| D5 | 0.313 ± 0.002 | 2.023±0.071 | 0.604±0.024 | 0.116±0.007 | 0.040±0.003 | 283.26±15.40 |
| B130 | 0.305 ± 0.006 | 2.045±0.181 | 0.677±0.058 | 0.109±0.015 | 0.040±0.002 | 314.11±73.15 |
| B184 | 0.310 ± 0.006 | 1.969±0.069 | 0.610±0.049 | 0.116±0.013 | 0.047±0.002 | 386.93±49.01 |

$\mu_{max}$: maximum specific growth rate (1/h) on glucose; $r_S$: specific glucose uptake rate (g/g-DCW·h); $r_E$: specific ethanol production rate (g/g-DCW·h); $r_G$: specific glycerol production rate (g/g-DCW·h); $r_A$: specific acetate production rate (g/g-DCW·h); $r_P$: specific α-amylase production rate (U/g-DCW·h).

The cell samples were harvested at $OD_{600}$≈1.0 for RNA-seq analysis. PCA showed strong reproducibility of the biological replicates and grouped the strains into three clusters (Fig. 20a). The clusters also showed the relationship between these strains, which was consistent with the evolutionary pedigree of the strains (Fig. 20b). Cluster 1 contained AACK and M715; Cluster 2 contained MH23 and F83; and Cluster 3 contained MH34, D5, B130 and B184. In the first round of mutagenesis, a small number of genes were significantly up- or down-regulated (M715 vs. AAC) (Fig. 20c). However, in the second and third rounds of mutagenesis, the transcript levels of many genes in the mutant strains were significantly affected. This result suggests that a global modulation of gene expression is required to achieve higher α-amylase secretion.

The common gene expression changes in Clusters 2 and 3 (MH23, F83, MH34, D5, B130 and B184) were identified. A total of 31 genes are listed in Fig. 21; their transcript levels were commonly changed among all the mutants. The genes *ANB1*, *TIR3*, *CYC7*, *DAN1* and *AAC3*, which are required under anaerobic or hypoxic conditions, were significantly up-regulated. The mutant strains displayed anaerobic characteristics even though the dissolved oxygen levels of all strains were above 90% at that time point.

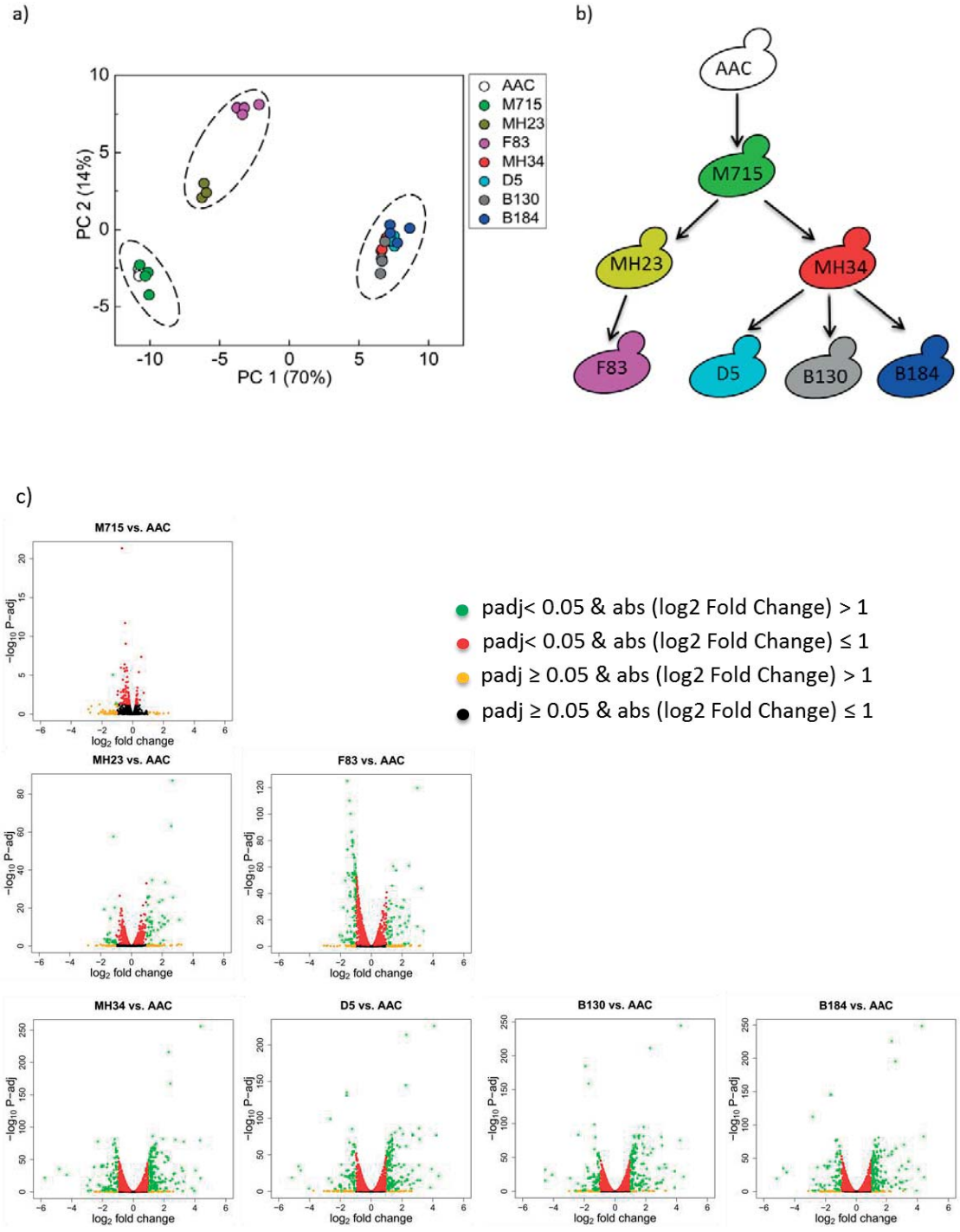


**Fig. 20** a) Principal component analysis (PCA) was performed by using expression profiles. b) Evolutionary relationships among strains. c) Transcriptional differences are presented in volcano plot.

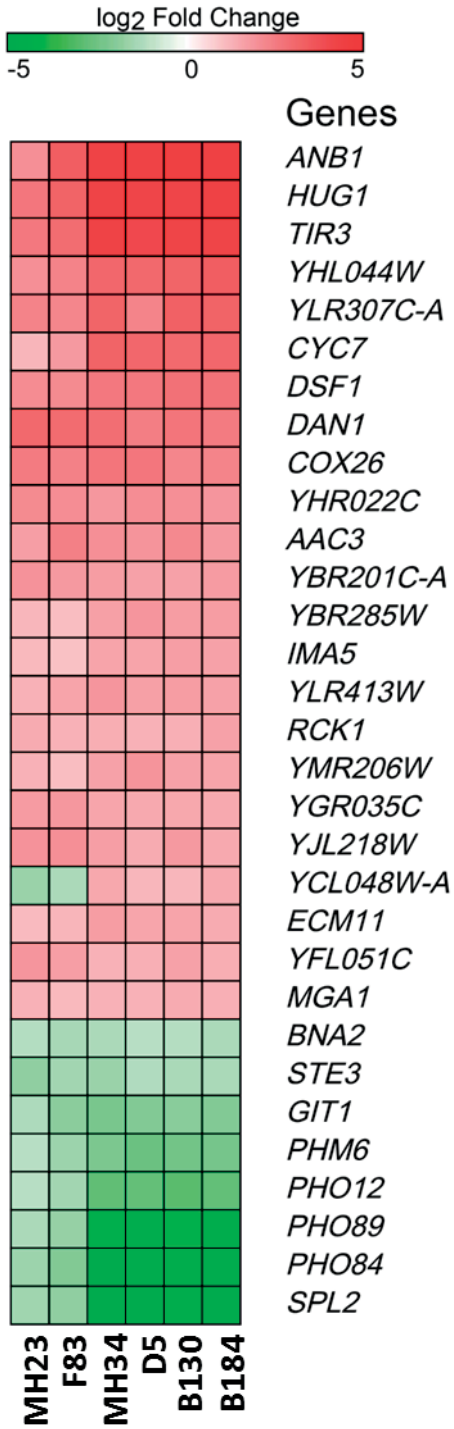


**Fig. 21 Expression levels of the 31 common significant differentially expressed genes in all mutant strains.**

Reporter TF analysis was performed in order to investigate the transcriptional regulatory responses in the mutant strains of Clusters 2 and 3. A total of 308 TFs were scored. The top 5 reporter TFs represented a dominant regulatory network

coordinated with increased protein secretion in each strain. Hypoxia-induced genes are negatively regulated by the TF Rox1p under aerobic conditions [78]. The TF analysis results showed that the expression levels of the genes negatively regulated by Rox1p in the six mutant strains were up-regulated, and the expression level of Rox1p itself was also down-regulated. Genes regulated by TUP1p, a subunit of the Tup1p-Cyc8p complex that negatively regulates anaerobic genes with Rox1p [79], were up-regulated in the mutant strains. Rox1p is activated by the TF Hap1p [80]. The genes regulated by Hap1p and the expression level of Hap1p itself were down-regulated. In addition, genes regulated by the Hap2p/3p/4p/5p complex, which is a transcriptional activator and global regulator of respiratory gene expression, were also down-regulated. These results indicated that mutant strains exhibited anaerobic/hypoxic behaviours even if the dissolved oxygen levels were high. This result is also consistent with the findings mentioned above. In addition to anaerobic metabolism, other perturbed cellular processes were identified, such as nutrient signalling, nucleotide synthesis and phosphate metabolism. The TFs Mss11p and Tec1p activate the expression of hundreds of genes in response to nutrient starvation [81]. The production of recombinant proteins competes with resources that could be used for cell growth. A slightly lower biomass was observed in the mutant strains. The competition of resources might result in the induction of nutrient starvation. The TF Bas1p encoded by *BAS1* is involved in regulating the basal and induced expression of genes of the purine and histidine biosynthesis pathways. Genes regulated by Bas1p were down-regulated in the mutant strains, which suggests that this change might be beneficial to recombinant protein secretion. Reporter TF analysis also indicated a change in the cell cycle since the genes regulated by Mbp1p and Swi4p were up-regulated. Mbp1p and Swi4p are the subunits of the MBF (Mbp1p/Swi6p-dependent cell cycle box Binding Factor) complex and SBF (Swi4p/Swi6p-dependent cell cycle box binding factor) complex, which mediates gene expression during the G1/S transition of the cell cycle [82].

To investigate whether the increase in protein secretion resulted from the regulation by the TFs mentioned above, we tested several TFs for further evaluation. Previous studies showed that deletion of *ROX1* and *HAP1* could increase recombinant protein secretion [83, 84], which confirmed our findings. To evaluate the impact of other TFs on protein secretion, we deleted *TUP1*, *BAS1, HAP2* and *HAP4* and over-expressed *MBP1, MSS11, SWI4* and *SUT1*. All of the engineered strains showed increased protein secretion except for *SWI4* (Fig. 22). These results indicate that efficient protein secretion could be achieved by engineering the target TFs.

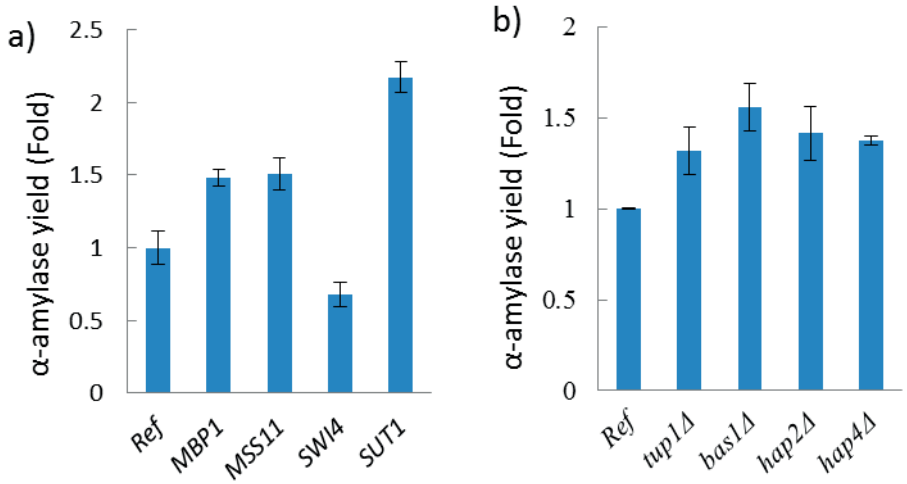


**Fig. 22 Enhanced production of amylase by overexpression of TFs (a) or deletion of TFs (b).**

Increased glucose uptake rate and ethanol production rate in the mutant strains were observed during batch fermentation (Table 5). GO term analysis also revealed that the respiration, generation of precursor metabolites and energy metabolism in mutant strains were down-regulated. The expression level of the low-affinity glucose transporter Hxt1p, which is highly expressed under high-glucose conditions, was down-regulated in Cluster 3, whereas the transcript levels of other high-affinity hexose transporters, which were induced under low glucose conditions, were up-regulated. In addition, the glucose uptake rate in these strains increased (Table 5). This result implies that the glucose-sensing system in the strains in Cluster 3 was affected. The expression levels of the genes involved in the citric acid cycle in the mutant strains were down-regulated together with reduced respiration. Genes involved in glycolysis were also down-regulated, which is inconsistent with the increased glucose uptake rate. However, the glycolytic flux is well known to not be strongly correlated with the transcriptional level [85].

According to the results of batch fermentation, the mutant strain B184 showed the best protein secretory capacity; thus, we performed a more detailed analysis of the transcriptome of this strain. We found that the transcripts levels of the genes involved in thiamine biosynthesis in B184 were significantly up-regulated; this result was also observed in strain F83, D5 and B130. In an effort to determine whether thiamine is required for efficient protein secretion, additional thiamine was added to the medium. However, there was no significant increase in α-amylase secretion in AACK and B184 (Fig. 23a). We further studied the transcriptional regulation pattern of thiamine biosynthetic genes. The thiamine biosynthetic pathway is regulated by the Thi2p/3p complex and the Thi2p/Pdc2p complex, and the transcription of *THI2* is negatively controlled by the intracellular thiamine level [86]. Thus, the intracellular thiamine might be low in B184 owing to the up-regulated transcript levels of *THI2*,

which implied that a low level of thiamine might benefit protein secretion. The low thiamine level activates the thiamine response mechanism, inducing the expression of the genes involved in thiamine biosynthesis. Therefore, we deleted *THI2*, *THI3* and *THI4* in both AACK and B184 to reduce thiamine biosynthesis. All the deletions had a positive effect on protein secretion (Fig. 23b), indicating that a low thiamine level can increase protein secretion.

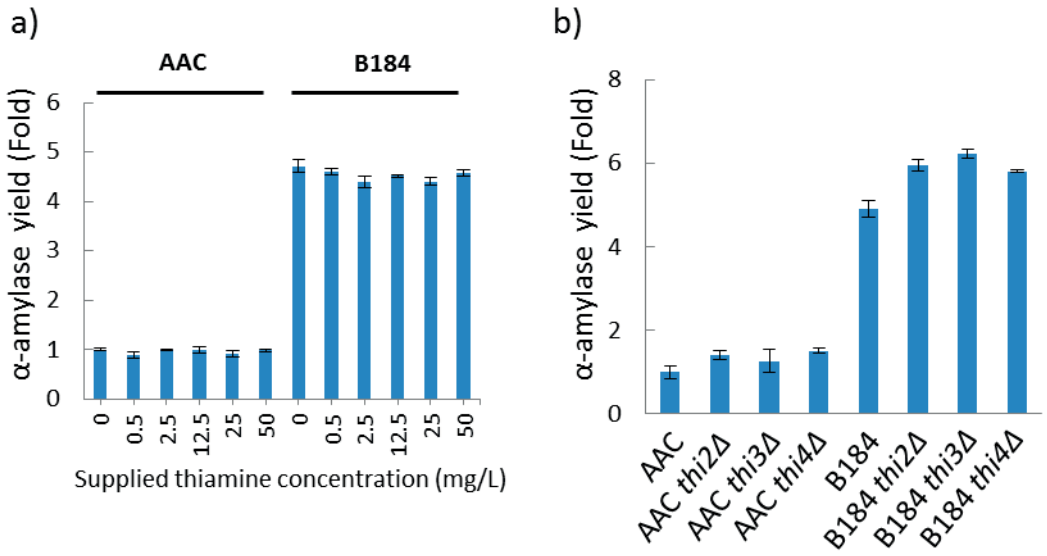


**Fig. 23 a) Supplied thiamine to the medium b) Deletion of *THI2*, *THI3* and *THI4* enhances amylase production.**

Recombinant protein production normally introduces extra ROS into cells because of protein folding in the ER [63]. The UPR is activated by arising oxidative stress in the ER to assist in reducing cellular stress. Hac1p is a key UPR-induced TF for transcriptional activation of ER chaperone-encoding genes, including *KAR2,* and *ERO1* [87]. Reduced ER stress was found in the strains in Cluster 3 (Fig. 24a). Based on the results of transcriptome analysis, the expression levels of *HAC1, ERO1* and *KAR2* in Cluster 3 were down-regulated. Interestingly, we found the transcript levels of *PDI1* and *EMC1*, which are responsible for protein folding in the ER, were up-regulated in Cluster 3, probably because of the huge demand for protein folding. Meanwhile, we also investigated ROS accumulation in the cells; all mutant strains showed lower ROS accumulation (Fig. 24b).

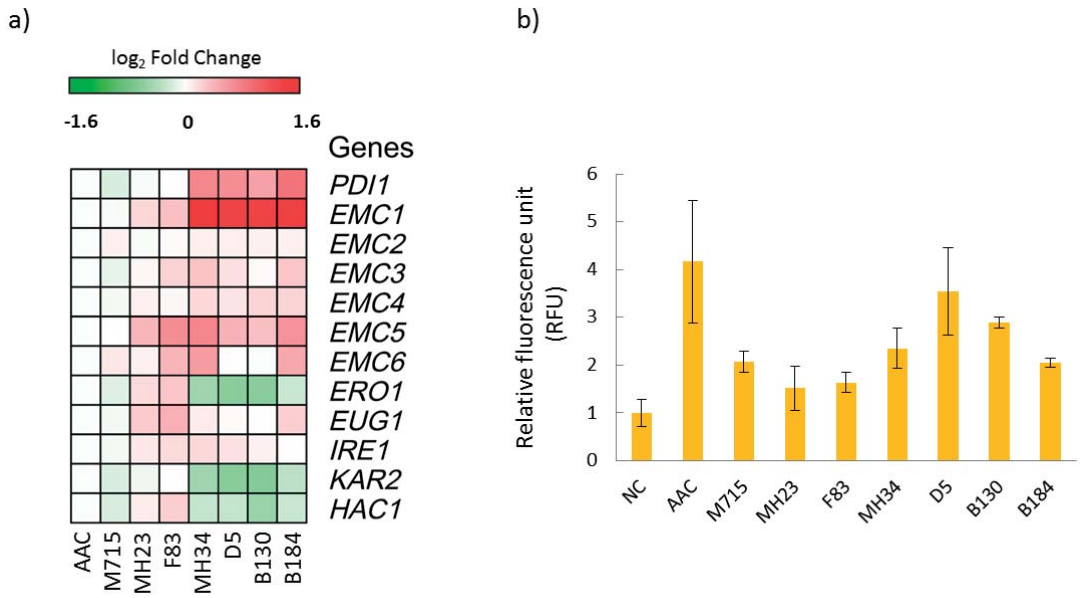


**Fig. 24 a) Expression levels of genes related to protein folding in ER. b) Quantification of ROS in strains by DHR123 staining**

Here, we studied some of the underlying mechanisms of efficient protein secretion through comparative systems biology analysis of efficient α-amylase secretion mutant strains and a reference strain. From genome-wide transcription analysis, we found that the majority of genes related to glycolysis and the citric acid cycle were down-regulated in the mutant strains, but the final biomass yield on glucose only slightly decreased, and the maximum specific growth rate increased. A previous study revealed that the enzymes involved in glycolysis represent a large proportion (30%-60%) of the soluble proteins in the cell [88]. In addition, there are strong redundancies in glycolytic enzymes; therefore, reducing the level of several glycolytic proteins has only a minor impact on yeast growth [89]. This might be due to the fact that the mutant cells re-allocated proteome mass and protein synthesis capacity for recombinant protein secretion. Furthermore, reduced cellular stress was observed in the mutant cells, resulting in a higher growth rate compared with the reference strain. Faster growth is associated with increased ethanol production and therefore a slightly lower biomass yield.

It is noteworthy that many identified reporter TFs were related to anaerobic conditions. Hypoxic genes controlled by reporter TFs were up-regulated in the mutant strains, whereas respiratory genes were down-regulated. This was consistent with reporter GO terms analysis, which showed that cellular respiration and mitochondrial function were down-regulated. Our results emphasize the importance of reducing oxidative stress associated with protein production regardless of the pathway used for this. In addition to alteration of intracellular processes by changes

in gene expression, cells can also alter processes by regulating the activity of enzymes [90, 91], and our results showed that several pathways catalysed by thiamine-dependent enzymes were affected by a low thiamine status in the mutant strains [92], which may provide another solution to tune the activity of metabolic pathways.

## Paper IV: Yeast secretion assay platform for biomedical and biotechnological applications

To allow for screening of drugs that can cure secretory-related diseases we developed a yeast secretion platform strain by expressing β-galactosidase in a secreted form. The strain can be used for evaluation of chemicals that cause secretion disturbance and hence can be further developed to discover drugs for curing secretory-related diseases while also revealing secretory limitations in the production of recombinant proteins, which will aid in the identification of targets for modification in the rational design of cell factories for protein production.

β-Galactosidase was used as a reporter protein to reveal the secretory pathway status of yeast in this study. Different *lacZ* cassettes were constructed using standard molecular biology procedures and inserted into CPOTud for β-galactosidase expression (Fig. 25a). The amount of secreted β-galactosidase is reflected by enzyme activity through X-gal hydrolysis, which will yield a blue colour on plates. A β-galactosidase-secreting strain is expected to report secretory disturbances through visible colour changes in the colony.

First, to achieve β-galactosidase secretion by yeast, the lacZ gene was fused with signal sequences. Because there are no general rules for determining which leader sequences will be best for the secretion of a specific protein, both a synthetic leader and the native yeast alpha factor leader were tested. Strain Y2 harbouring the plasmid pCP-sLacZ utilized the synthetic leader for β-galactosidase secretion. Similarly, strain Y3 harbouring pCP-aLacZ secreted β-galactosidase using the alpha factor leader. β-galactosidase secretion by these two strains was tested using X-gal plates (Fig. 25b). Y2 produced deeper blue colonies on X-gal than did Y3, indicating that the synthetic leader was more efficient for β-galactosidase secretion.

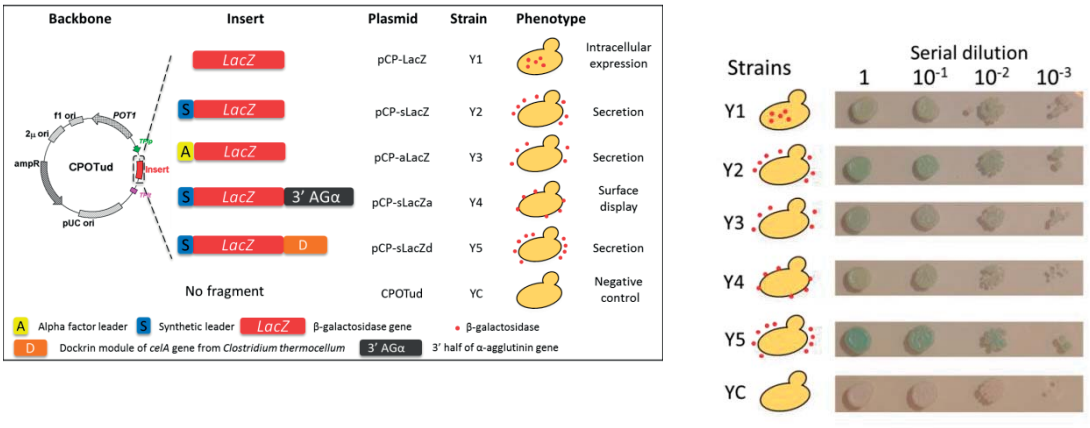


**Fig. 25 a) Construction of β-galactosidase expression strains.** Plasmids containing different inserts were transformed into *S. cerevisiae* CEN.PK 530.1D for β-galactosidase expression, resulting in different phenotypes. **b) Spot testing on X-gal plates to measure secretion efficiency.** Cells were collected from overnight cultures, washed with water and resuspended in water at $OD_{600}$ = 1. Then, the cells were spotted onto X-gal YPD plates in tenfold serial dilutions. Photographs were taken after 3 days of incubation at 30 °C and 1 day of storage at 4 °C. Y1: Intracellular expression of β-galactosidase; Y2: secretion of β-galactosidase with the synthetic leader; Y3: secretion of β-galactosidase with the alpha factor leader; Y4: surface display of β-galactosidase using the 3' half of the α-agglutinin gene (the synthetic leader was used as the secretion signal peptide); Y5: secretion of β-galactosidase fused with the dockrin module (the synthetic leader was used as the secretion signal peptide); YC: Empty plasmid as a negative control.

As efficient secretion should provide a wider dynamic range in response to disturbances, the synthetic leader was selected for use throughout this study. Surface display, or the localization of the protein/enzyme to cell surface, may also increase the concentration of the protein around the cell and extend the life of the protein. To enhance the hydrolytic capacity per unit of β-galactosidase, we tested the surface display with β-galactosidase by utilizing the 3' half of α-agglutinin (3'AGα), which is widely used for protein immobilization on the cell surface [93]. The surface-display strain Y4, which harboured the plasmid pCP-sLacZa, was then tested on X-gal plates. However, Y4 colonies did not appear deeper blue than the Y2 colonies, indicating no improvement in X-gal hydrolysis. One possible explanation is that the secreted enzyme remained in the vicinity of the colony on solid media rather than diffusing away. In other words, the cells growing on agar were more or less equivalent to the surface display. Alternatively, a dockerin module from the *C. thermocellum* celS gene, which has the ability to enhance secretion, was employed. β-Galactosidase fused with dockerin at the C-terminus was expressed in strain Y5, which showed a deeper blue colour on X-gal than did the other strains, indicating more efficient β-galactosidase secretion.

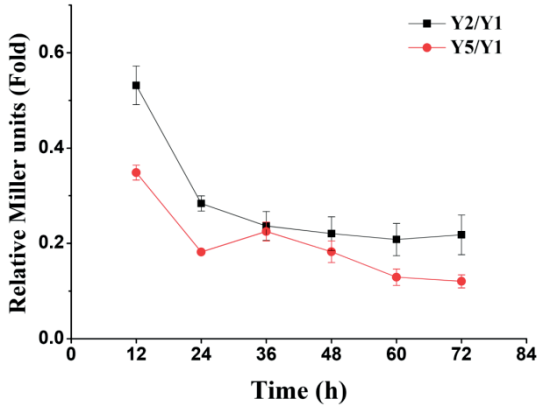


**Fig. 26 Less intracellular β-galactosidase retention in strains Y2 and Y5 than in strain Y1.** Cells were cultured in SD-2 × SCAA medium in shake flasks at 30 °C, 200 rpm.

We attempted to measure β-galactosidase activity in the supernatant from YPD liquid cultures. However, only minimal β-galactosidase activity was detected in the supernatant. Instead, intracellular β-galactosidase activity was measured and expressed in Miller units. Compared with intracellular expression strain Y1, much lower β-galactosidase retention was detected in strains Y2 and Y5 throughout the entire cultivation process (Fig. 26). In addition, the amount of intracellular β-galactosidase in strain Y2 was only 22% of that in strain Y1 at 72 hours and was even lower – only 12% – in strain Y5 (Fig. 26). This finding was in agreement with the X-gal plate results, in which strain Y5 showed the highest secretory capacity. Hence, strain Y5 was used as the yeast secretion platform in subsequent studies.

The efficient β-galactosidase secretion strain Y5, constructed above, was tested on plates containing different chemicals to assess its responses to chemicals that affect the secretory pathway (Fig. 27a).

L-Azetidine-2-carboxylic acid (AZE) is a proline analogue that is competitively incorporated into proteins and causes protein misfolding [94]. Yeast cells treated with AZE showed no significant growth defects (Fig. 27b). However, AZE reduced the amount of β-galactosidase secreted by strain Y5, as indicated by lighter blue colonies. Glutathione (GSH) is an important reductant in all cells that acts as an efficient antioxidant and maintains cytosolic redox homeostasis [95]. Strain Y5 produced white colonies instead of blue colonies on X-gal plates containing GSH. Because adding GSH to plates may alter GSH homeostasis in the cell, the formation of disulfide bonds during protein folding can be prevented by the elevation of the intracellular reducing environment due to excess GSH. L-Buthionine-sulfoximine (BSO) is an inhibitor of γ-glutamylcysteine synthetase, which is involved in GSH biosynthesis, and adding BSO to the medium has been shown to reduce intracellular GSH levels in yeast cells [96]. Interestingly, although excess GSH inhibits protein folding, colonies of

strain Y5 on BSO plates were similar in colour to those on control plates (without additional chemicals), suggesting that decreasing GSH did not affect β-galactosidase production. This result was consistent with a previous study in which oxidative protein folding in the ER did not require GSH [97].

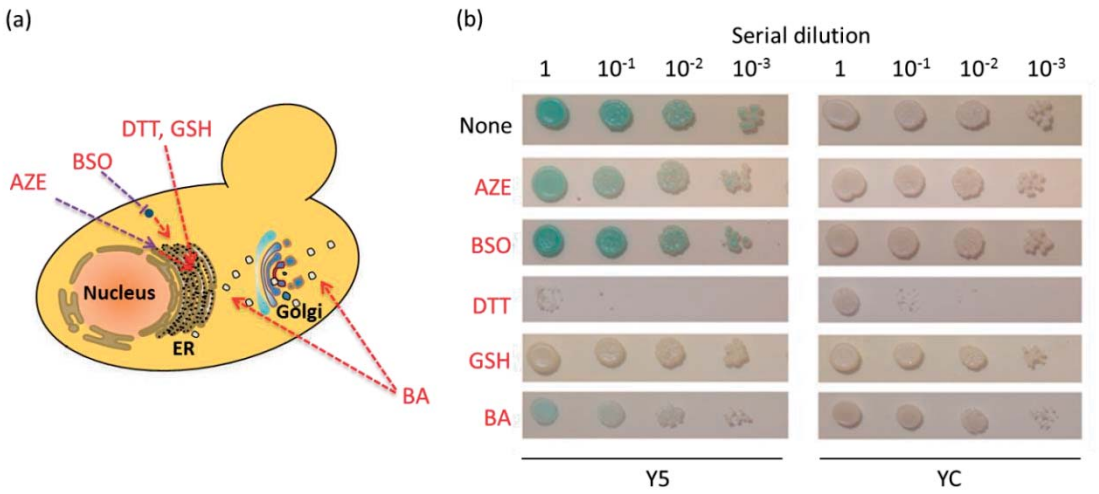


**Fig. 27 The YSAP showed differential secretory pathway activity when exposed to different chemicals.** (a) An illustration of affected targets by chemicals in *S. cerevisiae*. AZE: azetidine-2-carboxylic acid; BSO: L-buthionine sulfoximine; DTT: dithiothreitol; GSH: glutathione; BA: benzoic acid. (b) The YSAP strain Y5 showed a wide dynamic range in response to different chemicals. Cells were collected from overnight cultures, washed with water and resuspended in water at $OD_{600}$ = 1. Tenfold serial dilutions of the cell suspension were the spotted onto X-gal YPD plates containing different chemicals. Photographs were taken after 3 days of incubation at 30 °C and 1 day of storage at 4 °C.

Benzoic acid (BA) decouples gradients across the cell membrane and is widely used as a food preservative [98]. Protein secretion involves protein folding and PTMs in membrane-bound compartments (e.g., the ER and the Golgi apparatus) and vesicle trafficking between different compartments; hence, membrane integrity is important for protein secretion [98]. Strains YC and Y5 showed less growth on plates containing BA. In addition to this restrained growth, β-galactosidase secretion by strain Y5 was greatly reduced on BA plates. Dithiothreitol (DTT) is a strong reducing reagent that prevents disulfide bond formation; hence, misfolded proteins will accumulate in the ER and activate the UPR [87]. When we added DTT to plates, not only was β-galactosidase expression affected, but the growth of both strains was also severely impaired. The densest spot of strain 5, corresponding to OD=1, showed growth inhibition compared with strain YC, indicating that strain Y5 was sensitive to secretory pathway disturbances.

These results show that strain Y5 can report secretory disturbances. By introducing pathogenic factors (e.g., alpha-synuclein and amyloid-β peptide [99, 100]), strain Y5 can be developed in a pathogenic model and used as a drug screening platform to identify potential chemicals for rescuing the pathogenic phenotype.

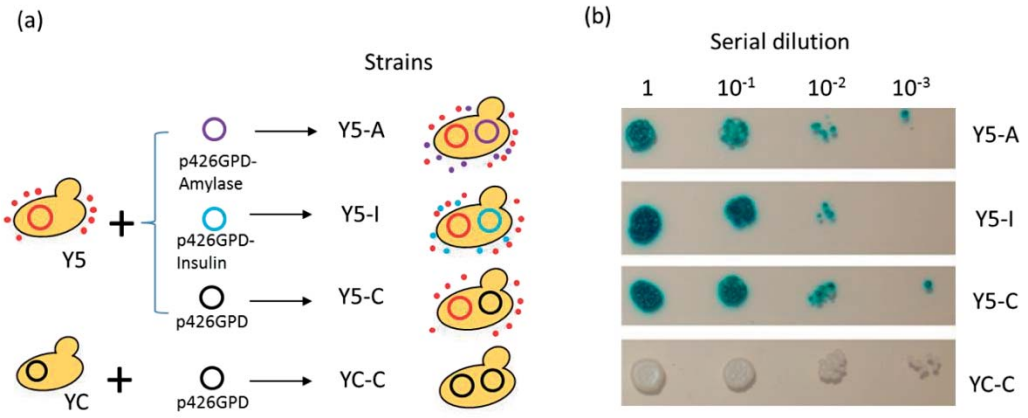


**Fig. 28 The YSAP showed differential reporting from the secretory pathway in cells expressing different proteins.** Cells were collected from overnight culture, washed with water and resuspended in water at $OD_{600}$ = 1. Ten-fold serial dilutions of the cell suspensions were then spotted onto SD-2 × SCAA plates with X-gal. Photographs were taken after 3 days of incubation at 30 °C. Y5-A: co-expression of α-amylase in strain Y5; Y5-I: co-expression of insulin precursor in strain Y5; Y5-C: strain Y5 harboring an empty plasmid as a reference; YC-C: strain YC harboring an empty plasmid as a negative control. Compared with Y5-C and Y5-I, Y5-A showed pale blue colonies on plates, indicating a blockade of the secretory pathway.

Above, we showed that strain Y5 can respond to secretory disturbances caused by chemicals. We were also interested in determining whether strain Y5 could indicate differences following the expression of different proteins. Two proteins, α-amylase and proinsulin, were chosen for testing in strain Y5 (Fig. 28a). When we introduced the amylase expression plasmid p426GPD-amylase into the Y5 strain, the resulting strain, Y5-A, produced pale blue colonies on X-gal plates, indicating reduced β-galactosidase secretion (Fig. 28b). By contrast, expressing proinsulin had no obvious impact on β-galactosidase secretion. Relative to proinsulin, α-amylase is a large protein [101], and proper protein folding is necessary for amylase function. Previous studies observed limitations in the secretory pathway in cells expressing amylase, including insufficient protein folding and ER-to-Golgi trafficking, which were not affected in cells expressing proinsulin [102, 103]. Decreased β-galactosidase secretion in strain Y5-A was likely affected by the increased secretory burden caused by the co-expression of amylase. This result showed that strain Y5 can respond to changes in the secretory burden caused by the co-expression of proteins. Hence, Y5 can be used to determine whether limitations in the secretory capacity would present major obstacles to protein production, allowing modification strategies for

the secretory pathway to be considered first. For proteins that do not burden secretory capacity, targets for modification can initially focus on optimizing transcriptional and/or translational levels before encountering problems with secretory burden.

In this study, we utilized secreted β-galactosidase as a reporter to reveal the status of secretion in yeast. The efficient β-galactosidase secretion strain Y5 was able to respond to different chemicals through changes in colony colour on X-gal plates. Strain Y5 can be developed into a platform for screening potential drugs to treat secretion-related diseases. Strain Y5 can also respond to secretory pathway burden caused by the expression of heterologous proteins, thereby helping to determine which aspects of protein production should be first considered as targets for modification.

# Summary and perspectives

The yeast *S. cerevisiae* is widely used for recombinant protein secretion. So far, many efforts have been made to increase the yeast secretory capacity.

In paper I, we reported that moderate over-expression of *SEC16* increases the recombinant protein secretory capacity of yeast. Meanwhile, we also observed a reduction of ER stress and more ERESs in the *SEC16* moderate over-expression strain, which means that the *SEC16* moderate over-expression strain has good conditions for recombinant protein synthesis, folding and secretion. However, the gross ROS accumulation in the *SEC16* moderate over-expression strain increases remarkably. The fewer mitochondria in the *SEC16* moderate over-expression strain may result from the high level of ROS accumulation.

In paper II, we engineered retrograde trafficking based on the *SEC16* strain constructed in paper I. The idea is based on the hypothesis that the excess ER membrane proteins might be transported from the ER to the Golgi with the increased anterograde trafficking in the *SEC16* strain. Hence, we separately overexpressed two GAP proteins, Gcs1p and Glo3p, in the *SEC16* strain. We found that the protein secretory capacity could be further increased when the retrograde trafficking flow increased. However, the secretory capacity was impaired when overexpressing only the GAP proteins in the strain with normal anterograde trafficking flow. This result suggests that retrograde trafficking is not limited in normal yeast cells, whereas increasing the retrograde flow has a negative effect on the protein secretory capacity; moderate over-expression of *SEC16* indeed increased the anterograde trafficking flow, resulting in retrograde trafficking being the limiting step, in this case, enlarging the retrograde flow could solve this problem.

In paper III, we performed transcriptome analysis of seven high α-amylase production strains that were isolated from three rounds of UV mutation. We found that the ROS accumulation in mutated strains is lower than in the reference strain even if the titres of α-amylase are higher. We also found that tuning down thiamine biosynthesis and up-regulating the expression of the hypoxic genes could increase the recombinant protein secretory capacity of yeast.

In paper IV, we constructed a yeast secretory assay platform by expressing β-galactosidase in a secreted form. This platform was responsive to secretion disturbance by chemicals through changes in colony colour on X-gal plates and hence can be further developed to discover drugs to cure secretory-related diseases while also revealing secretory limitations in the production of recombinant proteins. This will aid in the identification of targets for modification of the rational design of cell

factories for protein production.

The protein secretory pathway is a complicated system. The effects of the same engineering strategies on the secretion vary among recombinant proteins, which depends on the size of the protein, the numbers of disulfide bonds, the glycosylation pattern, etc. The limited step in the secretory pathway can be different according to properties of the recombinant proteins, and it can also be shifted once the preliminary limitation step is eliminated.

In paper I, we overexpressed several different genes involved in the anterograde trafficking processes, ER-to-Golgi and Golgi-to-PM. We found that the secretory limitation step in the vesicle trafficking processes for α-amylase occurs in the transport from the ER to the Golgi. In paper II, the protein secretory capacity was further increased by engineering the retrograde Golgi-to-ER trafficking in the ER-to-Golgi transport enlarged strain. However, improving the retrograde trafficking in the cell with normal anterograde trafficking did not further increase the secretion of the recombinant protein. This indicated that the limited step was changed by the engineering strategies. Therefore, in the future we can focus on the engineering of the vesicle trafficking from the Golgi to the PM in the vesicle trafficking improved strain. In paper I, we found that lowering the cellular stress can increase the protein secretion by adding moderate amount of vitamin C into the media. It has been reported that NADPH can counteract ROS potentially [104], so increasing the pool of NADPH might have a positive effect on the recombinant protein secretion by e.g. engineering the pentose phosphate pathway.

From analysis of several mutants having improved protein secretion capacity we identified several targets which could be engineered to increase recombinant protein secretion (paper III). In the future, we could integrate these targets, e.g., deletion of *BAS1*, overexpression of *SUT1*, and deletion of *THI2/3/4,* together to further increase the protein secretory capacity. Since the functions of some genes or proteins are still poorly understood, some targets and factors could be neglected in the rational design. The useful targets could be identified unbiasedly by using large scale cell based screening which benefit the secretion. However, this requires an indicator which can reflect the secretion of the protein through colour, halo, etc. These targets can also be applied in mammalian cell factories to improve the protein secretion.

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