THE EFFECTS OF NATIVE *SACCHAROMYCES CEREVISIAE* SNARE GENE OVEREXPRESSION ON HETEROLOGOUS CELLULASE SECRETION

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DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

This dissertation contains two original papers published in peer-reviewed journals, an additional experimental chapter and a review article covering aspects of some of the published work described within the manuscript. The development and writing of these papers was, unless indicated otherwise, solely my responsibility and external contributions are declared where applicable.

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Declaration by the candidate:

With regards to the entirety of the dissertation, my contributions were as follows:

Nature of contribution	Extent of contribution
Planning, experimental work and preparation of manuscript draft	92%

The following co-authors have contributed to the manuscript:

Name	E-mail address	Nature of contribution	Extent of contribution
Dr. R den Haan		Construction of two initial parental strains and assistance with densitometry work	6%
Dr. H Kroukamp		Construction of an initial pBHD1 plasmid vector	2%
Prof. WH van Zyl		Intellectual input as promoter	Non-quantifiable

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Declaration by co-authors:

The undersigned hereby confirm that:

1. The declaration above accurately reflects the nature and extent of the contributions of the candidate and co-authors.
2. No other co-authors, other than those specified, have contributed to the completion of this manuscript.
3. Potential conflicts of interest have been revealed to all interested parties.

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SUMMARY

The budding yeast *Saccharomyces cerevisiae* has been successfully utilized in several industrial sectors and has over the last decade emerged as a promising host for the production of valuable heterologous proteins. As with the development of most biologically-based production systems, there are invariably hurdles to overcome, the most pressing being the sub-optimal production yields for many heterologous proteins. The low protein secretion capacity of *S. cerevisiae* has been attributed to a great number of factors including various unknown secretory bottlenecks within the secretion pathway that collectively result in secretory titers that are often lower than 1% of the theoretical estimates. Increased secretory titers for the industrially significant fungal cellulases in the *S. cerevisiae* protein production host would greatly contribute to the economic feasibility of second generation bioethanol production. Improved titers will also benefit the production of commercially important biopharmaceutical proteins.

SNAREs (Soluble NSF (N-ethylmaleimide-sensitive factor) Attachment REceptor proteins) represent a class of membrane proteins that are required for the majority of membrane fusion events in the cell, including fusion of the protein secretory vesicles with the *cis*-Golgi and the plasma membrane. In this study, we attempted to elucidate whether the over-production of some of these SNARE components at the *cis*-Golgi interface (*BOS1, BET1, SEC22* and *SED5*) and at the plasma membrane (*SNC1, SNC2, SSO1, SSO2* and *SEC9*) could increase the efficiency of the protein secretion process in *S. cerevisiae* for two industrially significant fungal cellulases – the *Saccharomycopsis fibuligera* Cel3A (β-glucosidase) and the *Talaromyces emersonii* Cel7A (cellobiohydrolase I). Our investigation further attempted to elucidate other physiological effects that these genetic modifications could bring about, both in terms of growth vigor and response to secretory stress.

The exocytic t-SNARE Sso1p yielded the most improved secretory phenotype for *Sf*-Cel3A, with an improvement of approximately 43%, whilst the Snc1p v-SNARE component yielded the largest improvement in *Te*-Cel7A secretion of 71% (relative to the parental strain). The improvements for this reporter protein could be semi-quantitatively illustrated using SDS-PAGE and densitometry analysis. Simultaneous overexpression of exocytic SNARE genes led to a moderate improvement of 52% and 48% for the secretion of *Te*-Cel7A and *Sf*-Cel3A, respectively, whilst simultaneous SNARE-overexpression in the strains producing the *Sf*-

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Cel3A led to measurable decreases in ethanol and osmotolerance, as well as a decreased growth vigor. For the Endoplasmic Reticulum (ER)-to-Golgi SNAREs, it was the t-SNARE Sed5p that yielded the biggest improvements in the secretion of *Sf*-Cel3A (22%) and *Te*-Cel7A (68%). However, overexpression of Sed5p did lead to decreases in ethanol and osmotolerance for strains harboring either of the heterologous cellulases expressed on episomal plasmids, in addition to slight decreases in growth vigor. Simultaneous ER-to-Golgi SNARE overexpression led to less significant secretory improvements for *Te*-Cel7A and decreased secretory titers for *Sf*-Cel3A, whilst the yeast could not maintain cell viability upon simultaneous overexpression of the ER-to-Golgi SNAREs in the presence of the before-mentioned reporter protein. Co-overexpression of the most promising ER-to-Golgi and exocytic SNARE components identified for the improvement of *Sf*-Cel3A secretion (*SED5* and *SSO1*, respectively) led to a significant improvement in extracellular activity of 130%.

The production of *Sf*-Cel3A led to a measurably increased unfolded protein response (UPR), a mechanism proportionately induced by the buildup of folded and misfolded proteins in the ER. When Sed5p, which led to an improved secretion phenotype for *Sf*-Cel3A, was overexpressed in conjunction with the aforementioned reporter protein, the UPR activation was notably diminished. This suggests that a higher dosage of Sed5p may improve ER-to-Golgi protein transport to such an extent that the UPR response diminished. Overexpression of the exocytic SNAREs proved more effective for the improvement of native invertase secretion, with Sso1p and Snc1p leading to improvements of 53% and 32%, respectively. However, Sed5p only yielded a 15% improvement.

This study suggests that SNAREs fulfill a prominent role within a larger cascade of secretory pathway components that hold potential as secretory-enhancing factors for the *S. cerevisiae* heterologous protein production host. The positive effects that overexpression of SNAREs introduced for the secretion of heterologous and native proteins (such as invertase) indicate that these components may be implicated in secretory bottlenecks at the *cis*-Golgi and/or plasma membrane interface.

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OPSOMMING

Die gis *Saccharomyces cerevisiae* is oor die afgelope dekade suksesvol vir die produksie van 'n groot verskeidenheid heteroloë proteïene in verskeie industriële sektore benut. Soos vir die ontwikkeling van ander biologiese produksiesisteme, is daar altyd struikelblokke om te oorkom en een van die mees prominente uitdagings t.o.v. hierdie gis is sy suboptimale heteroloë sekresiekapasiteit. Die lae heteroloë sekresiekapasiteit in *S. cerevisiae* is al aan 'n reeks veranderlikes toegeskryf, insluitende 'n groot hoeveelheid onbekende sekresiebottelnekke wat gesamentlik tot sekresieopbrengste lei wat dikwels laer as 1% van die teoretiese skatting is. Verbeterde opbrengste vir waardevolle heteroloë sellulases in *S. cerevisiae* sal 'n merkwaardige bydrae tot die realisering van 'n bekostigbare tweede-generasie bio-etanol alternatief lewer, terwyl 'n verbetering in dié produksieorganisme 'n bydrae tot die produksie van kommersiële biofarmaseutiese proteïene kan lewer.

SNAREs ("Soluble NSF (N-ethylmaleimide-sensitive factor) Attachment REceptor proteins") verteenwoordig 'n klas membraanproteïene wat essensieël vir die meerderheid van membraan samesmeltingsreaksies in die sekresiepadweg van die eukariotiese sel is. Hierdie reaksies sluit die samesmeltings van die proteïensekresievesikels met die *cis*-Golgi en die plasmamembraan, onderskeidelik, in. In hierdie studie het ons probeer bepaal of die oorproduksie van sommige van hierdie SNARE-komponente by die *cis*-Golgi intervlak (*BOS1*, *BET1*, *SEC22* en *SED5*) en plasmamembraan (*SNC1*, *SNC2*, *SSO1*, *SSO2* en *SEC9*) die effektiwiteit van die sekresieproses in *S. cerevisiae* vir twee heteroloë sellulases, naamlik die *Saccharomycopsis fibuligera* Cel3A (β-glukosidase) en *Talaromyces emersonii* Cel7A (sellobiohydrolase I), verbeter. Verder het ons ook probeer bepaal watter ander fisiologiese effekte dié genetiese modifikasies kon meebring, beide in terme van groei en sekresiestres.

Die eksositiese t-SNARE-komponent Sso1p het die grootste impak op die sekresie van *Sf*-Cel3A gelewer, met 'n verbetering van 43%, terwyl die Snc1p v-SNARE-komponent die grootste verbetering vir *Te*-Cel7A (71%) (relatief tot die ouerras) gelewer het. Die verbeteringe vir die *Te*-Cel7A kon semi-kwantitatief m.b.v. SDS-PAGE- en densitometrie-analiese geïllustreer word. Gesamentlike ooruitdrukking van eksositiese SNARE gene het matige verbeteringe van 52% en 48% vir onderskeidelik die *Te*-Cel7A and *Sf*-Cel3A opgelewer, hoewel dié strategie tot vertraagde groei en verlaagde etanol- en osmotoleransie gelei het. Die ER-to-Golgi t-SNARE Sed5p- komponent het tot die grootste

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verbetering in die sekresie van *Sf*-Cel3A (22%) en *Te*-Cel7A (68%) gelei. Die ooruitdrukking van Sed5p het ook tot ‘n verswakking in groeikapasiteit gelei, asook verlaagde etanol- en osmotoleransie in rasse wat een van die die heteroloë sellulases vanaf episomale plasmiede uitgedruk het. Gesamentlike ooruitdrukking van die ER-tot-Golgi-SNAREs het tot slegs matige verbeteringe in die sekresie van *Te*-Cel7A gelei, maar het die sekresie van *Sf*-Cel3A negatief beïnvloed. Die gis kon ook nie die gelyktydige ooruitdrukking van al vier komponente onderhou in kombinasie met die laasgenoemde heteroloë proteïen. Die gelyktydige ooruitdrukking van die mees effektiewe ER-tot-Gogli en eksositiese SNARE-komponente vir die verbetering van *Sf*-Cel3A produksie (*SED5* en *SSO1*) het tot ‘n merkwaardige verbetering van ongeveer 130% gelei.

Die heteroloë produksie van *Sf*-Cel3A het tot ‘n beduidende induksie van die ontvouproteïenreaksie (UPR) gelei, ‘n spesifieke reaksiemeganisme wat die gis onder ER-stres-geïnduseerde omstandighede aktiveer wanneer die opeenhoping van gevoude en misgevoude proteïene in die ER toeneem. Wanneer Sed5p, wat *Sf*-Cel3A sekresie verbeter, tesame met *Sf*-Cel3A ooruitgedruk word, is ‘n verlaging in hierdie UPR waargeneem, wat aandui dat verbeterde proteïenverkeer tussen die ER en die Golgi apparaat deur ‘n verhoogde dosis van Sed5p bewerkstellig is, met ‘n gevolglike verlaging in die UPR-reaksie. Ooruitdrukking van die eksositiese SNAREs (Snc1p en Sso1p) was meer effektief vir die verbetering van die gis se eie invertasesekresie in vergelyking met die mees prominente ER-to-Golgi alternatief, (Sed5p) met verbeteringe van 32%, 53% en 15%, onderskeidelik.

Hierdie studie stel voor dat SNAREs ‘n prominente rol binne ‘n breë kaskade van sekresiewegkomponente uitmaak wat potensiële voordele vir die heteroloë sekresiekapasiteit van *S. cerevisiae* kan inhou. Die positiewe effekte van SNARE-ooruitdrukking op die sekresie van heteroloë sellulases en eie proteïene soos invertase, impliseer dat SNAREs ‘n rol in die sekresiebottelnekke by die *cis*-Golgi en plasmamembraan speel.

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PREFACE

This dissertation is presented in an anthology format, with the majority of the results component having been published in two peer-reviewed journal articles and some components utilized for the publication of a review article. A third research component, covering additional work extrapolating on results published in the afore-mentioned journal articles, concludes the investigation. The literature review has been specifically divided into subsections that will, upon reading, allow for a progressively focused introduction to the subject matter.

Peer-reviewed journal articles:

Van Zyl JHD, **Den Haan R, Van Zyl WH** (2014) Overexpression of native *Saccharomyces cerevisiae* exocytic SNARE genes improved heterologous cellulase secretion. Appl Microbiol Biotechnol 98 (12): 5567-5578

Van Zyl JHD, Den Haan R, Van Zyl WH (2015) Overexpression of native *Saccharomyces cerevisiae* ER-to-Golgi SNARE genes improved heterologous cellulase secretion. Appl Microbiol Biotechnol DOI 10.1007/s00253-015-7022-2

Contribution to review article:

Den Haan R, Kroukamp H, Van Zyl JHD, Van Zyl WH (2013) Cellobiohydrolase secretion by yeast: Current state and prospects for improvement. Proc Biochem 48: 1-12

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LIST OF ABBREVIATION

AMP	Adenosine Monophosphate
AFEX	Ammonia Fibre Expansion
Asn	Asparagine
ATP	Adenosine-5'-triphosphate
BGL	β-Glucosidase
BSA	Bovine Serum Albumin
CBH	Cellobiohydrolase
CBM	Carbohydrate Binding Module
CBP	Consolidated Bio-Processing
cDNA	Complementrary DNA
COG	Conserved Oligomeric Golgi
COP	Coat Proteins
CPY	Carboxypeptidase Y
CTAB	Cetyltrimethylammonium Bromide
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNS	3,5-Dinitrosalicylic acid
EMEA	European Medicines Agency
ER	Endoplasmic Reticulum
ERAD	ER-associated Degradation
FDA	Food and Drug Administration
GC	Golgi Complex
GDP	Guanosine-5'-diphosphate
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GPI	Glycosylphosphatidylinositol
GRAS	Generally Regarded As Safe
GTP	Guanosine-5'-triphosphate
HGH	Human Growth Hormone
HPV	Human Papilomavirus
LB	Luria Bertani
MTC	Multisubunit Complexe
NSF	N-ethylmaleimide-sensitive factor
Nu	Nucleus
ORF	Open Reading Frame
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PDI	Protein Disulfide Isomerase
PKA	Protein Kinase A
PM	Plasma Membrane
***p*NPC**	*p*-nitrophenyl-β-D-cellobioside
***p*NPG**	*p*-nitrophenyl-α-D-glucopyranoside
RNA	Ribonucleic Acid
RT-qPCR PCR	Quantitative Reverse Transcription
SC	Synthetic Complete
scFvs antibodies	Single Chain Variable Fragment
SDS	Sodium Dodecyl Sulphate
Ser	Serine
SM	Sec1/Mun-18
SNAPs	Soluble NSF Attachment Proteins
SNAREs proteins	Soluble NSF Attachment Receptor
TGN	Trans-Golgi Network
Thr	Threonine
UPR	Unfolded Protein Response
USA	United States of America
VAMP Proteins	Vesicle-Associated Membrane
YPD	Yeast Extract Peptone Dextrose

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Chapter 1:

General introduction and project aims

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Chapter 1:

General introduction

The budding yeast *Saccharomyces cerevisiae* has been extensively intertwined with human societal development, playing an essential role in the evolution of our baking, brewing and wine-making practices for millennia (Legras *et al.,* 2007; Borneman *et al.,* 2013). The early 1980s brought about further exploitation of this industrially versatile yeast, with the advent of large-scale production of extracellular proteins of microbial, plant and animal origin (Romanos *et al.,* 1992; Romanos *et al.,* 1995). In keeping with the vigorous expanses in the field of molecular biology, *S. cerevisiae* was soon being molded to serve as a production host for recombinant and native proteins for the agricultural, biopharmaceutical and commercial enzyme industries (Demain & Vaishnav, 2009).

With the environmental damage of global fossil fuel consumption and the problematic socio-economic effects of energy security becoming more evident, the renewable energy sector subsequently latched onto *S. cerevisiae*'s impressive fermentative capabilities in order to produce carbon neutral biofuels. *Saccharomyces cerevisiae* emerged as a prime candidate microorganism for the production of renewable fuels from plant biomass and today, a large number of strains have been engineered to produce fungal cellulases for the breakdown and fermentation of plant biomass to ethanol (Van Rooyen *et al.,* 2005; Den Haan *et al.,* 2007; Illmén *et al.,* 2011). Until recently, approximately 90% of the biofuels market consisted of first generation biofuels, i.e. bioethanol fermented from corn starch or cane sugar and biodiesel esterified from edible vegetable oils and animal fats (Zverlov & Shwarz, 2007). In order to effectively compete with fossil fuels with respect to economic viability, these biomass feedstocks would demand the large-scale diversion of farmland and crops, encroaching heavily on global food security (Mohr & Raman, 2013).

An alternative feedstock to those used for first generation biofuels is lignocellulosic biomass from non-food energy crops or agricultural waste products, which contain a sizeable cellulose-fraction that can be enzymatically broken down to fermentable sugars through the action of fungal cellulases and hemicellulases. The downside to lignocellulosic feedstocks is their notorious recalcitrance, which leads to the requirement for energy-intensive pre-treatment processes and large amounts of expensive cellulolytic enzymes, regarded as the third most

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expensive price component in lignocellulosic ethanol production (Stephen *et al.,* 2012; Isola, 2013). A promising strategy that could improve the efficiency and overall economics of lignocellulosic ethanol production is a single step hydrolysis and fermentation scheme, or consolidated bioprocessing (CBP) approach, whereby cellulase production, cellulose hydrolysis and fermentation of the resulting hexose and pentose sugars to ethanol are combined in a single reaction vessel (Van Zyl *et al.,* 2007).

Despite the vast number of advantages that *S. cerevisiae* offers as an ethanol producer, it is still not as highly regarded as other yeast expression systems such as *Pichia pastoris* for heterologous protein production, due to its Crabtree-positive nature and sub-optimal heterologous protein secretion titers, with many proteins being produced at 1% (or less) of the theoretical estimates (Müller *et al.,* 1998; Liu *et al.,* 2014). This reduced secretory capacity for heterologously expressed fungal cellulases is one of the major barriers to the successful application of *S. cerevisiae* as CBP-host (Ilmén *et al.,* 2011). An increase in the presently attainable titers for heterologous cellulases would therefore vastly improve the efficiency of lignocellulose breakdown to fermentable sugars, which would in turn improve the financial viability of lignocellulosic ethanol production (Kitagawa *et al.,* 2010). Rationally designed engineering strategies focusing on components associated with protein folding, glycosylation and vesicular fusion reactions within the secretion pathway, have yielded moderate success. Improvements remain largely peptide-specific and a holistic understanding of the collection of bottlenecks contributing to the sub-optimal heterologous secretion phenotype has remained elusive (Hou *et al.,* 2012; Kroukamp *et al.,* 2013; Van Zyl *et al.,* 2014; Tang *et al.,* 2015; Van Zyl *et al.,* 2015).

Soluble NSF (N-ethylmaleimide-sensitive factor) attachment receptor proteins (SNAREs) have become a topic of significant interest over the last decade, with pioneering work by James E. Rothman, Randy W. Schekman and Thomas C. Südhof culminating in them jointly receiving the 2013 Nobel Prize in Physiology or Medicine (http://www.nobelprize.org/nobel_prizes/medicine/laureates/2013/). SNAREs are a class of membrane proteins that are required at the vast majority of membrane fusion events in the cell, facilitating the targeted fusion of protein transport vesicles between the various membrane-enclosed organelles in the secretory pathway and the plasma membrane (Weber *et al.,* 1998; Grote *et al.,* 2000). Hou *et al.* (2012) illustrated that overexpression of SNARE-interacting proteins can have favourable effects on heterologous protein secretion, whilst

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Ruohonen *et al.* (1997) also reported that the overexpression of some SNAREs have a positive effect on protein production. With this in mind, we have investigated the effects of SNARE-overexpression on heterologous cellulase production, focusing on the components facilitating vesicular fusion at the *cis*-Golgi and plasma membrane, in an attempt to elucidate whether these components could contribute to favourable secretion phenotypes.

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Aims of the study:

The general aims of the study were to determine whether **(1)** the overproduction of SNARE proteins involved in anterograde vesicle fusion reactions has an effect on the heterologous secretion capacity of *S. cerevisiae;* **(2)** to identify the most effective SNARE components facilitating possible improvements; and **(3)** to investigate some of the physiological effects of SNARE overexpression on the yeast, both in terms of growth and response to secretory stress.

The objectives set out to assist in addressing these aims were:

1. To investigate whether overexpression of native ER-to-Golgi and exocytic SNARE genes could improve the heterologous secretion capacity of *S. cerevisiae* for two heterologously produced cellulases, i.e. the *Saccharomycopsis fibuligera* Cel3A and the *Talaromyces emersonii* Cel7A.
2. To determine whether any secretion improvement trends extended to native *S. cerevisiae* proteins such as invertase.
3. To identify the key SNARE components at the *cis*-Golgi and plasma membrane interface that produced an enhanced secretory phenotype.
4. To determine whether simultaneous overexpression of cognate SNARE complex components and inter-complex components could improve secretion in an additive or synergistic manner.
5. To determine whether SNARE overexpression-related improvements were dependent on gene dosage.
6. To evaluate the physiological effects of SNARE gene overexpression, both singularly and simultaneously, on the basal growth capability of the yeast.
7. To determine whether SNARE overexpression effected the tolerance of the yeast to general growth stresses.
8. To evaluate the effects of SNARE overexpression on ER-stress induction and the unfolded protein response (UPR).

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Chapter2:

Literature Review

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Chapter 2:

Literature Review

2.1 Biomass as renewable energy source: potential and barriers

Since the early 1920s, petroleum has established itself as the fuel of choice to supply energy to much of the planet (Aristidou & Penttilä, 2000). Since it takes millions of years for biomass to be converted to these fossil fuels, they are not considered renewable within the timeframe that we utilize them. Therefore, plant biomass is currently the only foreseeable source of sustainable organic fuels and chemicals for the future. The environmentally damaging effects of fossil fuel combustion have been well documented, with the CO_2 released during the process playing a prominent role in global warming (Branduardi *et al.,* 2008). Ethanol is a clean-burning fuel which is greenhouse gas neutral since the CO_2 that is produced during combustion is sequestered from the atmosphere by photosynthetic plants and incorporated into biomass (Nitin *et al*., 2008).

Bioethanol (i.e. ethanol produced from biomass) is considered one of the most important renewable fuels on earth and its large scale production from the waste products of non-food energy crops, rich in lignocellulose, represents a low-cost alternative to fossil fuels (Purwadi *et al.,* 2007). In 2007, approximately 90% of the biofuels market consisted of first generation biofuels, i.e. bioethanol produced from corn starch or cane sugar and biodiesel esterified from edible vegetable oils and animal fats (Zverlov & Shwarz, 2007). For the realistic displacement of petroleum with first generation biofuels, there would have to be a large scale diversion of farmland and crops for biofuel production, which would in turn lead to additional constraints on the world food supply whilst generating additional economic and ethical issues.

Advanced biofuels, i.e. ethanol produced from cellulosic biomass, provide the most attractive environmentally sustainable solution for the partial or complete replacement of petroleum (Aristidou & Penttilä, 2000). A large variety of biomass feedstocks can be utilized for the production of bioethanol and other hydrocarbon-based chemicals. These include agricultural waste products such as corn stalks, wheat straw and potato waste, wood harvesting residues,

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specifically cultivated energy crops such as sugarcane and corn as well as non-food energy crops such as Switchgrass and Giant Miscanthus.

The major downside to lignocellulosic feedstocks (non-food energy crops and agricultural byproducts) is their notoriously recalcitrant nature, making them more difficult to convert to fermentable sugars than first generation alternatives (van Maris *et al.,* 2006). Plant biomass, or lignocellulose, consists primarily of a combination of lignin, cellulose and hemicellulose (**Fig. 1**) (Breen & Singleton, 1999). It is an inexpensive and copious raw material of which a notable fraction can be converted to fermentable sugars for ethanol production. Cellulose, a major constituent of lignocellulose, is the most abundant naturally occurring renewable biopolymer on Earth and has been extensively applied in a variety of areas, including the textile industry, the paper and pulp industries and the biofuels sector (Mansfield *et al.,* 1999; Bhat, 2000; Alriksson, 2006). It is remarkably strong and plays a major role as a structural component in plant cell walls, consisting of extensive, unbranched linear homopolymers of D-glucose units that are linked by β-1,4-glucosidic bonds, where adjacent D-glucoses are flipped, making cellobiose the fundamental repeating unit (Mansfield *et al.,* 1999; Sun & Cheng, 2002). Although cellulose is generally present in biomass as crystalline fibers that are notably resistant to hydrolysis, it nevertheless makes up a larger percentage in biomass than its lignin and hemicellulosic counterparts, making cellulases (cellulolytic enzymes) the key enzymatic target for bioethanol production (Ragauskas *et al.,* 2006).

Cellulolytic enzymes are able to carry out cellulolysis, i.e. the hydrolysis of cellulose, and are naturally produced by fungi, bacteria and protozoans. For the complete hydrolysis of crystalline cellulose, as found in nature, three groups of enzymes are required, namely endoglucanases, exoglucanases and β-glucosidases (Juhász *et al.,* 2005). Endoglucanases attack low-crystallinity (amorphous) areas of cellulose fibres, generating free chain-ends from which exoglucanases (CBH1 (Cel7A) and CBH2 (Cel6A)) can remove cellobiose groups, whilst β-glucosidases (BLG1 (Cel3A)) hydrolyze the cellobiose disaccharides into its two glucose constituents (Sun and Cheng, 2002). The importance of sufficient β-glucosidase activity in commercially applied enzyme cocktails has been well described (Xin *et al.,* 1993; Pitson *et al.,* 1997; Han & Chen, 2008; Zhang *et al.,* 2010; Gurgu *et al.,* 2011), whilst the problematic nature of high-level cellobiohydrolase secretion in yeast has also been well documented (Illmén *et al.,* 2011; Den Haan *et al.,* 2013a).

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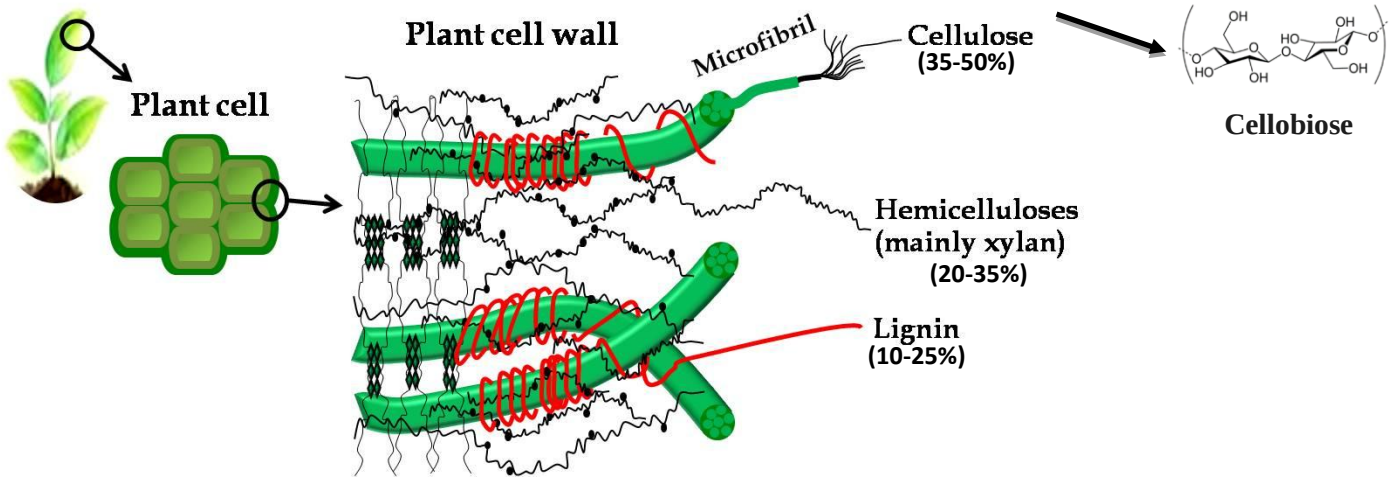

Fig. 1: The relationship between the various components in lignocellulose is represented, with the repeating unit (cellobiose) of cellulose illustrated within its structural context (Adapted from Tomme *et al.,* 1995).

Methods for the hydrolysis of lignocellulose to fermentable sugars can be divided into a concentrated-acid hydrolysis, a dilute acid hydrolysis or, alternatively, an enzymatic hydrolysis process (Alriksson, 2006). The concentrated-acid hydrolysis of lignocelluloses, with a concentrated acid such as H_2SO_4 administered at relatively modest temperatures, efficiently releases fermentable sugars, but results in the unwanted build-up of residual acid (Alriksson, 2006). Using the dilute-acid hydrolysis approach, H_2SO_4 is added at high temperatures, which leads to prompt reaction times coupled with low acid consumption. However, it also produces a low sugar yield, results in the degradation of lignocelluloses, the build-up of fermentation inhibitors and requires relatively high treatment temperatures. The current state of chemical and physiochemical pretreatment methodologies for lignocellulose hydrolysis has been extensively reviewed by Brodeur *et al.* (2011) and will not be covered further in this review.

Enzymatic hydrolysis revolves around the use of specific cellulase enzymes, first discovered in the filamentous fungus *Trichoderma reesei* during the Second World War, to hydrolyze lignocellulose and as early as 1964 extracellular enzyme preparations from this fungus were already being produced commercially (Mandels *et al.,* 1964; Sheehan & Himmel, 1999). Enzymatic hydrolysis is usually accompanied by some sort of pre-treatment step, whether steam explosion, dilute-acid treatment, alkaline hydrolysis, milling, irradiation or Ammonia Fibre Expansion (AFEX), to remove lignin and hemicelluloses and increase the structural

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susceptibility to enzymatic breakdown, which in turn enhances cellulose hydrolysis. An in-depth review, covering the various pretreatment methodologies associated with enzymatic hydrolysis, has been published by Chiaramonti *et al.* (2012) and these will not be discussed in further detail in this manuscript. The advantages of enzymatic hydrolysis are high fermentable sugar yields, moderate treatment temperatures, as well as the production of low levels of undesired by-products (Alriksson, 2006).

The production of bioethanol from lignocellulosic substrates using recombinant yeast strains is still not financially viable as an alternative fuel source, when compared to fossil fuels produced at large scale in mature refineries (Stephen *et al.,* 2012). Two of the major price components contributing to the current lack of financial feasibility include: 1) the soaring energy cost of lignocellulosic pre-treatment at high temperatures; and 2) the high production cost of the commercial cellulases required to release sugars from the substrate. The production cost of commercial cellulases makes the process increasingly expensive and alternative strategies are consistently being sought to make the process more financially viable (Kitagawa *et al.,* 2010). In fact, the cost of enzymatic saccharification is regarded as the third most expensive price component in the production of lignocellulosic bioethanol, behind capital and feedstock costs (Pu *et al*., 2008; Aden & Foust, 2009; Stephen *et al.,* 2012; Isola, 2013).

There have been four major suggestions to address the impeding effects of enzyme production costs, including: 1) Improving the efficacy of pretreatment procedures to increase the digestibility of the cellulosic feedstock (Mosier *et al.,* 2005; Alvira *et al.,* 2010; Galbe & Zacchi 2012); 2) decreasing the cost of enzyme production (Merino & Cherry, 2007; Wilson, 2009); 3) increasing the specific activity of the enzymes (Darias & Villalonga, 2001; Boer & Koivula, 2003); and 4) successfully recycling enzymes for successive rounds of hydrolysis (Tu *et al.,* 2006; Tu *et al.,* 2007). Over the last decade, the cost of enzyme production has been lowered by a reported 72-80% (Isola, 2013), though it remains a major financial hurdle to overcome in the pursuit of an economically viable second generation bioethanol solution.

The pinnacle of what has been sought is a CBP (Consolidated Bioprocessing) approach by which cellulase production, cellulose hydrolysis and fermentation of the resulting hexose and pentose sugars to ethanol are combined in a single step (Lynd *et al.,* 2005). There are several shortcomings that must be overcome when considering *Saccharomyces cerevisiae* as a CBP host, including enabling pentose sugar fermentation and high-level heterologous cellulase

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production (Van Zyl *et al.,* 2007). An additional issue with regards to cellulase production is the fact that *S. cerevisiae* is generally not capable of secreting sufficiently high levels of heterologous proteins (Ruohonen et *al.,* 1997). Heterologous protein production must therefore be enhanced in yeasts to increase the efficiency of lignocellulose degradation to fermentable sugars, which will in turn improve the financial viability of lignocellulosic ethanol production (Kitagawa *et al.,* 2010).

2.2 *Saccharomyces cerevisiae* as commercial protein production host

Yeasts have been utilized successfully for the large-scale heterologous production of intracellular and extracellular human, animal and plant proteins since the early 1980s, whilst specific species such as *S. cerevisiae* have been extensively applied in brewing and winemaking practices for centuries (Romanos *et al.,* 1992; Romanos, 1995). Besides a high ethanol yield and tolerance, the budding yeast *S. cerevisiae* provides numerous advantages when considered for laboratory and industrial use. It is non-pathogenic and single celled, whilst having acquired a GRAS (Generally Regarded As Safe) status. Budding yeast are also easily cultured and genetically manipulated and have been used extensively as a eukaryotic model system for the study of a variety of cellular processes (Drubin, 1989). The immense amount of research on *S. cerevisiae* has led to major insights into crucial eukaryotic processes such as transcription, RNA processing, cell cycle regulation, cell aging, vesicle trafficking and cell signaling (Siggers & Lesser, 2008). It was also the first eukaryote of which the genome was completely sequenced (Goffeau *et al.,* 1996). Unlike many bacterial expression systems, yeast also provide the added benefit of not possessing the risk of containing pyrogens, viral or oncogenic DNA (Toikkanen, 1999).

Saccharomyces cerevisiae is also capable of high-density fermentations, whilst allowing cultivation in small culture vessels, enabling easier laboratory manipulation (Ostergaard *et al.,* 2000; Idiris *et al.,* 2010). Furthermore, yeasts in general provide a relatively efficient means to modify and secrete heterologous proteins according to a eukaryotic processing scheme. Some of the essential eukaryote-specific post-translational modifications that yeasts are able to perform include proteolytic processing, specialized folding, disulfide bridge formation,

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acetylation and glycosylation (Eckart & Bussineau, 1996). Yeast (e.g. *S. cerevisiae*) generally secrete very few proteins and only approximately 0.5% of the total repertoire of native yeast proteins are secreted into the extracellular environment (Panchal, 1990). Therefore, when engineered to produce extracellular heterologous proteins, these could theoretically be readily purified without excessive purification steps.

Several yeast species have been engineered specifically as heterologous protein expression systems over the last few decades (Gellissen *et al.,* 1995; Müller *et al.,* 1998; Piontek *et al.,* 1998; Idiris *et al.,* 2010). Some of these yeast secretion systems that are currently being investigated include the fission yeast *Schizosaccharomyces pombe* (Takegawa *et al.,* 2009), methylotrophic species such as *Pichia pastoris* (Ilgen *et al.,* 2005), *Hansenula polymorpha* (Kang & Gellissen, 2005) and *Pichia methanolica* (Raymond *et al.,* 1998), as well as the dimorphic yeast *Yarrowia lipolytica* (Madzak *et al.,* 2005). Whilst the majority of yeasts, such as *S. cerevisiae* and *P. pastoris,* are outcompeted in terms of productivity by filamentous fungi such as *Aspergillus* and *Trichoderma* species, or bacterial protein production systems such as *Bacillus* and *Escherichia coli,* they nevertheless offer a safe, reliable and high-density fermentative option to heterologous protein production (Payne *et al.,* 2008).

The current market for United States Food and Drug Administration (US FDA)-approved therapeutic proteins and antibodies now exceeds $100 billion per annum according to BIOPHARMA® (*http://www.biopharma.com/approvals_2012*). Of the 151 recombinant US FDA and European Medicines Agency (EMEA)-approved biopharmaceuticals in production in 2009 (Ferrer-Miralles *et al.,* 2009), yeast and bacteria constituted approximately 50% of protein production hosts, with 20% of the biopharmaceutical repertoire produced in *S. cerevisiae.* Some of the therapeutic proteins and antibodies produced in yeast include insulin, albumin, human growth hormone (HGH), human papillomavirus (HPV) vaccines and hepatitis B surface antigen, illustrating that the optimization of yeast secretion systems is widely applicable (Harford *et al.,* 1987; Schmidt, 2004; Rader, 2007; Idiris *et al.,* 2010; Hou *et al.,* 2012, Nielsen, 2013). In 2008, the biopharmaceutical market outperformed the pharmaceutical market with an average annual growth of 19%, largely due to increases in yields of up to 100-fold in eukaryotic expression systems (Schröder *et al.,* 2008).

The heterologous enzyme industry emerged in the early 1980s and has flourished over the last few decades with the advent of microbial enzyme production (Demain & Vaishnav, 2009).

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Some of the major initial limitations the industry faced, whilst enzymes were predominantly derived from plant and animal sources, were low yields, expensive production costs and low levels of product availability in nature. Microbial enzyme production, in conjunction with the increased ease of genetic manipulation of production hosts associated with rapid expanses within the field of molecular biology, resulted in a significant increase in production yields and, in doing so, the variety of products (Falch, 1991). Within the industrial enzyme production sphere, over half of the current industrial enzyme repertoire is produced by yeasts and molds and as early as 1994, over 50% of the enzymes in production were produced using recombinant methodologies (Hodgson, 1994). In this study, it is the utilization of the budding yeast *S. cerevisiae* as a production host for cellulolytic enzymes required for second-generation bioethanol production that is of particular interest, with wild type *S. cerevisiae* bereft of natural cellulolytic capabilities (Harford *et al.,* 1987; Schmidt, 2004; Rader, 2007; Idiris *et al.,* 2010; Hou *et al.,* 2012).

2.3 The *S. cerevisiae* secretion conundrum

The production of heterologous proteins in *S. cerevisiae* is often largely restricted by the inefficiency of product secretion and many recombinant proteins are produced at an estimated 1% (or less) of their theoretical capacity (Liu *et al.,* 2014). Sub-optimal heterologous protein secretion in yeasts has been largely attributed to a low protein folding rate, although many factors such as transcriptional and translational blocks could contribute (Gasser *et al.,* 2007). Factors that affect heterologous protein secretion in yeast include: 1) the copy number of the expression cassette; 2) the site and mode of integration of the expression cassette; 3) promoter strength; 4) nature of the secretion signal; 5) translational start codon context (AUG); 6) glycosylation; 7) endogenous protease activity; 8) folding requirements in the endoplasmic reticulum (ER); 9) secretion pathway bottlenecks; 10) host strain physiology as well as media, growth conditions and fermentation parameters (Payne *et al.,* 2008). The correct choice of an appropriate host strain, induction medium and expression plasmid can alleviate some of the secretory limitations, but the aforementioned factors ultimately limit secretion titers severely (Liu *et al.,* 2014).

Yeast, as eukaryotes, contain membrane-enclosed, chemically distinct organelles that have specialized functions and contain unique combinations of proteins, lipids and cofactors

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(Toikkanen, 1999). The secretion pathway in eukaryotes essentially consists of two organelles, the ER and the Golgi apparatus, which function in tandem to maintain the fidelity of protein synthesis, maturation and secretion (**Fig. 2**) (Lodish, 2000). The majority of proteins destined for organelles cannot enter them directly from the cytosol and many therefore have to be sorted accordingly before being transported to their destination (Nunnari & Walter, 1996). Proteins are initially translated from nuclear mRNAs by ribosomes on the rough ER and subsequently translocated into the ER – the entry point to the secretory pathway. Transport of vesicles amid the various components of the secretory pathway and to the extracellular environment is facilitated by the fusion of vesicles and membranes (Schekman, 1982). Membrane and vesicle fusion are fundamentally important and intricately regulated cellular processes that are essential to the transport of proteins, lipids and metabolites in all eukaryotes (Strop *et al.,* 2008). Yeast have been invaluable in the study of vesicle fusion and vesicular transport due to the ease of their genetic and biochemical manipulation, and over 100 genes involved in yeast protein secretion have been identified (Toikkanen, 1999).

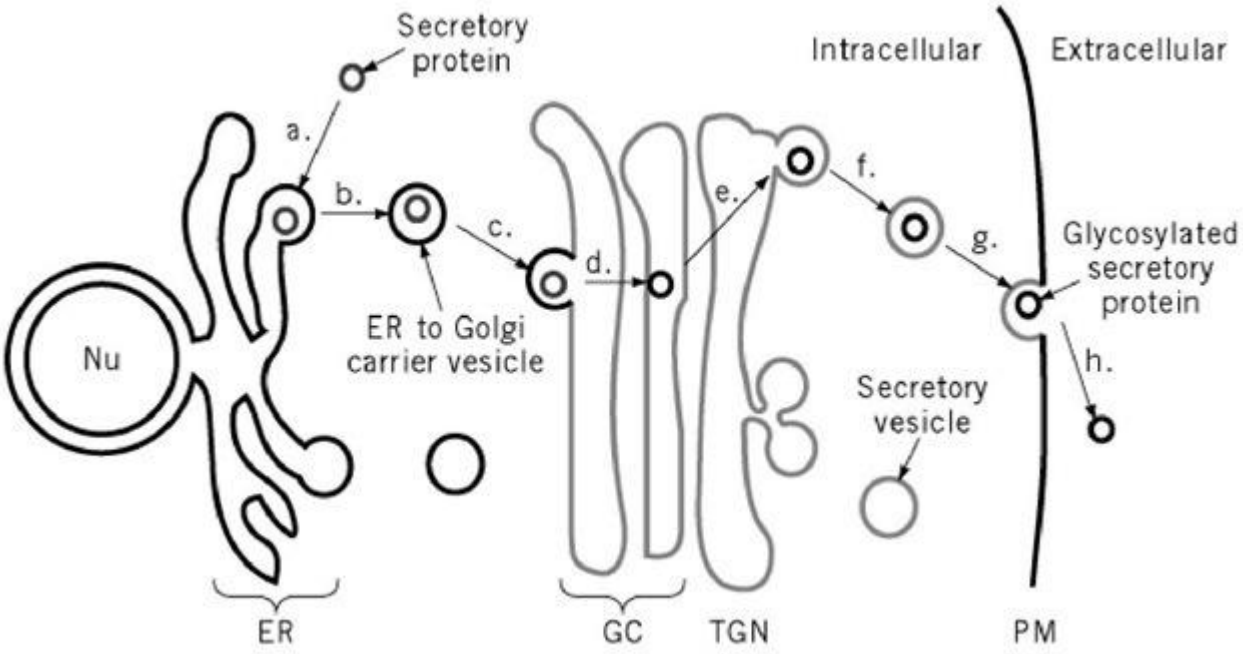

Fig. 2: A simplified overview of some of the most prominent components within the anterograde secretion pathway, including the secretory vesicles, the ER, the Golgi Complex (GC), the trans-Golgi Network (TGN) and the plasma membrane (PM), with the nucleus (Nu) also illustrated. Sequential reactions are indicated alphabetically (a-h). (http://what-when-how.com/molecular-biology/protein-secretion-molecular-biology/).

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Genes within the secretory pathway can be classified into functional groups based on the segment of the pathway within which their protein performs their function (Schekman, 1982). These steps include: 1) ER entry; 2) budding from the ER; 3) targeting to the Golgi complex; 4) budding from the Golgi; 5) targeting to the plasma membrane; as well as 6) exocytosis from the cell. A previous model of the secretory pathway in yeast postulated that only budding cells are actively secreting extracellular proteins (Schekman, 1982), whilst approximately 80% of the proteome is expressed during basal growth conditions (Ghaemmaghami *et al.,* 2003). This translates to the minority of cells, approximately only 30% of an exponential yeast culture, that are actively secreting a protein of interest (Vośišek, 2000). The secretory pathway in yeast is one of a number of intricately regulated systems that are in place to ensure the correct localization and compartmentalization of intracellular lipids and proteins (Franzusoff *et al.,* 1991). In *S. cerevisiae,* the protein secretory pathway includes several membrane-enclosed organelles and facilitates the passage of organelle-derived protein secretory vesicles through the ER, Golgi, post-Golgi network and endosome, followed by delivery to the extracellular space, cell membrane or vacuole (Hou *et al.,* 2012). A large number of proteins therefore make their way, via successive vesicular transport steps, to the cell surface of *S. cerevisiae* and are either integrated into the cell-wall structure, or secreted to the extracellular environment (Nombela *et al.,* 2006).

2.4 Improving heterologous protein secretion in *S. cerevisiae*

Several strategies have been employed to increase the production capacity for heterologous proteins in *S. cerevisiae* including: 1) the engineering of molecular chaperones and foldases (Hackel *et al.,* 2006; Carla Fama *et al.,* 2007; Hou *et al.,* 2012); 2) engineering of the peptide leader sequence (Kjaerulff & Jensen, 2005); 3) optimization of the gene copy number (Ilmén *et al.,* 2011); 4) manipulation of promoter strength (Alper *et al.,* 2005); 5) engineering of the heterologous protein of interest (Huang & Shusta, 2005; Kim *et al.,* 2006; Den Haan *et al.,* 2013a); and 6) optimization of the expression conditions (Wedekind *et al.,* 2006). Although each of these approaches can lead to improvements in heterologous protein secretion capacity, the majority of improvements tended to be protein-specific (Huang *et al.,* 2008; Den Haan *et al.,* 2013a). Reviews of the literature relating to engineering strategies employed

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specifically to enhance secretory levels in *S. cerevisiae* have been compiled and the reader is directed there for further reading (Idiris *et al.,* 2010; Hou *et al.,* 2012).

In the majority of "super-secretory" yeast strains obtained through random mutagenesis, processes other than secretion have been altered, making the majority of these phenotypes pleiotropic. This makes it difficult to apply them to industrial strains, which are mostly polyploid or aneuploid (Smith *et al.,* 1985; Shuster *et al.,* 1989; Wingfield & Dickinson, 1993). The difficulties relating to secretory improvements can be partially attributed to the fact that the secretion process for each protein needs to be tuned to the specific physical properties and requirements of the heterologous polypeptide being produced, e.g. the number of disulphide bonds, the size of the protein, the hydrophobicity of the peptide and the extent of glycosylation (Hou *et al.,* 2012). Processes such as folding, disulphide bond formation, glycosylation and vesicle trafficking all need to be accomplished without interrupting quality control feedback loops, as cellular homeostasis must be maintained.

Using our current understanding of these processes, an increasingly attractive approach to improve heterologous protein secretion in yeast is to utilize genetic engineering, combined with a systems biology approach (Graf *et al.,* 2009). The in-depth characterization of a variety of gene products required for endogenous protein folding, translational events and cell growth in *S. cerevisiae* have effectively assisted in identifying candidate target genes that may improve the secretion capacity of this specific yeast (Brauer *et al.,* 2008; Jonikas *et al.,* 2009).

Mature proteins, heterologous or native, often require the action of specialized signal peptidases and co-factors for the removal of their respective signal sequences (Strausberg & Strausberg, 2001). Therefore, when secreted proteins are overexpressed, these co-factors and proteases may become limiting, occasionally resulting in the accumulation of the precursor protein, which ultimately limits secretion titers (Haguenauer-Tsapis & Hinnen, 1984). An important bottleneck that has been suggested to significantly limit heterologous protein secretion in yeast expression systems is the exit of the nascent polypeptide from the ER and its subsequent transport to the *cis*-Golgi (Lodish *et al.,* 1983; Shuster, 1991; Robinson & Wittrup, 1995). Overexpression of co- and post-translational translocation components as well as ER-chaperones at this particular membrane interface has led to notable, protein-specific increases in the *S. cerevisiae* secretion phenotype, illustrating the potential location of a contributory secretory bottleneck (Tang *et al.,* 2015).

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The overexpression of many heterologous proteins in *S. cerevisiae* often leads to the intracellular accumulation of folded and misfolded proteins as various components of the secretory pathway become saturated (Shusta *et al.,* 1998; Kauffman *et al.,* 2002; Huang & Shusta, 2006). Quality control machinery and chaperones are in place to ensure that misfolded or immature proteins do not escape the ER and enter the rest of the secretory pathway (Hurtley & Helenius, 1989). Once there is an accumulation of folded and misfolded proteins in the ER, as is the case when heterologous proteins are being produced at high enough titers, the yeast activates the Unfolded Protein Response (UPR) or ER-associated Degradation (ERAD), two quality control mechanisms specifically induced in relation to ER-stress (Kimata *et al.,* 2003). These mechanisms have differential outcomes, occurring within various time frames, that depend on subtle variations in the spacial organization of cellular compartments (organelles) and ultimately alter the intracellular localization and concentration of selected proteins (McCracken & Brodsky, 2003; Midelfort & Wittrup, 2006; Brodsky, 2007).

The removal of misfolded proteins through ERAD occurs via ubiquitin-conjugating enzymes associated with the ER, followed by translocation and subsequent degradation by the proteasome in the cytoplasm (Nakatsukasa & Brodsky, 2008). Subtle perturbations in the local concentration of unfolded proteins within the ER activates the UPR, which is broadly defined as the global cytoprotective signaling cascade that transcriptionally upregulates transcription of genes encoding ERAD components (Travers *et al.,* 2000; Otte & Barlowe, 2004). Several chaperone genes within the UPR, such as *PPI,* are responsible for the transcriptional attenuation of genes encoding secretory proteins (Schröder & Kaufman, 2005), which partially shuts down the secretion pathway as part of the UPR regulation process (Chang *et al.,* 2004). Induction of the UPR is detrimental to the protein secretion process (Kauffman *et al.,* 2002), however, constitutive overexpression of specific components of the UPR (*HAC1*) have also yielded increased secretion titers of up to 70% for several heterologous proteins in yeast and filamentous fungi (Valkonen *et al.,* 2003). The aforementioned illustrates the often protein-specific nature of secretion enhancement interventions.

Glycosylation is one of the major covalent modifications that modulate the structure and function of secretory and membrane glycoproteins in eukaryotic cells, while the biosynthetic steps mediating glycosylation are intricately coupled to the secretory pathway (Herscovics & Orlean, 1993). Both N- and O-linked glycosylation of proteins are initiated in the ER and processing is further continued in the Golgi. N-glycosylation entails the addition of N-linked

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core oligosaccharides to the amide group of the asparagine residues that are part of "asn-x-ser/thr" tripeptides, with "x" representing any amino acid other than proline (Orlean, 1997). O-linked glycosylation entails the addition of O-linked glycans, which consist of mannose residues, to serine and threonine residues and it is thought that the collective effect of these types of glycosylation significantly affects secretion in *S. cerevisiae* as many candidate proteins are hyperglycosylated (Herscovics & Orlean, 1993; Ilmén *et al.,* 2011).

Engineering strategies focusing on the post-translational folding of nascent polypeptides, have also yielded positive heterologous protein secretion improvements (Zhang *et al.,* 2006; Gasser *et al.,* 2007; Tang *et al.,* 2015). The overexpression of the protein disulfide isomerase (PDI), involved with protein folding and isomerization reactions in the ER and other chaperones (Kar2p, Sec63p) led to measurable heterologous protein secretion improvements, adding credence to the suggestion that folding becomes a limiting factor when proteins are being heterologously expressed (Tang *et al.,* 2015). Heterologous proteins are often successfully folded in *S. cerevisiae*, only to accumulate intracellularly and result in relatively low secretion titers (Hou *et al.,* 2012).

Many non-ER checkpoints in the protein secretion pathway play a significant role in determining the rate of secretion for a specific heterologous protein and it has been strongly suggested that various stages in the secretory pathway, such as quality control sorting or the fusion of secretory vesicles at the Golgi and the plasma membrane, could limit the rate of secretion for properly folded heterologous proteins (Huang *et al.,* 2008). Specific stages in the secretory pathway could therefore additively limit secretion titers and it is thought that a diverse array of bottlenecks within the secretion pathway become limiting for different heterologously produced proteins. Consequently, the overexpression of genes associated with components within the secretory pathway, promoting anterograde reactions such as vesicle fusion and targeting, have been successfully applied to increase heterologous protein secretion, with varying degrees of success (Ruohonen *et al.,* 1997; Idiris *et al.,* 2010; Hou *et al.,* 2012; Van Zyl *et al.,* 2014; Tang *et al.,* 2015; Van Zyl *et al.,* 2015). An element of the secretory pathway that is notably intertwined with the rate and efficiency of a protein's journey to the extracellular environment, is the vesicular fusion reactions of the transport vesicle within which it is contained. These vesicles, which are initially enveloped by the COPII vesicle coat proteins as they emerge from the ER-membrane, face a complex and intricately regulated

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journey to the plasma membrane, having to undergo targeted, directional membrane fusion with the various membrane-enclosed organelles in the secretory pathway.

2.5 SNAREs and membrane fusion

Membrane fusion is an essential and fundamental process required for protein/lipid trafficking, hormone secretion and organelle morphogenesis in all eukaryotic cells (Furukawa & Mima, 2014). Soluble NSF (N-ethylmaleimide-sensitive factor) attachment receptor proteins (SNAREs) are required at the majority of membrane fusion events during intracellular transport, and play crucial roles in facilitating protein trafficking between the variety of membrane-enclosed organelles and the plasma membrane (Weber *et al.,* 1998; Grote *et al.,* 2000; Hou *et al.,* 2012). Though SNAREs do not solely facilitate membrane fusion, they play an essential role in catalyzing vesicular and membrane fusion events, in addition to the recruitment of other components that modulate said membrane fusion processes (Ungermann *et al.,* 1998b; Weber *et al.,* 1998).

SNAREs are involved in many, if not all fusion events along the endocytic and secretory pathways in eukaryotes, with members of this protein family having been identified on the ER, the Golgi membrane, the vacuole/lysosome, the plasma membrane and the vesicles that are derived from their respective membranes (Burri & Lithgow, 2004; Malsam *et al.,* 2008). In fact, the only intracellular eukaryotic compartments that do not depend on the functioning of SNAREs to drive their fusion reactions are chloroplasts and mitochondria, an observation in keeping with the endosymbiotic theory (Malsam *et al.,* 2008). The importance of SNAREs to the yeast's native physiology is epitomized by the fact that, as the budding daughter cell grows in *S. cerevisiae,* it requires polarized vesicle fusion at the bud tip (Hattendorf *et al.,* 2007). Furthermore, in order for the cell to effectively proliferate, it depends on the functioning of its polarized actin cytoskeleton and its array of lipid bilayer vesicles for transfer of essential macromolecules to sites of cellular expansion (Heider & Munson, 2012).

SNAREs, which constitute a compartment-specific protein family, were originally identified in yeast during an extensive search for specific receptors that are able to bind SNAPs (soluble NSF attachment proteins) (Söllner *et al.,* 1993). Genome-wide analysis has subsequently identified at least 25 SNAREs in yeast, similar to the numbers found in other well studied eukaryotes such

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as fungi and protists (Gupta & Heath, 2002; Dacks & Doolittle, 2002; Malsam *et al.,* 2008). Through extensive genetic screening of *S. cerevisiae* secretory mutants, the majority of yeast SNARE proteins have now been described (Kienle *et al.,* 2009). A graphic representation of the yeast SNARE proteins, including their roles in retrograde and anterograde protein transport is provided in **Fig. 3.**

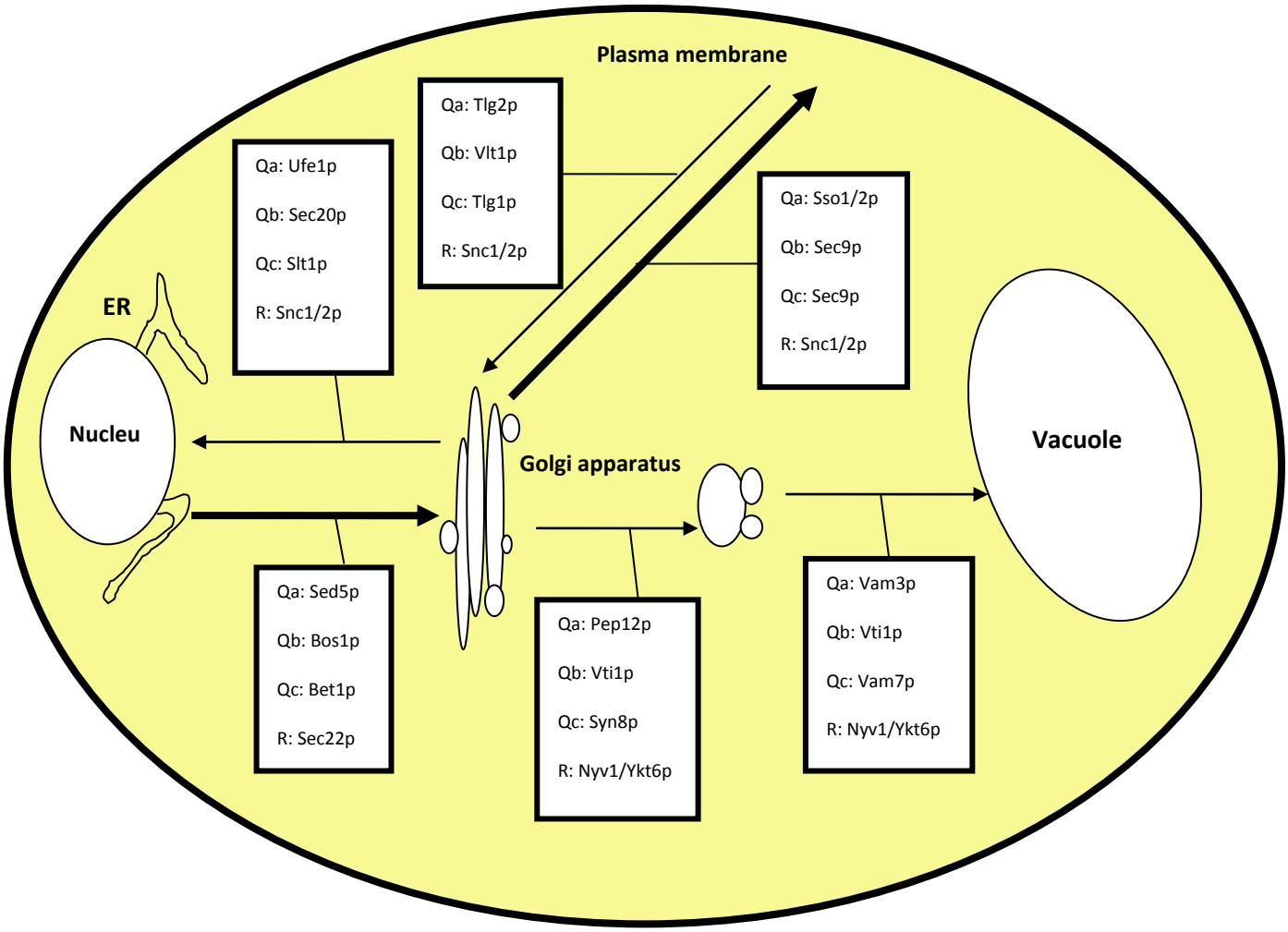

Fig. 3: A graphic illustration of some of the various collections of cognate SNARE proteins facilitating retrograde (reverse) and anterograde (forward) protein traffic in yeast (adapted from Kienle *et al.,* 2009).

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2.5.1 The basic SNARE machinery

SNAREs generally contain three domains, a conserved SNARE motif that helps to mediate self-assembly of the tetrameric SNARE complex, a C-terminal transmembrane domain and a variable N-terminal region (Sutton *et al.,* 1998). The large majority of SNARE proteins are type II membrane proteins with the before-mentioned C-terminal segment of polypeptides that serves as the membrane anchor and a short ~ 70 amino acid alpha-helical SNARE-motif, which distinguishes SNAREs from each other (Paumet *et al.,* 2004). It is worth noting that SNAREs can possess one or two of these SNARE motifs, with the primary sequence of the motifs being characterized by a distinct heptad pattern of hydrophobic amino acids (Munson & Hughson, 2002). The bulk of the protein is situated in the cytoplasm, while a single membrane-spanning region along with a small number of amino acids face either the extracellular side or the lumen of a cellular compartment (Söllner *et al.,* 1993; Jahn & Scheller, 2006; Malsam *et al.,* 2008). Some SNAREs do not possess a membrane spanning region and are simply attached to their particular membrane via post-translational acyl modifications such as palmitoylation and farnesylation (Malsam *et al.,* 2008). In addition, certain SNAREs such as Vam7p have been identified that are able to associate with organelle membranes via a lipid binding domain (Cheever *et al.,* 2001). The cytoplasmic portion of a SNARE consists of two domains: an N-terminal domain and a SNARE motif, with the latter representing the before-mentioned coiled-coil domain of ~70 amino acids which is essential to establishing SNARE specificity (Paumet *et al.,* 2004).

SNAREs are divided into two classes depending on the localization of their activity, with different vesicle trafficking steps deploying different v-/t-SNARE complexes (Malsam *et al.,* 2008). Those present on target membranes are referred to as t-SNAREs while those present on the transport vesicles themselves are known as v-SNARES. Cognate v- and t-SNAREs have the fundamental characteristic of being able to assemble at distinct trafficking steps and can subsequently lead to the formation of membrane-specific SNARE complexes, which facilitate SNARE-mediated membrane fusion (Pelham *et al*., 1995). Thus, collectively, the intracellular distribution of SNARE proteins is able to provide a tentative roadmap of yeast membrane traffic (**Fig. 3**) (Malsam *et al.,* 2008).

V-SNARES interact in *trans* with t-SNARES on specific target membranes, resulting in the formation of a complex that bridges the two membranes. This SNAREpin, or *trans*-SNARE

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complex, consists of an α-helical coil, with each of the SNARE components contributing a single SNARE motif (Malsam *et al.,* 2008). This in turn facilitates the movement of the respective bilayers into close proximity, which is an energetically favorable state for fusion (Rothman & Warren, 1994; Malsam *et al.,* 2008). Bilayer convergence is facilitated by N- to C-terminal zippering of the coiled-coil SNARE bundle, which in turn brings the membranes into close apposition, which deforms and dehydrates the respective bilayers, leading to compartmental mixing and the emergence of the fusion pore (**Fig. 4**) (Hanson *et al.,* 1997; Nichols *et al.,* 1997; Sutton *et al.,* 1998; Li *et al.,* 2007; Domanska *et al.,* 2009; Schwartz & Merz, 2009; Walter *et al.,* 2010). The SNARE ratio between the donor and acceptor membranes is usually 1:3, although it can also be 2:2 (Cao & Barlowe, 2000).

At the core of the SNARE bundle, located in the central '0' layer, each SNARE-related α-helix typically displays either an arginine (R) or glutamine (Q) residue and a distinct, structure-based classification of SNAREs has subsequently been devised accordingly (Fasshauer *et al.,* 1998). SNAREs are therefore further sub-classified based on their interacting amino acid residue (Glutamine (Q) or Arginine (R)) at the core of the bundle, i.e. Qa-, Qb-, Qc- and R-SNAREs (Fasshauer *et al.,* 1998; Bock *et al.,* 2001). The Qa-SNARE is often referred to as the t-SNARE heavy chain while the Qb- and Qc-SNAREs are usually regarded as the t-SNARE light chains. Q-SNAREs generally function as t-SNAREs whilst R-SNAREs often serve as v-SNAREs (Malsam *et al.,* 2008).

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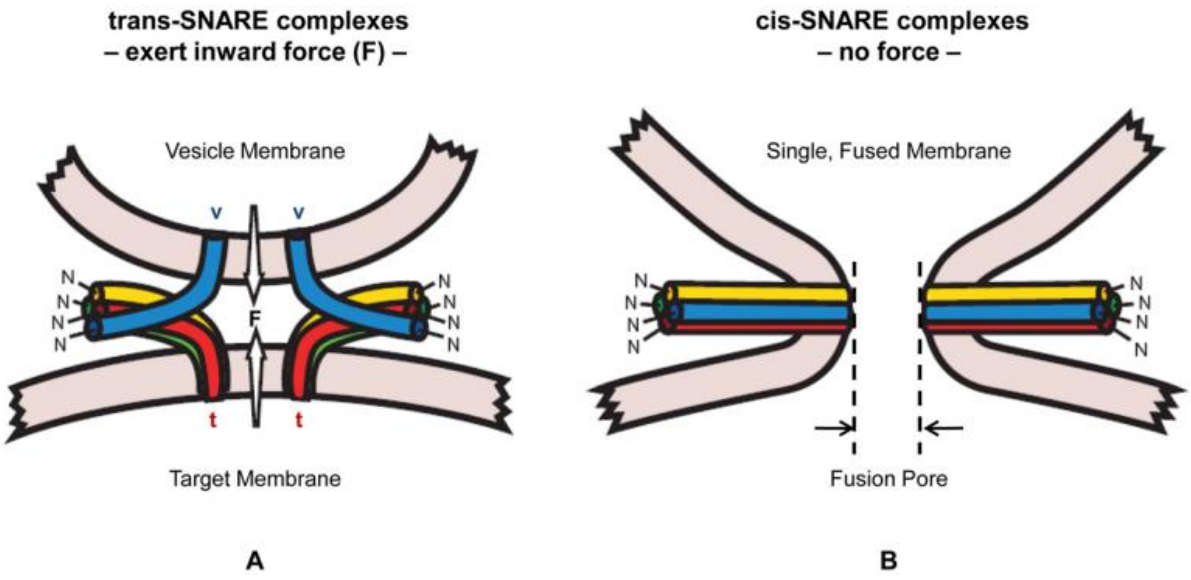

Fig. 4: (A) An illustration of the four-helical SNARE bundle, consisting of a v-SNARE and, in this example, three t-SNAREs within a force-generating *trans*-SNARE complex. Membrane-anchored C-terminals and N-terminal transmembrane domains are indicated, along with **(B)** the structural progression during N- to C-terminal zippering during formation of the *cis*-SNARE complex, which facilitates the creation of the fusion pore (Südhof & Rothman, 2009).

An essential prerequisite for the eventual formation of the *trans*-SNARE complex (SNAREpin) is the formation of a functional t-SNARE ternary complex, consisting of three t-SNAREs assembled on the target membrane, which is in turn able to act as template for cognate v-SNARE binding (Malsam *et al.,* 2008; Laufman *et al.,* 2013). The formation of the functional t-SNARE complex is tightly regulated, with regulatory components steering assembly towards functional complexes that can serve as the basis for the formation of fusogenic SNAREpins. The assembly of the *trans*-SNARE complex (SNAREpin), consisting of three t-SNAREs and a single v-SNARE motif assembled into a twisted parallel four-helix bundle, is the driving force behind membrane fusion and these structures are highly conserved among eukaryotes (Weimbs *et al.,* 1997; Bock *et al.,* 2001; Laufman *et al.,* 2013). This assembly succeeds in bringing the target membranes and vesicles into close proximity, which in turn acts as a catalyst for their subsequent fusion (Jahn *et al.,* 2003; Hong, 2005; Malsam *et al.,* 2008).

SNAREpin assembly takes place in a stepwise manner, with the reaction initiating at the amino-terminal (membrane distal) end of the SNARE motif and proceeding towards the membrane proximal end (Hanson *et al.,* 1997; Pobbati *et al.,* 2006). It is these membrane proximal regions,

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which are initially largely unstructured, that serve as targets for specific regulatory components that delay or accelerate SNAREpin formation (Xu *et al.,* 1999; Margittai *et al.,* 2001). During and after membrane fusion, the membrane-spanning *trans*-SNARES (constituting SNAREpins) are converted to stable, fusion inactive *cis*-SNARE complexes, within which all the SNARE proteins are entrapped with a single membrane (**Fig. 5**) (Ungar & Hughson, 2003). The number of SNAREpins required to successfully facilitate membrane fusion is unknown, though the lipid/protein composition of the respective compartments and the presence of lipid-bilayer perturbing factors could have a significant effect (Malsam *et al.,* 2008).

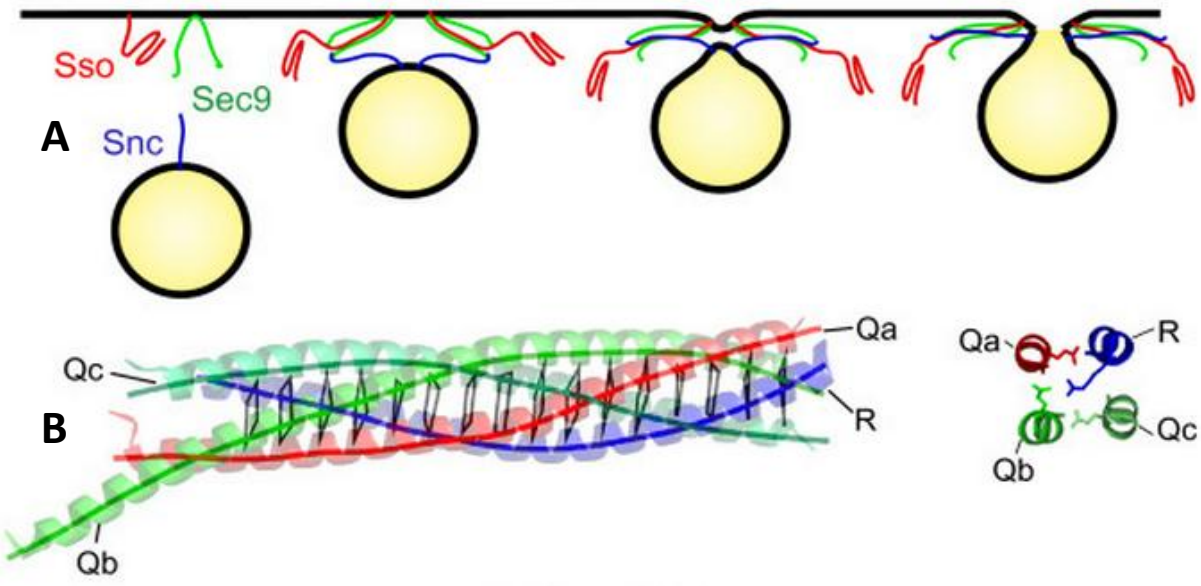

Fig. 5: (A) The SNARE mediated membrane fusion reaction at the plasma membrane, illustrating the structural relationships between the various exocytic SNARE components as they progress from a dissociated state into a *trans*-SNARE complex, with its characteristic four-helical bundle **(B)**, and finally into a *cis*-SNARE complex within which all components are associated with a single lipid bilayer (Kienle *et al.,* 2009).

As opposed to the conserved SNARE motifs, there exists significant variety exists amongst the t-SNARE N-terminal domains, many of which are able to form a three-helix bundle, or β-strand/α-helix structure, which can regulate SNARE assembly when folded back onto the SNARE motif (Munson *et al.,* 2000; Tochio *et al.,* 2001; Dulubova *et al.,* 2001; Antonin *et al.,* 2002). Whilst the SNARE motifs are therefore largely responsible for establishing SNARE

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specificity, these N-terminal domains are likely involved in controlling the rate of complex formation and fusion and it is thought that these domains specifically interact with additional regulatory proteins (Parlati *et al.,* 1999; Paumet *et al.,* 2004). These N-terminal regulatory domains have also been shown to be able to impose a kinetic block on SNARE assembly *in vivo.* For example, the N-terminal regulatory domain of Sso1p (t-SNARE) is able to slow it's assembly into its relevant SNARE complex by up to three orders of magnitude and it is thought that regulatory domains such as these are in place to prevent promiscuous trans-SNARE complex assembly (Nicholson *et al.,* 1998; Munson *et al.,* 2000; Tochio *et al.,* 2001).

The latter auto-inhibitory, amino-terminal domain of the Sso1/2p t-SNAREs binds to Sec1p, an SM family protein required for the regulation of SNARE complex assembly and function at the plasma membrane (Jahn *et al.,* 2003; Toonen & Verhage, 2003). Similarly, the N-terminal domains of the Ufe1p and Sed5p SNAREs are able to recruit the SM (Sec1/Munc18-like) protein Sly1p to the ER and Golgi, respectively (Burri & Lithgow, 2004). Another example of the N-terminal domain of SNAREs recruiting SM proteins is Tlg1p, which is able to recruit Vps45p to the SNARE complexes facilitating membrane fusion between the vacuolar compartments. Not all SNAREs actively recruit SM proteins and their specific N-terminal domains likely serve other, distinctive functions (Teng *et al.,* 2001; Gonzalez *et al.,* 2001).

Whilst the majority of yeast SNAREs contain a large N-terminal domain proximal to the SNARE motif, the PHOX domain of Vam7p, a Q-SNARE mediating vesicle transport to the vacuole, is the only clear example where this domain is used to localize the specific SNARE to its site of action (Cheever *et al.,* 2001). It is also possible that the SNARE motifs, being domains based on protein-protein interaction, are able to assist in containing proteins in their appropriate compartments within the endomembrane system, effectively docking them to landmarks within that membrane (Burri & Lithgow, 2004). Each of these may serve as a landmark for another, collectively diminishing their flux from their site of action. General trafficking of SNARE proteins appears to occur according to the same scheme applicable to the majority of tail-anchored proteins (Burri & Lithgow, 2004).

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2.6 SNARE regulation and specificity

SNAREs have long been established as being able to solely drive membrane fusion *in vitro* (Weber *et al.,* 1998), but *in vivo* membrane fusion requires a selection of cofactors and regulatory components, including tethering factors, Rab proteins and SM proteins (Jackson & Chapman, 2006; Wickner & Schekman, 2008; Yu & Hughson, 2010; Jahn & Fasshauer, 2012). It is therefore not surprising to find that many SNARE proteins are able to self-assemble into a host of different complexes in solution, though only a small fraction of these are fusogenic (McNew *et al.,* 2000). The stringency of SNARE specificity seems to be enhanced during the early secretory pathway (ER-to-Golgi, intra-Golgi and endosomal complexes), with late secretory complexes, particularly the vacuolar SNAREs, illustrating significantly higher levels of SNARE binding promiscuity (Furukawa & Mima, 2014). The *in vitro* self-assembly of the majority of physiologically cognate SNARE complexes appears to be remarkably inefficient (Ohya *et al.,* 2009; Stroupe *et al.,* 2009), which raises two essential questions: 1) how are SNAREs efficiently assembled into fusogenic complexes *in vivo*; and 2) how is the production of non-fusogenic SNARE complexes on target membranes prevented?

2.6.1 Tethering factors

Tethering factors are a group of proteins and protein complexes that are required to link transport vesicles to their appropriate, cognate target membranes, providing a kinetic advantage for subsequent SNAREpin assembly (Malsam *et al.,* 2008; Laufman *et al.,* 2013). To ensure that vesicle fusion is specifically targeted to distinct membrane sub-compartments, vesicle tethering is locally restricted (Malsam *et al.,* 2008). Tethers are therefore able to directly interact with SNARE proteins and, in doing so, add another layer of specificity whilst contributing to high-fidelity fusion (Cai *et al.,* 2007). Tethering proteins are relatively heterogeneous and are classified into two broad categories: long coiled-coil proteins and multisubunit complexes (MTCs) (Whyte & Munroe, 2002; Bröcker *et al.,* 2012). Both classes seem to interact with t-SNARE components, with a single representative identified that wholly stimulates SNARE complex assembly (Shorter *et al.,* 2002). Vesicle tethering precedes SNARE complex formation, whilst literature strongly suggest that tethering proteins, in particular MTCs, play an active role in mediating SNARE complex assembly (Sapperstein *et al.,* 1996; Shorter *et al.,* 2002; Pérez-Victoria & Bonifacino, 2009). Tethering factors have been shown to

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interact with Rab GTPases, SNAREs and vesicle coats, and thus fulfill a critical role in vesicular capturing and docking to specific target membranes (Cai *et al.,* 2007; Angers & Merz, 2010). In fact, most tethering factors that have been identified at the majority intracellular membrane transport steps employ Rab proteins and some of their effectors (Grosshans *et al.,* 2006).

2.6.2 Rab GTPases and effector proteins

Rab proteins have been assigned to various cellular processes, including vesicle formation, motility, budding and uncoating, organelle motility and identity, and the tethering of vesicles to their target membranes (Zerial and McBride, 2001; Stenmark, 2009). These molecules are highly conserved with regards to their organelle compartmentalization, whilst they've been especially helpful in determining transport specificity. Rabs constitute the largest branch of the Ras GTPase superfamily, which employs the guanine nucleotide-dependent switch mechanism to effectively regulate the four major membrane trafficking steps: vesicle budding, delivery, tethering and fusion with the target membrane (Grosshands *et al.,* 2006). Rab proteins are small, compartment-specific GTPases that actively cycle between the cytosol, where they remain in an inactive GDP-bound state, and the membranes, where they function in an active GTP-bound state (Malsam *et al.,* 2008). The binding of membranes by activated Rab proteins at distinct intracellular membrane interfaces is followed by the recruitment of effectors, which is temporally restricted by GTP hydrolysis. Rab proteins are able to recruit a number of functionally diverse effectors that assist in cargo sorting, vesicle motility, tethering, regulation of SNARE activity and modeling of the membrane subdomains. These effector proteins include sorting adaptors, tethering factors, kinases, phosphatases and motor proteins, whilst cross-talk between a range of Rab GTPases (through shared effectors) recruits selective Rab-activators that ensure the spatiotemporal regulation of vesicle traffic (Stenmark, 2009). Thus, through the orchestrated actions of Rabs and their effectors, Rabs appear to coordinate the sequential steps involved in intracellular membrane trafficking.

2.6.3 SM (Sec1/Mun18) proteins

SM (Sec1/Mun18) proteins interact with SNAREs at every vesicle fusion event in the cell, playing not only a mediating role in vesicle tethering and fusion, but contributing greatly to the specificity of vesicle fusion (Südhof & Rothman, 2009). SM proteins, which are clasp-shaped

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(arch-shaped) and characterized by three domains, are soluble 60-70 kDa cytosolic SNARE-interacting proteins that bind to their cognate *trans*-SNARE complexes (SNAREpins) to direct and accelerate the basal fusogenic reaction (**Fig. 6**) (Hata *et al.,* 1993; Bracher & Weissenhorn, 2002; Shen *et al.,* 2007 Südhof & Rothman, 2009). Within the cell, several isoforms are expressed, with each individual SM protein functioning at distinct intracellular transport steps (Malsam *et al.,* 2008). SM proteins are exclusively located at fusion sites, as opposed to having a broad membrane distribution (Carr *et al.,* 1999).

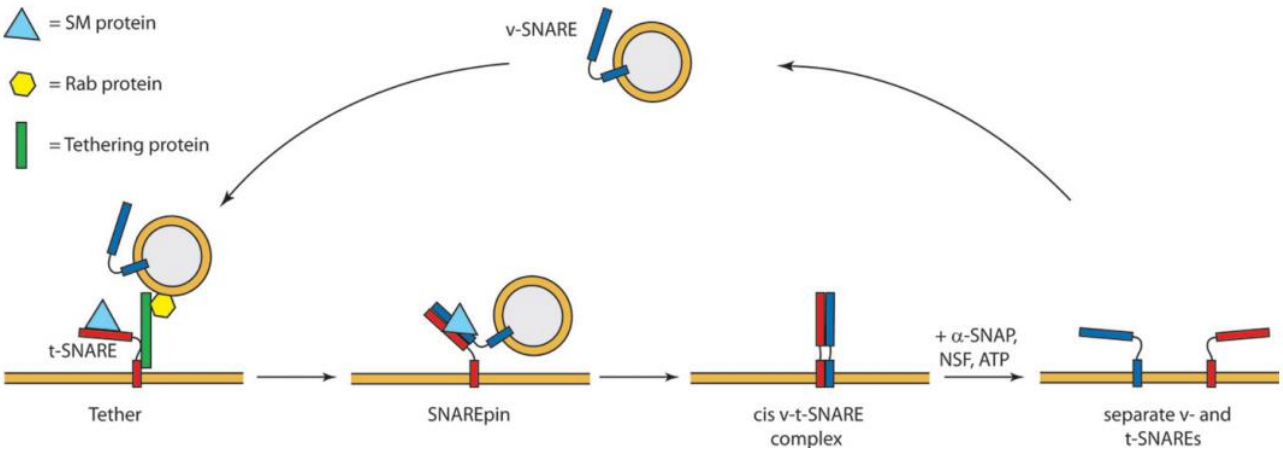

Fig. 6: The basic components facilitating SNARE regulation, assembly and disassembly. The initial interactions between the individual vesicle and its target membrane is facilitated by tethering proteins and a small Rab GTPase, after which v- and t-SNARE proteins form a cognate *trans*-SNARE complex with SM proteins fulfilling a mediating role. These successive events culminate in the formation of a *cis*-SNARE complex, which can then be dissociated and recycled through the action of NSF (Sec18p) and α-SNAP (Sec17p) in the presence of ATP (Malsam *et al.,* 2008).

Several SM proteins have been identified in the secretion pathway of *S. cerevisiae* and their importance with regard to heterologous protein secretion was underlined by Hou *et al.* (2012), who established that overexpression of certain SM proteins led to secretory improvements. The importance of SM proteins is further accentuated by the fact that wherever a specific transport step is essential for cell survival and the associated SM protein is deleted, cell death invariably ensues (Verhage *et al.,* 2000). *S. cerevisiae* possesses four differential SM proteins: Sec1p, Sly1p, Vps45p and Vps33p (Aalto *et al.,* 1993). While Sec1p mediates vesicle traffic between the Golgi and the plasma membrane, Sly1p plays a role in modulating protein traffic

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between the ER and the Golgi. Conversely, Vps45p fulfills a function in Golgi-late endosomal trafficking whilst Vps33p is important for endosome and vacuolar traffic. The conserved binding target of SM proteins is the core four-helix SNARE bundle, whilst this SNARE bundle is thought to be accommodated within the central cavity regions of SM proteins, which are fundamentally adapted to bind four-helical bundles (Bacaj *et al.,* 2010).

Although interactions between SM and SNARE proteins have been established, the intricate roles of SM proteins in the wider protein secretory pathway are yet to be elucidated as activatory and inhibitory roles have been reported (Yang *et al.,* 2000; Shen *et al.,* 2007). Once the v- and t-SNAREs have zippered up into a particular four-helical bundle that pulls the respective membranes into close proximity, the SM proteins bind to the *trans*-SNARE complex to direct its fusogenic reaction (Südhof & Rothman, 2009). Distinct combinations of SNARE and SM proteins establish vesicle fusion specificity between particular membrane compartments and, in a broader sense, these proteins are controlled by specific regulators that embed the SM-SNARE fusion machinery into its correct physiological context.

A variety of different protein classes, including SM proteins, Rab proteins, tethering complexes and additional SNARE-specific interactions all therefore contribute to increasing the specificity of SNARE-mediated vesicle fusion (Söllner *et al.,* 1993; McNew *et al.,* 2000; McNew, 2008). In fact, *in vitro* liposome fusion assays illustrated that out of 300 different combinations of yeast SNARE proteins, only 9 were fusogenic, illustrating the stringency with which membrane fusion is modulated (Malsam *et al.,* 2008). Lobingier *at al.* (2014) recently established that whilst SM proteins have long been assigned accelerative functions within the SNARE assembly repertoire, recent results indicated that some of them (Sly1p and Vps33) were able to directly shield their cognate SNAREs from Sec17p (α-SNAP) and Sec18p (NSF) disassembly, a process which will be covered in a later section of this dissertation.

2.6.4 Phosphorylation

Another group of proteins that actively interact with SNARE proteins and play a significant role in the regulation of their activity, are the kinases (Snyder *et al.,* 2006). These components are able to phosphorylate sites on both SNARE proteins and their interacting proteins, with a great deal of research having focused on these kinases in higher eukaryotes. Nevertheless, it has been illustrated that Sso1p and Sso2p, the exocytic t-SNARE orthologs in yeast, are

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phosphorylated both *in vivo* and *in vitro* by protein kinase A (PKA) (Marash & Gerst, 2001) – a protein kinase that is able to sense the external nutrient availability through signal transduction pathways modulating the intracellular levels of cyclic AMP (Adenosine Monophosphate) (Griffioen & Thevelein, 2002). Protein phosphorylation plays an essential role in signal transduction, leading to control over the membrane fusion machinery and exocytosis (Weinberger & Gerst, 2004). It appears that most examples of SNARE or SNARE-regulator (e.g. SM proteins) phosphorylation results in a decrease in SNARE-SNARE or SNARE-SNARE-regulator interaction and this phosphorylative effect is well conserved, having been described in yeast and mammals. Conversely, dephosphorylation generally enhances SNARE interactions and leads to enhanced membrane fusion in yeast (Marash & Gerst, 2001; Bryant & James, 2003). It is likely that temporal and spacial constraints help to allow for phosphorylative control of SNARE (and SNARE regulators) localization, recycling and assembly and the combinatorial effect is likely to promote, if not fine-tune, the overall secretory process (Weinberger & Gerst, 2004).

2.6.5 SNARE activation

Since SNARE proteins play such an essential role in directing vesicle traffic amongst the majority of cellular compartments, much focus has been placed on investigating the regulation of SNARE complex assembly (Munson & Hughson, 2002). Regulation of SNARE assembly is an essential prerequisite to prevent promiscuous pairing of non-cognate SNARE components and to prohibit cognate SNAREs from binding to one another before they have reached the correct target membranes (Gerst, 2003). The before-mentioned would assist in limiting the activity of pre- or post-Golgi SNAREs to within their areas of requirement. It also becomes important when keeping in mind that whilst SNARE proteins appear to be widely dispersed across resident compartments, only SNARE proteins that are adjacent or recruited to the membranes of interest need to be activated for imminent fusion. Certain components (e.g. SM proteins) assist in maintaining fusion specificity whilst enhancing the fusion process, whilst other factors (e.g. Vsm1p and phosphorylation) may limit SNARE activity distal to the fusion site. Investigation of the literature has revealed that the functional activity of SNAREs is greatly affected by their interactions with other proteins (Gerst, 2003).

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Both the yeast and mammalian SNAREs facilitating exocytosis are able to adopt "closed" or inactive conformations, where the α-helical NH_2-terminal region folds over onto the COOH-terminal SNARE binding domain. This forms an intramolecular four-helix bundle that prevents association with cognate partners, until an alteration of the intramolecular four-helix bundle allows for these associations to occur (Fernandez *et al.,* 1998; Nicholson *et al.,* 1998; Fiebig *et al.,* 1999). Many SNAREs are able to alternate between this "closed" conformation, which is favorable to SM-binding, and the "open" conformation, which is able to form the core complex (Nicholson *et al.,* 1998; Fiebig *et al.,* 1999). Additionally, SNARE proteins are able to adopt specific conformations that in turn control their entry into intermolecular complexes (Calakos *et al.,* 1994; Lerman *et al.,* 2000; Tochio *et al.,* 2001). Many studies have since confirmed the existence of these "closed" conformations within which SNARE motifs, through intramolecular interactions with a regulatory domain, are subsequently rendered inaccessible (Munson *et al.,* 2000; Tochio *et al.,* 2001).

2.6.6 Sec17p (α-SNAP), Sec18p (NSF) and SNARE disassembly

Another crucial aspect of SNARE proteins and their efficient mediation of vesicle fusion is their disassembly for recurring cycles of fusion. *Cis*-SNARE complexes are able to accumulate as products of preceding fusion reactions or, alternatively, through limited spontaneous re-association of separated SNAREs in *cis* (Söllner *et al.,* 1993; Mayer *et al.,* 1996). These complexes are subsequently reactivated, liberating individual SNAREs for subsequent re-assembly into *trans*-SNARE complexes (Ungermann *et al.,* 1998a; 1998b). Disassembly of SNARE complexes needs to be carried out in an active manner as these complexes are extremely stable, with some resisting SDS (Sodium dodecyl sulfate) and thermal denaturation at temperatures of up to 80˚C (Hayashi *et al.,* 1994). Despite their stability and extensive heterogeneity amongst different SNARE isoforms, there is one molecular component that is able to facilitate their disassembly: the AAA+ ATPase NSF (*N*-ethylmaleimide-sentitive factor)/(Sec18p) and its adapter protein α-SNAP (Soluble NSF attachment protein)/(Sec17p) (Lenzen *et al.,* 1998; Yu *et al.,* 1998; Fleming *et al.,* 1998; May *et al.,* 1999; Yu *et al.,* 1999). Sec17p and Sec18p have been shown to function downstream of fusion, disassembling *cis*-SNARE complexes that in turn allows v-SNAREs to be efficiently recycled (Grote *et al.,* 2000).

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2.7 SNAREs functioning between the ER and the Golgi

2.7.1 The t-SNARE (Qa-SNARE): Sed5p

The Sed5p (Suppressor of Erd2p deletion) t-SNARE (Qa-SNARE) is the heavy chain phosphoprotein involved in the ER- to-Golgi phase of protein transport in yeast, whilst also playing a role in intra-Golgi transport (Hardwick & Pelham, 1992). Sed5p is a homolog of the highly conserved Syntaxin family of neuronal membrane proteins (Banfield *et al.,* 1994). Sed5p combines cooperatively with two t-SNARE light chains, Bos1p (Qb-SNARE) and Sec22p (R-SNARE), to form the specific functional t-SNARE ternary complex that is able to receive vesicles from the ER (Parlati *et al.,* 2002). Interestingly, attempts to determine the number of possible Sed5p-containing SNARE complexes revealed that it is promiscuous, being able to form numerous different complexes *in vitro* (Tsui *et al.,* 2001). This correlates well with the fact that Sed5p plays a major role in several Golgi-related membrane transport events, including anterograde transport to the *cis*-Golgi (utilizing the Bos1p, Sec22p and Bet1p SNAREs), intra-Golgi transport (utilizing the Sft1p, Got1p and Ykt6p SNAREs), as well as endosome-to-Golgi transport (utilizing the Tlg1p, Vti1p and Gos1p SNAREs). Sed5p's ability to assemble into such a multitude of complexes may indicate that this specific protein is highly important in the maintenance of ER and Golgi structure and function as it localizes to the *cis*-Golgi (Hardwick & Pelham, 1992), whilst also cycling through the ER (Wooding & Pelham, 1998; Weinberger *et al.,* 2005). Therefore, the localization of Sed5p must be efficiently controlled to maintain a steady-state ER distribution (Hardwick & Pelham, 1992; Wooding & Pelham, 1998), while still being able to traverse the cisternae of the Golgi to interact with its other SNARE partners or be retrieved from the ER region.

The promiscuity of Sed5p is emphasized by the fact that it is known to bind at least nine different SNARE proteins, although the binding is not entirely selective in the majority of cases (Tsui & Banfield, 2000; Tsui *et al.,* 2001). The t-SNARE Sed5p has been shown to be able to interact with at least 7 v-SNAREs, whilst Gos1p (a yeast v-SNARE) is also thought to interact with other t-SNAREs. Therefore, t- and v-SNAREs that function at multiple transport steps are not uncommon (Lupashin *et al.,* 1997; Fischer von Mollard *et al.,* 1997). Parlati *et al.* (2002) determined that, although a multitude of distinct quaternary complexes can be formed in solution with Sed5p in combination with Bos1p, Sec22p, Bet1p, Gos1p, Ykt6p, Sft1p, Tlg1p and Snc1p, only two of these possible complexes were fusogenic: (1) The Sed5p/Bos1p, Sec22p

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complex with Bet1p containing vesicles; and (2) the Sed5p/Gos1p, Ykt6p complex with Sft1p containing vesicles.

Peng *et al.* (2002) established that when studied *in vivo,* the SM protein Sly1p was able to provide some level of specificity with regards to Sed5p binding partners, preventing non-cognate complex formation of non-physiological SNARE complexes, which were able to assemble to Sly1p-unbound Sed5p. They also established that, unlike the corresponding neuronal ER-to-Golgi SNARE complexes and similar to the yeast exocytic SNARE machinery, the yeast SNARE complexes functioning between the ER and the Golgi were associated with its specific Sec1 family protein, Sly1p. The importance of Sed5p to ER-to-Golgi SNARE assembly is emphasized by the fact that binding of its cognate SM protein, Sly1p, to Sed5p actively enhanced *trans*-SNARE complex assembly at the *cis*-Golgi (Kosodo *et al.,* 2002).

Deletion of Sed5p was characterized by the accumulation of small secretory vesicles, elaboration of ER membranes and a decrease in protein transport and cell viability (Hardwick & Pelham, 1992). The elaboration and expansion of the ER was associated with a block in protein export from the ER, which may have been caused by a block in retrograde transport from the Golgi. Interestingly, overproduction of Sed5p on a multicopy vector had an inhibitory effect on cell growth, and resulted in the accumulation of intracellular membranes and the secretion of ER resident proteins.

As with many SNARE proteins, a cycle of phosphorylation and dephosphorylation is necessary in order to maintain normal t-SNARE functioning for Sed5p (Weinberger *et al.,* 2005). The cycling of Sed5p phosphorylation and dephosphorylation was suggested to additionally choreograph Golgi ordering and dispersal as the phosphorylation state of this specific SNARE influenced the integrity of the Golgi so dramatically that a dynamic cycle of this t-SNARE's phosphorylation and dephosphorylation may be required for the Golgi to retain its structure. It has been suggested that a controlled mechanism for ordering and dispersal exists, with the latter likely being critical for Golgi assembly and disassembly during the mitotic cell cycle in higher eukaryotes, which also involves cycles of dephosphorylation and phosphorylation. The latter was highlighted by the fact that a lack of phosphorylation at specific amino acid positions within the Sed5p polypeptide induced a remarkably more structured Golgi, as opposed to the commonly observed dispersed structure in *S. cerevisiae*.

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2.7.2 The t-SNARE (Qb-SNARE): Bos1p

Bos1p is a t-SNARE (Qb-SNARE) protein that fulfills a role as one of two t-SNARE light chains (in conjunction with Sec22p) that are able to form a functional t-SNARE ternary complex with the t-SNARE heavy chain protein Sed5p at the *cis*-Golgi interface (Parlati *et al.,* 2002). Bos1p is a homolog of the highly conserved Synaptobrevin, or VAMP (Vesicle-Associated Membrane Proteins), family of neuronal proteins (Sacher *et al.,* 1997) and reportedly cycles between the ER and the Golgi in a COPI-dependent manner (Ossipov *et al.,* 1999).

Bet1p and Bos1p, having both been identified on ER-derived vesicles, are able to interact with their cognate Golgi-derived t-SNARE Sed5p, assisting in mediating vesicle fusion between the ER and the Golgi apparatus (Lian & Ferro-Novick, 1993; Tsui & Banfield, 2000). Newman *et al.* (1992) initially established that Bos1p co-purifies with carrier vesicles, Bet1p and the ER membrane and whilst both Bos1p and Sec22p have been detected in the Golgi; they cycle rapidly through the ER and can be considered dynamically localized (Cao & Barlowe, 2000; Parlati *et al.,* 2000). Bos1p and Sec22p only ever assemble into mutual complexes on ER-derived transport vesicles (Lian & Ferro-Novick, 1993; Lian *et al.,* 1994) and it is the pairing of these two SNAREs that directly modulates the function of Bos1p. Deletion analysis revealed that Sed5p was able to bind to both Bos1p and Sec22p (Sacher *et al.,* 1997), which was expected as it is a combination of these three SNAREs that essentially form the functional t-SNARE ternary complex at the *cis*-Golgi.

When Bos1p was present in multiple copies, it was able to suppress the growth and secretion deficiencies brought about by the deletion of Sec22p and Bet1p, mutations that disrupted transport between the ER and Golgi (Shim *et al.,* 1991). In fact, Bos1p was named according to a convention relating to the suppression of a Bet1p growth mutant (Bet one suppressor) (Newman *et al.,* 1990). Bos1p was found to be essential for cellular growth, whilst its deletion compromised the yeast's ability to transport pro-alpha-factor and carboxypeptidase Y (CPY) to the Golgi, leading to the accumulation of the extensive ER-network and transport vesicles (Shim *et al.,* 1991).

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2.7.3 The t-SNARE (R-SNARE): Sec22p

The Sec22p t-SNARE (R-SNARE) protein fulfills a role as one of two t-SNARE light chains, in conjunction with Bos1p (the other t-SNARE light chain) and Sed5p (t-SNARE heavy chain), forming a functional t-SNARE ternary complex that is able to receive its cognate v-SNARE (Bet1p) to form the ER-to-Golgi SNARE complex (Parlati *et al.,* 2002). Sec22p is a homolog of the highly conserved Synaptobrevin, or VAMP (Vesicle-associated Membrane Proteins), family of neuronal proteins (Sacher *et al.,* 1997). Sec22p cycles between the ER and the Golgi, whilst this process is thought to involve the cycling of retrograde COPI vesicles (Ballensiefen *et al.,* 1998). The localization of Sec22p means that it is required for both retrograde and anterograde fusion events between the ER and the Golgi (Spang & Schekman, 1998; Cao & Barlowe, 2000; Liu & Barlowe, 2002). *In vitro* data suggests that whilst Bos1p and Bet1p are mainly functional on ER-derived vesicles, with Sed5p usually acting on acceptor membranes (Cao & Barlowe, 2000), Sec22p seems to be able to act on both (Liu & Barlowe, 2002).

Disruption or mutation of *SEC22* diminished protein transport between the ER and the Golgi, though it was non-essential for growth (Dascher *et al.,* 1991). The viable phenotype following deletion of Sec22p resemble results relating to the SNARE proteins at the plasma membrane, where deletion of either of the R-SNAREs (v-SNAREs) Snc1/Snc2p yielded viable progeny (Protopopov *et al.,* 1993), but deletion of any of the Q-SNAREs (Sso1/Sso2p/Sec9p) did not. The dispensable nature of Sec22p, in contrast to the essential requirement for its ER-to-Golgi SNARE partners (Bos1p, Bet1p and Sed5) (Dascher *et al.,* 1991), was further exemplified by another SNARE protein, Ykt6p. The latter was able to substitute for Sec22p during anterograde protein transport, although cell growth was severely diminished in this scenario (Liu & Barlowe, 2002). The interchangeability of Sec22p and Ykt6p has been partially attributed to a longin domain within the variable N-terminal section of these SNAREs, which are structurally similar (Gonzalez *et al.,* 2001). The transport signals within the SNARE motif and longin domain of Sec22p assist in facilitating its COPII-dependent ER-export, contributing to the cycling of this protein within the ER-Golgi circuit (Liu *et al.,* 2004).

2.7.4 The v-SNARE (Qc-SNARE): Bet1p

The ER-to-Golgi v-SNARE (Qc-SNARE) Bet1p is an early Golgi protein that shares significant similarity to the neuronal Synaptobrevin, or VAMP, family of neuronal proteins (Newman *et al.,*

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1990; Ossipov *et al.,* 1999). Bet1p interacts directly with Bos1p, activating it and increasing its affinity for its t-SNARE receptor, Sed5p, which in turn assists in mediating the ER-to-Golgi phase of protein transport (Stone *et al.,* 1997). It was initially speculated that Bos1p, which cycles dynamically between the ER and the Golgi, is unable to recognize its t-SNARE counterpart Sed5p. However, as a vesicle is being formed, Bet1p interacts with Bos1p and this interaction may enable Bos1p to recognize and pair with its cognate t-SNARE counterpart (Sed5p), with the latter interaction regulated by the small GTP-binding protein Ypt1p.

An increase of Bet1p on ER-to-Golgi secretory vesicles may enhance the activity of Bos1p and it has been postulated that Bet1p actually modulates v-SNARE-t-SNARE interaction. Bet1p is therefore able to potentiate v-SNARE/t-SNARE interaction through direct contact with both Bos1p and Sec22p – the two t-SNARE light chains within its cognate t-SNARE sub-complex (Stone *et al.,* 1997). Therefore, though Bos1p, Sec22p and Bet1p function in concert with each other, the collective effects of Bet1p and Sec22p on Bos1p/Sed5p interactions are additive rather than synergistic, with these interactions being regulated by the ras-like GTP-binding protein Ypt1p (Lian *et al.,* 1994; Stone *et al.,* 1997). Bos1p therefore interacts with Bet1p in addition to Sed5p, whilst also interacting with Sec22p - with the latter reaction depending on the Rab-protein Ypt1p.

Bet1p is essential for secretion, with *bet1* mutations blocking the proper transport of many native yeast proteins including invertase, carboxypeptidase Y (CPY) and acid phosphatase, between the ER and the Golgi (Kipnis *et al.,* 2004). Bet1p is also essential to the secretion of glycosylphosphatidylinositol (GPI)-anchored cell wall mannoproteins, such as α-agglutinin, to the cell surface through vesicular transport pathways. Deletion of *BET1* led to secretion of α-agglutinin into the growth medium, compromising the ability of the yeast cell to incorporate α-agglutinin into its cell wall, leading to cell death (Kipnis *et al.,* 2004). Overexpressing Bet1p allowed the yeast to bypass the cell's requirement for the otherwise essential Sft1p (also a SNARE protein), required for intra-Golgi transport. This suggests that Bet1p participates not only in protein trafficking between the ER and the Golgi, but can also fulfill a role in retrograde intra-Golgi traffic (Tsui & Banfield*,* 2000). Liposomes possessing only the Bet1p v-SNARE were only able to fuse with liposomes bearing the cognate t-SNARE ternary complex consisting of Sed5p, Bos1p and Sec22p, correlating well with the molecular model describing the mechanics of the ER-to-Golgi SNARE complex (Parlati *et al.,* 2000, Paumet *et al.,* 2001; Parlati *et al.,* 2002).

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2.7.5 Overview: The ER-to-Golgi SNARE complex

Efficient, precise protein secretion requires that properly folded, newly synthesized proteins are selectively captured into ER-derived vesicles that deliver these proteins to downstream compartments in the secretory pathway (Lee *et al.,* 2004). These vesicles are generated by the before-mentioned COPII coat, a set of cytoplasmic coat proteins that function collectively. The exchange of GDP for GTP on the ER membrane results in the recruitment of Sar1p to the ER-membrane. The activated Sar1p then recruits the Sec23p/Sec24p heterodimer, which in turn binds and recruits specific cargo proteins into the nascent vesicle. In addition to the newly synthesized secretory proteins, the COPII coat, including the Sar1 – Sec23 – Sec24p pre-budding complex, must then also recruit the necessary machinery to facilitate the subsequent tethering and fusion of the vesicles at the Golgi apparatus. This machinery includes the SNARE proteins required for the fusogenic events between the ER and the *cis*-Golgi. Following thorough genetic, biochemical and structural investigation of the recruitment process facilitated by the COPII coat, the Sec24p subunit has been identified as the predominant subunit facilitating cargo selection at the ER (Miller *et al.,* 2003; Mossessova *et al.,* 2003). The Sec24p subunit possesses three independent cargo-binding sites: An “A-site” that interacts with a specific motif on the Sed5p ER-to-Golgi t-SNARE; a “B-site” that recognizes another motif on the before-mentioned Sed5p and a similar motif on the ER-to-Golgi v-SNARE Bet1p (Mossessova *et al.,* 2003); and a “C-site” that appears to specifically recognize a motif on another ER-to-Golgi t-SNARE, Sec22p (Miller *et al.,* 2003). The interaction between the Sec24p “A-site” and Sed5p is largely facilitated through hydrophobic interactions, whilst Sed5p seems to require the SM protein Sly1p in order to form a complex that is able to interact with Sec24p and hence be taken up into COPII vesicles (Miller *et al.,* 2005). The importance of the relationship between Sec24p and the ER-to-Golgi t-SNARE Sed5p is emphasized by the fact that mutational studies on the Sec24p YNNS**NPF** binding pocket, a specific motif located on Sed5p, severely reduced the ability of the yeast to package Sed5p into COPII vesicles. These vesicles, generated by the mutated Sec24p, were unable to fuse with the Golgi apparatus (Miller *et al.,* 2005), likely as a result of the depletion of its required t-SNARE (Sed5p). It is this specific NPF domain that is the dominant ER export signal on Sed5p, whilst being critical for Sed5p functioning. An illustration of the COPII components, within the broader ER-to-Golgi SNARE assembly reaction is given in **Fig. 7**.

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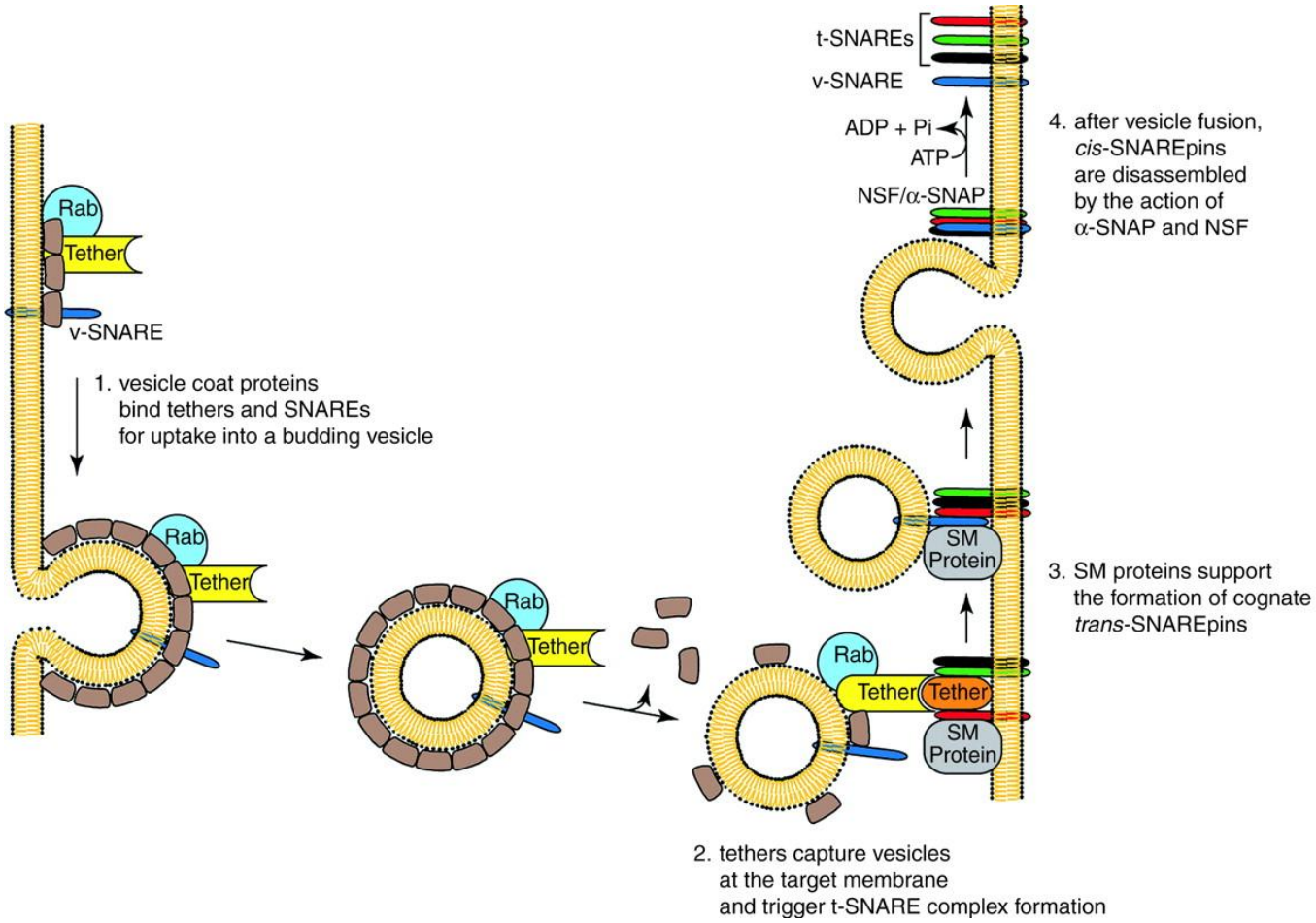

Fig. 7: An illustration of the various components that assist in facilitating the fusion of the ER-derived secretory vesicles (formation illustrated on the left), initially bearing the COPII coat proteins, with the *cis*-Golgi and the subsequent SNARE disassembly step. Other components that are illustrated are the previously mentioned Rab proteins, tethering factors, SM proteins, v- and t-SNARE components and the disassembly modulators NSF (Sec18p) and α-SNAP (Sec17p) (Malsam & Söllner, 2011).

Within the ER-to-Golgi subsection of the yeast secretion pathway, Sed5p, Sec22p, Bos1p and Bet1p fulfill roles of SNAREs, facilitating the targeting of anterograde vesicles to the *cis*-Golgi (Sacher *et al.,* 1997; Ossipov *et al.,* 1999). The yeast ER-to-Golgi SNARE complex consists of Bos1p, Sec22p and Sed5p, making up a functional t-SNARE, and small quantities of the v-SNARE Bet1p (Søgaard *et al.,* 1994; Stone *et al.,* 1997; Parlati *et al.,* 2002). The t-SNARE Sed5p combines cooperatively with the two light chains, Bos1p and Sec22p, to form the specific functional t-SNARE ternary complex that is able to receive vesicles from carrying the v-SNARE Bet1p from the ER (Parlati *et al.,* 2002). Anterograde ER-to-Golgi protein transport in *S.*

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cerevisiae therefore requires these four core SNARE proteins, in addition to the t-SNARE-associated SM protein Sly1p (Kaiser & Schekman, 1990; Newman *et al.,* 1990; Hardwick & Pelham, 1992; McNew *et al.,* 2000). Additional co-factors help to mediate SNARE assembly, such as the small GTP-binding protein Ypt1p, which allegedly impacts on the v-SNARE/t-SNARE complex formation, though it has not been identified as part of the complex itself (Lian *et al.,* 1994; Søgaard *et al.,* 1994). One important protein, Uso1p, is required for the *in vitro* targeting and fusion of ER-derived secretory vesicles to the Golgi and is required for the assembly of the ER-to-Golgi v-SNARE/t-SNARE complex *in vivo* (Sapperstein *et al.,* 1996). Additional proteins, including Sft1p, Ykt6p, Gos1p and Vti1p, mediate intra-Golgi and endosome-Golgi transport (Søgaard *et al.,* 1994).

All four SNARE proteins, Sed5p, Bos1p, Sec22p and Bet1p, are dynamically packaged into ER-derived secretory vesicles that are ultimately formed by the COPII coat (Cao & Barlowe, 2000). Mossessova *et al.* (2003) also proposed that the COPII coat actively selects only fusogenic forms of ER-to-Golgi SNAREs, resulting in fusogenic specificity encoded into the respective cognate v- and t-SNARE proteins (Parlati *et al.,* 2000). This specificity may therefore be deployed by the COPII coat to establish directionality that can control vesicles moving anterogradely in the ER-to-Golgi step, whilst preventing COPII vesicles from fusing back to the ER (Mossessova *et al.,* 2003).

In vitro assays indicate that Bos1p and Bet1p are functionally required on the vesicle membrane whilst the t-SNARE Sed5p is required on the acceptor membrane, even though all four ER-to-Golgi SNAREs are distributed and cycled similarly between these compartments (Cao & Barlowe, 2000). Therefore, each of these SNARE proteins are topologically restricted by design to function as either a v-SNARE or, alternatively, as part of the functional t-SNARE complex (Parlati *et al.*, 2000). Fusion only takes place when the v-SNARE Bet1p is located on one membrane, whilst Sed5p and its two light chains Bos1p and Sec22p are located on the other membrane, forming a functional t-SNARE. This is further supported by the fact that these SNAREs were only fusogenic when Bet1p was reconstituted into a single liposome, whilst Sed5p/Sec22p/Bos1p were situated in an opposing liposome (Parlati *et al.,* 2000). Each of the ER-to-Golgi SNAREs likely contribute a single α-helix (Sacher *et al.,* 1997), forming a four-helix bundle which in turn mediates the fusion reaction at the *cis*-Golgi (Weber *et al.,* 1998; Sutton *et al.,* 1998).

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2.8 SNAREs functioning between the Golgi and the plasma membrane

2.8.1 The v-SNAREs (R-SNAREs): Snc1p & Snc2p

The v-SNARE paralogs Snc1p and Snc2p (R-SNAREs) are 77% genetically identical and share 84% structural similarity. Genetic, morphological and biochemical data suggest a certain level of functional redundancy (Gerst *et al.,* 1992; Protopopov *et al.,* 1993; Aalto *et al.,* 1993). Both Snc1p and Snc2p are yeast paralogs that share significant sequence homology with members of the highly conserved Synaptobrevin, or VAMP, family of genes in higher eukaryotes (Gerst *et al.,* 1992). The Snc proteins cycle between the plasma membrane and other intracellular compartments, particularly on post-Golgi secretory vesicles, in keeping with their roles as the resident v-SNAREs, whilst they are able to physically interact with their cognate t-SNARE partners (Sso1p, Sso2p and Sec9p) at the plasma membrane interface (Protopopov *et al.,* 1993; Brennwald *et al.,* 1994; Rossi *et al.,* 1997).

Lewis *et al.* (2000) suggested that Snc1p is recycled for recurring fusion reactions at the plasma membrane via the Golgi, since Snc1p was observed to migrate from the cell surface to the Golgi when exocytosis was obstructed. The broad range of Snc1/Snc2p cycling, which extends from the plasma membrane to multiple cellular compartments, is dependent on a methionine residue within each of the respective SNARE helices (Snc1p-Met43 and Snc2-Met42) (Gurunathan *et al.,* 2000). Yeast cells lacking both *SNC* genes are viable, though the yeast is severely restricted with regards to its ability to transport proteins from the Golgi to the plasma membrane, leading to a build-up of secretory vesicles and restricted growth at non-permissive temperatures (Protopopov *et al.,* 1993). The permissive growth at certain temperatures led researchers to believe that some degree of vesicle fusion could still be taking place through the pairing of the Sso1/Sso2p and Sec9p t-SNARE components alone (Marash & Gerst, 2001).

A dualistic role was proposed for the Snc1/Snc2p proteins in both exocytosis and endocytosis, which is plausible as no endocytic v-SNARE has as yet been identified in yeast (Grote *et al.,* 2000; Gurunathan *et al.,* 2000). Direct roles for Snc1p and Snc2p have not yet been identified in sporulation per se, although a role for these v-SNAREs has been inferred by their close localization to the prospore membrane, a double-membrane structure that assists in isolating meiotic nuclei from the cytoplasm during sporulation (Neiman *et al.,* 2000).

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Vsm1p/Ddi1p, a ubiquitin receptor involved in cell cycle regulation, has been suggested as being a negative regulator of constitutive exocytosis in yeast (Lustgarten & Gerst, 1999; Gabriely *et al*., 2008). Vsm1p, which co-immunoprecipitates preferentially with Snc2p, led to increased secretion of native invertase when deleted and inhibited secretion when overexpressed – leading to the assumption that this protein fulfills a role as a negative regulator of exocytosis in yeast. Its ability to negatively regulate the latter stages of the secretory pathway arises from its ability to bind to exocytic v- and t-SNAREs such as Snc1/2p and Sso1/2p, respectively (Marash & Gerst, 2003).

Cells possessing disrupted or deleted *SNC1* or *SNC2* genes were severely deficient in the docking and fusion of membrane vesicles, which resulted in the accumulation of secretory vesicles at the plasma membrane and a notable loss in protein secretion competence as exocytosis became interrupted (Protopopov *et al.,* 1993). Mutations in *SNC1* or *SNC2* also led to decreased invertase secretion, though the viability of the yeast cells remained intact (Gerst, 1997). Additionally, the importance of the transmembrane segments of Snc1/2p and Sso1/2p was illustrated by replacing these segments with lipid anchors consisting of short peptide linkers (Grote *et al.,* 2000; McNew *et al.,* 2000). These short peptide insertions did not affect the targeting of secretory proteins or the assembly of the appropriate SNAREpins, but effectively blocked the final stages of membrane fusion as a result of the diminished ability of the particular SNAREpin to generate the force required to facilitate fusion (McNew *et al.,* 1999).

2.8.2 The t-SNARE (Qbc-SNARE): Sec9p

Sec9p represents the t-SNARE (Qbc-SNARE) heavy chain component at the plasma membrane interface and it is regarded as a yeast homologue of the mammalian SNAP-25 protein family (Jäntti *et al.,* 2002). Rather uniquely compared to the majority of SNARE complexes, the Sec9p t-SNARE component contributes two t-SNARE light chains, fulfilling the role of the Qb- and Qc-SNAREs and, in conjunction with the Sso1/2p Qa-SNARE subunit, it is able to form a functional binary SNARE complex prior to successful *trans*-SNARE complex assembly (Burri & Lithgow, 2004). Exceptions, such as the SNARE complex at the vacuole (consisting of five SNARE proteins) have also been identified, whilst the majority of functional t-SNARE complexes are ternary - consisting of three separate t-SNARE components. The t-SNAREs involved in

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exocytosis from the cell (Sec9p and Sso1/Sso2p), are localized along the entire plasma membrane, even in cells that have noticeably diminished buds (Brennwald *et al.,* 1994).

Sec9p is part of the SNAP-25 family of SNARE proteins in vegetative yeast cells and a homolog of this protein, Spo20p, interacts with the same binding partners in sporulating yeasts. Interestingly, Sec9p and Spo20p are only 37% identical; whilst they are both specialized to different developmental stages in *S. cerevisiae,* with Sec9p interacting with secretory proteins during vegetative growth and Spo20p required exclusively for sporulation (Kienle *et al.,* 2009). During sporulation in *S. cerevisiae,* the pro-spore membrane, that envelops each daughter nuclei during meiosis, is generated *de novo* by the SNARE machinery at a point adjacent to the spindle pole body. Both gene products, Sec9p and Spo20p, are required for post-Golgi vesicle fusion events, although they cannot substitute for one another (Neiman *et al.*, 2000).

An additional regulatory protein, Sro7p, has been shown to interact with the Sec9p subunit of the exocytic SNARE complex, whilst it has been suggested as an allosteric regulator of exocytosis (Hattendorf *et al.,* 2007). Additional physical and genetic interactions have also been identified between Sro7p and other exocytic proteins, including the exocyst and the Rab GTPase Sec4p, while genetic analysis revealed its function to be downstream of the aforementioned (Lehman *et al.,* 1999; Grosshans *et al.,* 2006).

Williams & Novick (2009) revealed that the level of exocytic SNARE complexes in a cell is directly controlled by the level of Sec9p, leading to the assumption that Sec9p is rate-limiting for SNARE complex formation. This is supported by the fact that Sec9p acts directly at the final step in exocytosis, with its binding to its Sso1/Sso2p t-SNARE counterpart to form the binary t-SNARE complex at this membrane interface considered the rate-limiting step (Nicholson *et al.,* 1998; Gasser *et al*., 2007). The functioning of Sec9p and SNARE complex formation is directly coupled to the physiological state of the cell (Williams & Novick, 2009) and any mutation or disruption in the *SEC9* gene manifests itself as a high temperature-sensitive phenotype.

2.8.3 The t-SNAREs (Qa-SNAREs): Sso1p & Sso2p

The t-SNARE (Qa-SNARE) Sso1p and its paralog Sso2p are 74% genetically similar and share 86% structural similarity, having initially been considered to be functionally redundant

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following their characterization as multicopy suppressors of a *sec1-1* mutant (Aalto *et al.,* 1993). These proteins are members of the highly conserved syntaxin family in eukaryotes, with seven other syntaxins having been identified in *S. cerevisiae* (Teng *et al.,* 2001). Both Sso1p and Sso2p, as well as the Snc1/Snc2p orthologs, are basally expressed during vegetative growth, which contributed to their initial putative classifications as being redundant (Aalto *et al.,* 1993).

Both Sso1p and Sso2p share significant sequence similarity with up to six other yeast proteins, including Sed5p, a crucial ER-to-Golgi t-SNARE protein involved in SNARE complex assembly at the Golgi (Aalto *et al.,* 1993). Following mutagenesis studies on *SSO1* and *SSO2,* it was determined that *SSO1,* but not *SSO2,* are required for sporulation. This functional distinction was further supported by the fact that, when *SSO1* and *SSO2* promoters were exchanged, it still did not render Sso2p functional in prospore membrane formation (Jäntti *et al.,* 2002; Oyen *et al.,* 2004). Further analysis, however, revealed that *SSO2*-overexpression could in fact suppress the sporulation deficiency brought about by *SSO1* deletion, adding further credence to the notion that *SSO1* is preferentially expressed in the cell compared to *SSO2* (Jäntti *et al.,* 2002). Therefore, though there exists a functional distinction with regards to their preferential utilization in sporulation processes, they are seemingly redundant during vegetative growth. Their importance to cellular growth is emphasized by the fact that a double deletion of both *SSO1* and *SSO2* was lethal, whilst cells depleted in Sso1p/Sso2p through promoter manipulation generally accumulated secretory vesicles at restrictive temperatures (Aalto *et al.,* 1993). Structurally, the Sso1p N-terminal domain has been identified as being essential to the regulation of SNARE assembly, whilst removal of this domain led to a three-fold increase in the rate of complex formation with Sec9p and Snc2p (Nicholson *et al.,* 1998).

2.8.4 Overview: The exocytic SNARE complex

Following transport of the Golgi-derived secretory vesicles to the plasma membrane, the action of a collection of proteins known as the exocyst complex is required for events leading to membrane fusion and exocytosis (Grote *et al.,* 2000). The exocyst is a large hetero-oligomeric protein complex, consisting of 8 subunits (Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p and Exo84p), that is essential for growth, secretion, exocytosis, endocytosis and cytokinesis (Munson & Novick, 2006; He & Guo, 2009; Nichols & Casanova, 2010). The multisubunit exocyst complex, which has also been implicated in the tethering of vesicles to sites of

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polarized secretion, is directly involved in the regulation of SNARE complexes and membrane fusion through interaction between its Sec6p subunit and the plasma membrane t-SNARE Sec9p (Morgera *et al.,* 2012). Sec6p, a subunit of the exocyst complex, is able to bind to the Sec9p t-SNARE subunit, dispersed throughout the plasma membrane. This binding prevents Sec9p from binding to its Sso1/2p t-SNARE partner – the rate-limiting step in SNARE complex assembly - effectively preventing premature or inappropriate SNARE complex assembly. The binding sites on Sec6p are similar for Sec1p and Sec9p, whilst the Sec6p-exocyst is thought to directly interact with Sec1p, the cognate SM protein of the exocytic SNARE complex, to coordinate SNARE complex assembly. It is possible that vesicle arrival coincides with Sec1p recruitment via the exocyst, after which Sec9p is released from Sec6p and is allowed to bind to Sso1/2p. This would provide a coordinated cascade wherein vesicle arrival and tethering coincides with the production of fusion-competent SNARE complexes (Morgera *et al.,* 2012). The Sec6p subunit is also crucial for anchoring the exocyst complex to sites of secretion on the plasma membrane. It has recently been proposed that exocyst-bound Sec6p is able to recruit Sec1p to sites of secretion, where it is transferred to newly formed ternary SNARE complexes for membrane fusion (Scott *et al.,* 2004; Morgera *et al.,* 2012). Hashizume *et al.* (2009) illustrated that Sec1p, the SM protein for the exocytic SNARE complex, stimulated vesicle docking and fusion to the plasma membrane, whilst another SM protein, Sly1p, promotes the assembly and disassembly of SNARE complexes between the ER and the Golgi (Peng & Gallwitz, 2002).

The exocytic SNARE complex is one of the most well-described SNARE-mediated fusion assemblages in yeast compared to its other anterograde counterparts. The SNARE complex at the plasma membrane is distinct, conserved between yeasts and humans and consists of a combination of only three proteins: the Qa-SNARE Sso1/2p, the Qb & Qc-SNARE Sec9p (possessing two SNARE motifs), both located on the target membrane, and the R-SNARE Snc1/2p that is located on the vesicle membrane (Burri & Lithgow, 2004).

The first step in exocytic SNARE complex assembly involves the binding of the Sec9p and Sso1/2p (t-SNAREs) subunits, forming a binary SNARE complex (Nicholson *et al.,* 1998). This interaction between Sso1/2p and Sec9p occurs with (1:1) stoichiometry (Strop *et al.,* 2008) and is considered the rate-limiting step in SNARE complex assembly at the plasma membrane (Nicholson *et al.,* 1998). Subsequent binding of the Snc1/2p (v-SNARE) located on the secretory vesicle forms a ternary complex that precedes exocytosis. The formation of the binary and

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ternary complexes is thought to result in the formation of additional alpha-helical structures in previously unstructured regions within the assembling SNARE complex (Fiebig *et al.,* 1999). The respective helical domains of Sso1/2p, Snc1/2p as well as both subunits of Sec9p, have been demonstrated to be aligned in parallel within the yeast exocytic SNARE complex, supporting the theory that the yeast exocytic SNARE complex essentially consists of the aforementioned four-helical bundle (Katz *et al*, 1998; Ungar & Hughson, 2003). Within the complex, two membrane-spanning SNAREs (Sso2p/Sso2p and Snc1p/Snc2p) each contribute one helical domain while Sec9p, which lacks a transmembrane anchor, contributes two helices (Katz *et al*, 1998). A graphic illustration depicting assembly of the four-helix bundle within the exocytic SNARE complex is given in **Fig. 8**, with the "closed" and "open" conformations of the Sso1p component also depicted. It is worth noting that Sec9p is usually present at levels 5-10 fold lower than its Sso1/2p and Snc1/2p counterparts (Lehman *et al.,* 1999).

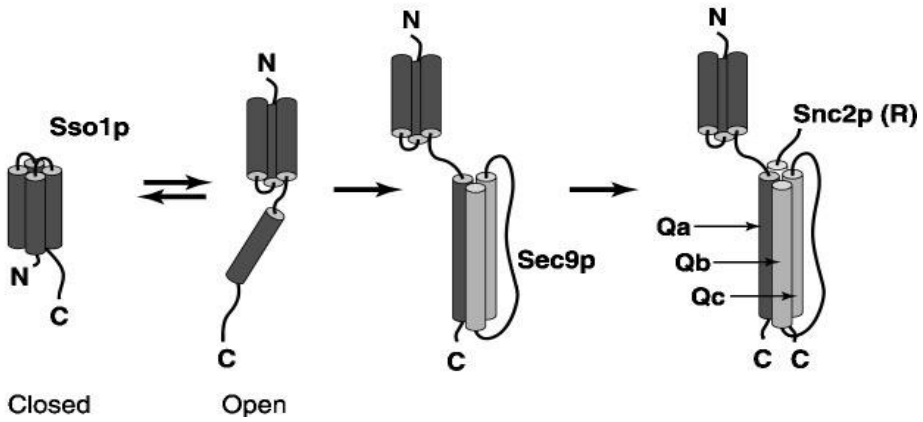

Fig. 8: The assembly reaction for the yeast exocytic SNARE proteins, including Sso1/2p, Sec9p and Snc2p. The "open" and "closed" conformations of the Sso1p/Sso2p N-terminal domain is illustrated and α-helices are represented by cylinders (Ungar & Hughson, 2003).

Although Sso1/2p (t-SNARE), Sec9p (t-SNARE) and Snc1/2p (v-SNARE) form the core of the yeast excocytic SNARE complex, many other protein interactions are necessary for its regulation and maintenance. The Rab-family GTP-binding proteins function as upstream regulators in SNARE mediated membrane fusion at the plasma membrane, while Mun18-1 binds with varying affinity to Snc1/2p, Sso1/2p and the assembled SNARE complexes in neuronal SNAREs (Xu *et al.,* 2010). Sec1p is the soluble, cytosolic, SM-family (Sec1/Munc-18) protein that interacts with assembled ternary SNARE complexes and regulates anterograde

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vesicle traffic between the Golgi and the plasma membrane (Carr *et al.,* 1999; Hou *et al.,* 2012). Mso1p, a Sec1p-interacting protein, also binds to the exocytic SNARE complex and plays an active role in mediating the functions of Sec1p (Castillo-Flores *et al.,* 2005). This specific protein has also been suggested to directly interact with the Rab protein, Sec4p, bridging the gap between Rab and SM-protein functions, leading to membrane fusion. Mso1p supposedly functions to coordinate the recruitment and subsequent removal of Sec1p in response to the GTPase cycle of the Rab protein Sec4p. Mso1p therefore interacts specifically with SNAREs associated with the exocytic complex, whilst it localizes to the plasma membrane under conditions that permit SNARE assembly. The latter was derived as its deletion leads to complete cessation in post-Golgi vesicle traffic, leading to an accumulation of Gogli-derived secretory vesicles (Salminen & Novick, 1987).

Sec4p apparently cycles between a form bound to the secretory vesicle or plasma membrane and another unbound, cytosolic analog (Goud *et al.,* 1988; Walworth *et al.,* 1989). The cycling between these respective forms is coupled to the sequential binding and hydrolysis of GTP and exchange of nucleotides, which in turn involves additional factors (Ferro-Novick & Novick, 1993; Novick & Brennwald, 1993). The exocyst plays a mediating role in determining the regulatory effect of Sec4p on the Sec9-Sso-Snc exocytic SNARE complex (Ferro-Novick & Novick, 1993; Brennwald *et al.,* 1994; Guo *et al.,* 1999). This essentially means that the exocyst is an effector for Sec4p, which effectively targets secretory vesicles to the site of exocytosis (Guo *et al.,* 1999). Toikkanen *et al.* (2003) also illustrated that the overexpression of Sec4p led to the increased production of secreted α-amylase, though the effect on heterologous protein secretion remains unknown. Another component of the exocyst complex, Sec3p, is localized to exocytic sites even in the absence of membrane traffic (Grote *et al.,* 2000). For the complete secretory vesicle fusion reaction to take place, at least 10 other late-acting *SEC* gene products are required. It is worth noting that many of these late-acting *SEC* genes are essential to facilitate sporulation as several different deletions have led to a loss of competence in this regard (Novick *et al.,* 1981).

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2.9 Engineering of *S. cerevisiae* ER-to-Golgi and exocytic SNAREs

Although the overexpression of native SNARE components in *S. cerevisiae* specifically for secretion enhancement has not been investigated beyond a handful of studies, hereafter follows a summary of the specific engineering strategies and their phenotypes relating to some of the components covered in this study (**Table 1**). The overexpression of a number of genes, including *SSO2,* had a positive effect on heterologous protein secretion in the yeasts *Pichia pastoris* and *S. cerevisiae* (Gasser *et al.*, 2007). Both yeast syntaxin paralogs *SSO1* and *SSO2* were reported to increase secretion of heterologous *Bacillus* α-amylase and native invertase by 4- to 6-fold when overexpressed in *S. cerevisiae* using a constitutive promoter (P_{ADH}) on multicopy plasmids (Ruohonen *et al.,* 1997). The Sso proteins are rate-limiting components in the secretory machinery and overexpression thereof is thought to enhance the secretory process itself, whilst prolonging secretion and enhancing delivery into the periplasmic space. Gasser *et al.* (2007) illustrated an increase of approximately 20% in secretion for human antibody Fab fragment in *P. pastoris* after having inserted only one additional copy of the *SSO2* gene. In contrast, depletion of the Sso2 proteins caused impaired growth and protein secretion as well as the accumulation of secretory vesicles at the budding site. It was suggested that overexpression of other components of the SNARE secretory machinery functioning at the plasma membrane, such as Snc2p, Snc1p and Sec9p, in addition to Sso1/2p, could further enhance protein secretion of proteins enclosed in Golgi-derived secretory vesicles (Ruohonen *et al.,* 1997).

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Table 1: A summary of the SNARE-related engineering strategies, focusing on the various intracellular SNARE complexes and their phenotypic responses in *S. cerevisiae.*

SNARE modification	Host organism	Phenotype	Reference
Anterograde ER-to-Golgi SNAREs			
bos1Δ	*S. cerevisiae*	• Non-viable progeny	Shim *et al.,* 1991
↓*BOS1*	*S. cerevisiae*	• Defective ER-to-Golgi transport • Secretory vesicle accumulation	Shim *et al.,* 1991
↑*BOS1*	*S. cerevisiae*	• Decreased protein secretion	Van Zyl *et al*., 2015
bet1Δ	*S. cerevisiae*	• Nonviable progeny	Newman *et al.,* 1990
bet1-1	*S. cerevisiae*	• Invertase accumulation • ER membrane accumulation • Temperature sensitivity	Newman & Ferro-Novick, 1987
bet1-1	*S. cerevisiae*	• Exaggerated ER-accumulation • Growth defects	Newman *et al.,* 1990
↑*BET1*	*S. cerevisiae*	• Secretory improvements	Van Zyl *et al.,* 2015
sec22Δ	*S. cerevisiae*	• Restricted growth • Temperature sensitivity	Dascher *et al.,* 1991
sec22-3	*S. cerevisiae*	• Exaggerated ER-accumulation • Growth defects	Newman *et al.,* 1990
sec22-3	*S. cerevisiae*	• Increased (defective) BiP (ER resident protein) secretion	Lewis *et al.,* 1997
↑*SEC22*	*S. cerevisiae*	• Secretory improvements	Van Zyl *et al.,* 2015
bet1-1 sec22-3	*S. cerevisiae*	• Non-viable progeny	Newman *et al.,* 1990
sed5-1	*S. cerevisiae*	• Temperature sensitivity	Weinberger et al*.,* 2005
sed5Δ	*S. cerevisiae*	• Nonviable progeny	Hardwick & Pelham, 1992
↓*SED5*	*S. cerevisiae*	• Buildup of ER membranes and vesicular structures • Accumulation of vacuolar CPY	Hardwick & Pelham, 1992
↑*SED5*	*S. cerevisiae*	• Secretion of ER-luminal Kar2p	Weinberger *et al.,* 2005

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Table 1: Continued

↑*SED5*	*S. cerevisiae*	• Secretory improvements • Reduced growth vigor • Reduced ethanol tolerance • Reduced osmotic tolerance • Reduction in heterologously-induced UPR induction	Van Zyl *et al.,* 2015
↑*SSO1*↑*SED5*	*S. cerevisiae*	• Secretory improvements • Reduced growth vigor • Reduced ethanol tolerance • Reduced osmotic tolerance	Van Zyl *et al.,* 2015
Retrograde Golgi-to-ER SNAREs			
ufe1-1	*S. cerevisiae*	• Temperature sensitivity	Lewis *et al.,* 1997
sec20Δ	*C. albicans*	• Membranous accumulation • Antifungal super-sensitivity • Reduced growth	Weber *et al.,* 2001
sec20-1	*S. cerevisiae*	• Temperature sensitivity • ER network accumulation • Secretory vesicle accumulation	Sweet & Pelham, 1992
slt1Δ	*S. cerevisiae*	• Lethality	Burri *et al.,* 2003
↓*SLT1*	*S. cerevisiae*	• Defective secretion of ER resident proteins	Burri *et al.,* 2003
Anterograde Golgi-to-Plasma membrane (Exocytic) SNAREs			
snc1Δ	*S. cerevisiae*	• No significant phenotypic effect	Gerst *et al.,* 1992
↑*SNC1*	*S. cerevisiae*	• Secretory improvements	Van Zyl *et al.,* 2014
sec9-4	*S. cerevisiae*	• Temperature sensitivity • Defective secretion at non-permissive temperatures	Novick *et al.,* 1980
sec9-7	*S. cerevisiae*	• Temperature sensitivity	Rossi *et al.,* 1997
sec9-Δ38	*S. cerevisiae*	• Recessive lethal phenotype	Rossi *et al.,* 1997
sec9-Δ17	*S. cerevisiae*	• Recessive lethal phenotype	Rossi *et al.,* 1997
↑*SEC9*	*S. cerevisiae*	• Secretory improvements	Van Zyl *et al.,* 2014
↑*SSO1*	*S. cerevisiae*	• Improved α-amylase secretion	Ruohonen *et al.,* 1997

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Table 1: Continued

↑*SSO1*	*S. cerevisiae*	• Secretory improvements • Reduced growth vigor	Van Zyl *et al.,* 2014
sso2-1	*S. cerevisiae*	• Temperature sensitivity	Jäntti *et al.,* 2002
↑*SSO2*	*S. cerevisiae*	• Improved α-amylase secretion	Ruohonen *et al.,* 1997
↑*SSO2*	*Pichia pastoris*	• Secretory improvements	Gasser *et al.*, 2007
↑*SSO2*	*S. cerevisiae*	• Variable secretory improvements	Van Zyl *et al.,* 2014
sso1Δ sso2-1	*S. cerevisiae*	• Temperature sensitive • Accumulation of secretory vesicles at restrictive temperatures • Cytokinesis & budding defects	Jäntti *et al.,* 2002
sso2Δ sso1-2	*S. cerevisiae*	• Temperature sensitive	Jäntti *et al.,* 2002
sso1Δ/sso2Δ	*S. cerevisiae*	• Sporulation deficient	Jäntti *et al.,* 2002
Sso1Δ sso2Δ	*S. cerevisiae*	• Non-viable progeny	Katz & Brennwald, 2000
Retrograde Plasma membrane-to-Golgi SNAREs			
Snc1ΔSnc2Δtlg2Δ	*S. cerevisiae*	• Reduced cell growth • Diminished doubling time	Gurunathan *et al.,* 2000
Snc1ΔSnc2Δtlg1Δ	*S. cerevisiae*	• Reduced cell growth • Temperature sensitivity	Gurunathan *et al.,* 2000
tlg1Δ	*S. cerevisiae*	• Reduced cell growth • ER-Golgi transport blocks • Vesicle accumulation • Vacuole fragmentation	Coe *et al.,* 1999
Vti1-1	*S. cerevisiae*	• Miss-sorting of proteins	Fischer von Mollard *et al.,* 1997
Vti1-11	*S. cerevisiae*	• Growth defects • ER & early Golgi protein accumulation	Fischer von Mollard *et al.,* 1997

The following shorthand was employed: **Small caps** – gene mutated/disrupted or deleted (Δ); ↑ - gene overexpressed; ↓ - gene expression repressed.

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Initial results reported by Hardwick & Pelham (1992) indicated that deletion of Sed5p, the ER-to-Gogli t-SNARE heavy chain component, prevented *S. cerevisiae* from effectively transporting CPY to the Golgi, whilst cells cessated in growth following the dramatic accumulation of ER membranes and vesicles. Furthermore, when Sed5p was overexpressed on a multicopy vector, it was reported to reduce the efficiency of ER-to-Golgi protein transport, though an increase in the number of putative transport vesicles was observed. Multicopy overexpression of Sed5p in strains not expressing a heterologous reporter protein also led to detectable morphological effects, including an increase in cell size and an increase in the number of apparent vesicular structures.

Hou *et al.* (2012) illustrated that the overexpression of either of the two SM proteins Sly1p and Sec1p, playing differential roles in SNARE assembly at the Golgi and plasma membrane, respectively, resulted in notable increases in native invertase and α-amylase secretion. Whilst Sec1p is involved in the regulation of vesicle transport and SNARE assembly at the plasma membrane interface, Sly1p is responsible for the corresponding processes at the *cis*-Golgi, with both playing mediating roles as clasp-shaped SM proteins. Their findings also illustrated that invigorating the ER-to-Golgi and Golgi-to-plasma membrane protein trafficking steps is a novel and feasible approach for improving the heterologous protein production capacity of *S. cerevisiae.* Sec1p interacts in a particular manner with the components of the exocytic SNARE complex (Snc1/2p, Sso1/2p, and Sec9p) which, at overexpressed levels, also led to notable heterologous protein secretion improvements (Van Zyl *et al.,* 2014). It was therefore plausible to postulate that similar to Sly1p, the SM protein functioning at the ER-to-Golgi phase of secretion that leads to secretory improvements at overexpressed levels (Hou *et al.,* 2012) – similar results could be expected when its cognate SNARE components functioning between the ER and Golgi (Sed5p, Bos1p, Sec22p and Bet1p) are overexpressed (Van Zyl *et al.,* 2015). Though much of the literature to date has focused on understanding the fundamental roles of SNAREs within both a physiological and secretory context, investigations into their application for secretory engineering have been relatively scarce. Nevertheless, a summary of some of the SNARE-related engineering results that have been published in literature, including SNARE components not investigated in this study and their respective phenotypic responses, is presented in **Table 1**.

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Engineering components of the secretory pathway to improve heterologous protein secretion has been successfully applied in *S. cerevisiae* over recent years to varying degrees of success (Ruohonen *et al.,* 1997; Hou *et al.,* 2012; Kroukamp *et al.,* 2013; Tang *et al.,* 2015). With this in mind, it was postulated that engineering this yeast to over-produce SNARE components that facilitate the fusion of anterograde protein transport vesicles at both the *cis*-Golgi and plasma membrane interface, respectively, could relieve potential secretion bottlenecks and allow for improved secretory titers for heterologous proteins, in particular cellulolytic enzymes.

With the majority of SNARE-related research having focused on characterizing their specific roles in the secretory pathway at varying membrane fusion steps, this work focused on identifying specific SNARE components that can enhance the heterologous protein secretion phenotype. In this study, we investigated for the first time whether overexpression of ER-to-Golgi (Bos1p, Bet1p, Sec22p and Sed5p) and exocytic (Snc1p, Snc2p, Sso1p, Sso2p and Sec9p) SNARE components, facilitating vesicle fusion at the *cis*-Golgi and plasma membrane, respectively, could increase heterologous protein secretion. Specific cellulolytic reporter proteins (*Saccharomycopsis fibuligera*-Cel3A and *Tallaromyces emersonii*-Cel7A) were selected for expression in *S. cerevisiae.* The challenge of high level cellobiohydrolase production in *S. cerevisiae* (Van Zyl *et al.,* 2013a), coupled with the critical role that β-glucosidases play in cellulase hydrolysis for bioethanol production (Xin *et al.,* 1993; Han & Chen, 2008; Singhania *et al.,* 2013) and the bottlenecks they impose on lignocellulosic fuels (Sørensen *et al.,* 2013), contributed to the selection of *Te*-Cel7A and *Sf*-Cel3A as reporter proteins.

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Chapter 3:

Overexpression of native *Saccharomyces cerevisiae* exocytic SNARE genes increased heterologous cellulase secretion

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Abstract:

SNAREs (soluble NSF (N-ethylmaleimide-sensitive factor) attachment receptor proteins) are required at the majority of fusion events during intracellular membrane transport and play crucial roles in facilitating protein trafficking between the various membrane-enclosed organelles and the plasma membrane. We demonstrate increases in the secretion of the *Tallaromyces emersonii* Cel7A (a cellobiohydrolase) and the *Saccharomycopsis fibuligera* Cel3A (a β-glucosidase), through the separate and simultaneous overexpression of different components of the exocytic SNARE complex in *Saccharomyces cerevisiae.* Overexpression of *SNC1* yielded the biggest improvement in *Te*-Cel7A secretion (71%), whilst *SSO1* overexpression led to the highest increases in *Sf*-Cel3A secretion (43.8%). Simultaneous overexpression of differential combinations of these SNARE components yielded maximal increases of ~52% and ~49% for the secretion of *Te*-Cel7A and *Sf*-Cel3A, respectively. These increases generally did not cause deleterious growth effects, whilst differential improvement patterns were observed for the two reporter proteins (*Sf*-Cel3A and *Te*-Cel7aA). Simultaneous overexpression of up to three of these components, in strains secreting the more efficiently expressed *Sf*-Cel3A, led to a slight decrease in osmotic tolerance at elevated NaCl concentrations, as well as a detectable decrease in ethanol tolerance at increased concentrations. This work illustrates the potential of engineering components of the anterograde secretory pathway, particularly its SNARE components, for the improvement of heterologous cellulase secretion.

Keywords: SNAREs, secretion, improvement, cellulases, yeast

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Introduction:

Since the early 1980s, yeasts have been utilized successfully for the large-scale production of intracellular and extracellular human, animal, plant, viral and fungal proteins, with the most widely utilized being *Saccharomyces cerevisiae* and *Pichia pastoris* (Romanos *et al.,* 1992; Romanos, 1995; Demain & Vaishnav, 2009). The advantages that yeast expression systems provide include simple handling in inexpensive media formulations and rapidly reaching high cell densities, whilst simultaneously being devoid of pyrogens, pathogens or viral inclusions (Çelik & Çalik, 2012). *Saccharomyces cerevisiae* is the most commercially exploited microbe in traditional fermentation processes. However, its major limitations as a commercial protein production host are its relatively low protein yields and tendency to hyperglycosylate certain heterologous proteins, which contribute to a reduced secretion rate (Romanos *et al.,* 1992).

The production of bioethanol from lignocellulosic substrates using recombinant yeast strains is still not financially viable as an alternative fuel source, when compared to fossil fuels produced at large scale in mature refineries (Stephen *et al.,* 2012). Two impeding cost factors are (i) energy cost of pre-treatment at high temperatures and (ii) high cost of commercial cellulases to release sugars from lignocellulosics. Recombinant *S. cerevisiae* strains, able to hydrolyze cellobiose and amorphous cellulose to ethanol, have been reported (McBride *et al.,* 2005; Van Rooyen *et al.,* 2005; Den Haan *et al.,* 2007). The pinnacle of what is being sought is a yeast capable of consolidated bioprocessing (CBP) that produces cellulases in sufficient quantities to hydrolyze cellulose and ferment the resulting hexose and pentose sugars to ethanol, in one step (Lynd *et al.,* 2005).

The limited cellulase secretion capacity of *S. cerevisiae* is one of the most significant barriers to its successful application as a CBP host for second generation bioethanol production (Ilmén *et al.,* 2011; Den Haan *et al.,* 2013). An increase in heterologous cellulase secretion could significantly increase the rate and efficiency of lignocellulose conversion to fermentable sugars, increasing the overall economics of the process. In addition, the current market for Food and Drug Administration (FDA)-approved therapeutic proteins, such as insulin, glucagons, GM-CSF (granulocyte macrophage colony stimulating factor) and hepatitis B surface antigen produced in *S. cerevisiae*, illustrates that the optimization of yeast secretion systems has a wider significance (Harford *et al.,* 1987; Schmidt, 2004; Rader, 2007; Idiris *et al.,* 2010; Hou *et al.,* 2012). It is therefore clear that an increase in the heterologous protein secretion capacity of *S. cerevisiae* would benefit not only the renewable energy

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sector, but the biopharmaceutical protein industry as well. The expression and secretion of heterologous proteins in *S. cerevisiae* are subject to several bottlenecks that collectively result in sub-optimal yields. Therefore, to improve the cell-specific productivity of this yeast, the bottlenecks for heterologous protein secretion have to be identified and resolved (Schröder, 2007).

SNAREs (soluble NSF (N-ethylmaleimide-sensitive factor) attachment receptor proteins) belong to a class of small type II membrane proteins that drive intracellular membrane traffic, whilst simultaneously contributing to the specificity thereof (Malsam *et al.,* 2008; Kloepper *et al.,* 2008). SNAREs are divided into two classes depending on the localization of their activity, with different vesicle trafficking steps deploying different v-/t-SNARE complexes (Jahn & Scheller, 2006). SNAREs present on target membranes are referred to as t-SNAREs, whilst those present on the transport vesicles are known as v-SNARES. V-SNARES interact in *trans* with cognate t-SNARES, on specific target membranes, resulting in the formation of a complex that bridges the two membranes. This *trans*-SNARE complex, or SNAREpin, which usually consists of four SNARE motifs bundled together in an α-helical coil, succeeds in bringing the respective bilayers into close proximity, which is an energetically favourable state for fusion (Malsam *et al.,* 2008). SNARE proteins have been identified on the Endoplasmic Reticulum (ER), the Golgi membrane, the vacuole/lysosome, the plasma membrane, as well as the vesicles that are derived from these respective membranes. In eukaryotes, these proteins are also involved in many, if not all, membrane fusion events along the endocytic and secretory pathways (Burri & Lithgow, 2004; Malsam *et al.,* 2008). Thus, collectively, the intracellular distribution of SNARE proteins is able to provide a tentative roadmap of yeast membrane traffic (**Fig. 1**).

The *trans*-SNARE complex at the plasma membrane is distinct, conserved between yeasts and humans and consists of a combination of only three proteins: Sso1/2p, Sec9p (possessing two SNARE motifs) and Snc1/2p (Burri & Lithgow, 2004). The v-SNARE paralogs Snc1p and Snc2p are 79% identical and have been suggested to be functionally redundant (Protopopov *et al.,* 1993; Shen *et al.,* 2013). The t-SNARE Sso1p and its paralog Sso2p (74% identical) were initially considered to be functionally redundant, although they have now been distinguished, with Sso1p playing a central role in sporulation (Jäntti *et al.,* 2002). Both the Snc1/2p and Sso1/2p proteins contribute only one α-helical domain to the exocytic SNARE complex, whilst Sec9p contributes two helical domains, a characteristic that makes the exocytic SNARE complex somewhat unique within the larger cascade of SNARE proteins (Burri & Lithgow, 2004), whilst exceptions such as the SNARE complex at the vacuole (consisting of five SNARE proteins) have also been identified.

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Ruohonen *et al.* (1997) illustrated a 1.5-fold (~50%) increase in native invertase secretion in *S. cerevisiae* following the overexpression of *SSO2*, on a multicopy plasmid. Gasser *et al.* (2007) obtained similar results through engineering of the methylotrophic yeast *Pichia pastoris*, illustrating an increase of 1.2-fold (20%) in secretion of human antibody (Fab) following the overexpression of the *SSO2* ORF. More recently, Xu *et al.* (2013) illustrated an increase of 10% in *Trichoderma reesei* Cel7A secretion, following the overexpression of the *SSO1* ORF in *S. cerevisiae.* Overproducing Sec1p, the SM (Sec1/Mun18-like) protein that plays a mediating role in facilitating SNARE complex assembly at the plasma membrane, leads to secretory improvements in *S. cerevisiae* (Hou *et al.,* 2012).

In this study, we have demonstrated that the overexpression of components of the exocytic SNARE complex (Snc1/2p, Sso1/2p and Sec9p) in *S. cerevisiae*, singly and simultaneously, was able to improve the secretion titers attainable for two cellulolytic reporter proteins (*T. emersonii* Cel7A and *S. fibuligera* Cel3A).

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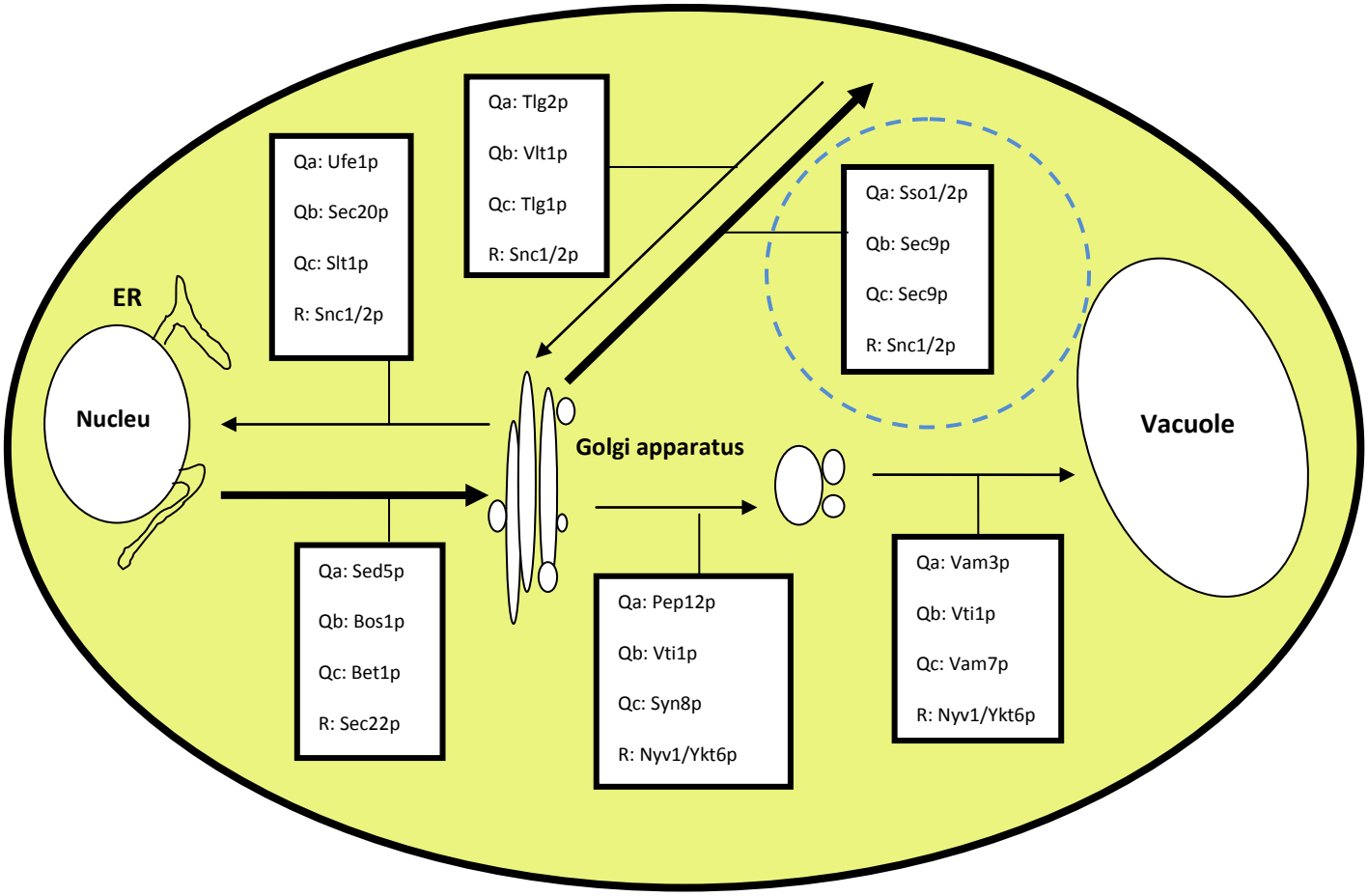

Fig. 1: A graphic summary of some of the SNARE proteins involved in different stages of protein trafficking in *S. cerevisiae.* The collection of proteins making up the SNARE complexes between the Golgi and plasma membrane are encircled, being of particular interest to this study, whilst the thick directional arrows indicate the anterograde (forward) transport steps (adapted from Burri & Lithgow, 2004).

Materials and Methods

Media and culturing conditions

Saccharomyces cerevisiae strain Y294 (*MATα*; *his3Δ*; *leu2Δ*; *lys2Δ*; *ura3Δ*) (ATCC 201160) was utilized as background strain. Yeast cells were routinely cultivated at 30˚C in YPD (Yeast Extract Peptone Dextrose) (1% yeast extract (Merck – Darmstadt, Germany), 2% peptone (Merck – Darmstadt, Germany), 2% glucose (Merck – Darmstadt, Germany)) medium. All *S. cerevisiae* transformants were selected on YPD agar supplemented with 200 µg mL^{-1} of the appropriate antibiotic (G418 disulphate (Melford Laboratories – Ipswich, United Kingdom), hygromycin B (Calbiochem – San Diego, USA) and zeocin (Melford – Ipswich, United Kingdom)), whilst liquid cultures were cultivated on a rotary shaker

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(200 rpm) at 30˚C. For protein studies yeast strains were grown on 2xSC^{-ura} (double strength synthetic complete) medium (3.4 g L^{-1} yeast nitrogen base (Difco - Sparks, USA) with all amino acids except uracil, 2% succinate (Sigma – St. Louis, USA), 1% ammonium sulfate (Merck - Darmstadt, Germany) and 2% glucose (Merck - Darmstadt, Germany), pH adjusted to pH 6.0 using 10 N sodium hydroxide). *Escherichia coli* DH5α was used for general cloning procedures and strains were routinely cultivated in Luria Bertani (LB) broth (0.5% yeast extract (Merck - Darmstadt, Germany); 1% tryptone (Merck - Darmstadt, Germany); 1% NaCl (Merck - Darmstadt, Germany)) supplemented with 100 µg mL^{-1} ampicillin (Roche – Johannesburg, South Africa) at 37˚C.

Plasmid and strain construction

Standard DNA manipulation protocols were followed (Sambrook & Russel, 2001). Initial PCR products were amplified using the Phusion® High-Fidelity DNA Polymerase (Thermo Scientific - Waltham, USA) on an Applied BioSystems 2720 Thermocycler, as instructed by the manufacturer, using forward and reverse primers that include *Pac*I and *Asc*I restriction sites for subsequent directional cloning into the pBKD1 (McBride *et al.,* 2008), pBHD1 (Kroukamp *et al.,* 2013) and pBZD1 (McBride *et al.,* 2008) yeast expression vectors. These vectors harboured differential antibiotic selection markers (*KanMX* for G418 disulphate resistance, *hph* for hygromycin B resistance and *Sh Ble* for zeocin resistance, respectively) and the constitutive *PGK1* gene promoter and terminator sequences. Initial PCR products were first ligated into the pCloneJET 1.2 commercial vector (Fermentas – Sankt Leon-Rot, Germany), as instructed by the manufacturer, which includes the *AmpR* gene for ampicillin selection.

PCR products/DNA fragments were routinely separated on 1% (w/v) agarose (Lonza – Rockland, USA) gels and fragments of appropriate sizes isolated using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, CA, USA). Sequence verification was carried out using the dideoxy chain termination method, with an ABI PRISM™ 3100 genetic analyzer (Central Analytical Facility, Stellenbosch University). Since the *SNC1* ORF contained a 114-bp intron, primers were utilized to successfully isolate and clone the exon using overlap-PCR. The total collection of PCR primers used in the study is summarized in **Table 1**.

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Table 1: PCR and qPCR primers utilized in the study.

Primer	Primer sequence
SNC1-L	5'-GATCTTAATTAAATGTCGTCATCTACTCCCTTTG-3'
SNC1a-R	5'-CCCACGGTATCATCAATTTCAGCTTGTAGTTCCGCAGTCC-3'
SNC1b-L	5'-GGACTGCGGAACTACAAGCTGAAATTGATGATACCGTGGG-3'
SNC1-R	5'-GATCGGCGCGCCCTATCGACTAAAGTGAACAGCAATG-3'
SNC2-L	5'-GATCTTAATTAAATGTCGTCATCAGTGC-3'
SNC2-R	5'-GATCGGCGCGCCTTAGCTGAAATGGACG-3'
SSO1-L	5'-GATCTTAATTAAATGAGTTATAATAATCCGTACC-3'
SSO2-R	5'-GATCGGCGCGCCTTAACGCGTTTTGACAAC-3'
SEC9-L	5'-GATCTTAATTAAATGGGATTAAAGAAATTTT-3'
SEC9-R	5'-GATCGGCGCGCCCTATCTGATACCTGCCAAC-3'
PGK1-L	5'-GTCAAGCTTGAATTCAATTGGGGCCCTCCCTCC-3'
ALG9-L	5'-TGCATTTGCTGTGATTGTCA-3'
ALG9-R	5'-GCCAGATTCCTCACTTGCAT-3'
TFC1-L	5'-ACACTCCAGGCGGTATTGAC -3'
TFC1-R	5'-CTTCTGCAATGTTTGGCTCA-3'
GEN1-L	5'-CCGCGATTAAATTCCAACAT-3'
GEN1-R	5'-CGATAGATTGTCGCACCTGA-3'
ZEO2-L	5'-GACGCGTGTACGCATGTAAC-3'
ZEO2-R	5'-TTGCAAATTAAAGCCTTCGAG-3'
HYG2-L	5'-ATTTCGGCTCCAACAATGTC-3'
HYG2-R	5'-GATGTTGGCGACCTCGTATT-3'

Plasmid isolations were carried out using the cetyltrimethylammonium bromide (CTAB) method (Sambrook & Russel, 2001). All plasmids constructed and utilized in this study are summarized in **Table 2**. Yeast transformations were carried out using the LiOAc/DMSO-method (Hill *et al.,* 1991). Two Y294 *S. cerevisiae* strains were utilized as parental strains for the study, one expressing the *S. fibuligera CEL3A* (Kroukamp *et al.,* 2013; Genbank AEV40916.1) and the other expressing the *T. ermersonii CEL7A-CCBM* (Ilmén *et al.,* 2011; Genbank AAL89553) (**Table 3**). Overexpression of the respective ORFs in the latter two parental strains was facilitated by integrating the expression cassettes through homologous recombination with native delta sequences distributed throughout the yeast genome (Lee & Da Silva, 1997). Genomic DNA extractions were carried out using the method described by Hoffman & Winston, (1987) and transformants cultivated on selective YPD agar containing 200 µg mL^{-1} of the appropriate antibiotic (G418 disulphate (Melford Laboratories – Ipswich, United Kingdom), hygromycin B (Calbiochem – San Diego, USA) or zeocin (Melford – Ipswich, United Kingdom)). All transformants were confirmed using PCR amplifications with a *PGK*1 promoter-specific forward primer and a gene-specific reverse primer (**Table 1**). All strains constructed for this study are described in **Table 3**.

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Table 2: All plasmids utilized in this study.

Plasmid	Relevant genotype	Reference/source
pBKD1	*bla* δ-site $PGK1_P$-$PGK1_T$ *kanMX* δ-site	McBride *et al.* (2008)
pBKD1-*SNC1*	*bla* δ-site $PGK1_P$-*SNC1*-$PGK1_T$ *kanMX* δ-site	This work
pBKD1-*SNC2*	*bla* δ-site $PGK1_P$-*SNC2*-$PGK1_T$ *kanMX* δ-site	This work
pBKD1-*SSO1*	*bla* δ-site $PGK1_P$-*SSO1*-$PGK1_T$ *kanMX* δ-site	This work
pBKD1-*SSO2*	*bla* δ-site $PGK1_P$-*SSO2*-$PGK1_T$ *kanMX* δ-site	This work
pBKD1-*SEC9*	*bla* δ-site $PGK1_P$-*SEC9*-$PGK1_T$ *kanMX* δ-site	This work
pBZD1	*bla* δ-site $PGK1_P$-$PGK1_T$ *Sh Ble* δ-site	McBride *et al.* (2008)
pBZD1-*SSO1*	*bla* δ-site $PGK1_P$-*SSO1*-$PGK1_T$ *Sh Ble* δ-site	This work
pBZD-*SSO2*	*bla* δ-site $PGK1_P$- *SSO2*-$PGK1_T$ *Sh Ble* δ-site	This work
pBHD1	*bla* δ-site $PGK1_P$-$PGK1_T$ *hph* δ-site	Kroukamp *et al.* (2013)
pBHD1-*SEC9*	*bla* δ-site $PGK1_P$-*SEC9*-$PGK1_T$ *hph* δ-site	This work
fur1::LEU2 ySFI	*bla ura3/URA3* $PGK1_p$-*XYNSEC-S.f.cel3A*-$PGK1_t$	Van Rooyen *et al.* (2005)
fur1::LEU2 pMI529	*bla ura3/URA3* $ENO1_p$-*T.e.cel7A-cCBM*-$ENO1_t$	Ilmén *et al.* (2011)

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Table 3: The collection of strains utilized in this study.

Yeast strain	**Relevant genotype**	**Reference/source**
S. cerevisiae Y294	*MATα ; his3Δ; leu2Δ; lys2Δ; ura3Δ*	ATCC 201160
S. cerevisiae Y294 (*CEL3A* Parental)	*ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t fur1::LEU2*	Den Haan *et al.* (2007)*
Y294_*CEL3A_SNC1* (Overexpressed)	*ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-his3/HIS3-PGK1p-SNC1-PGK1t-kanMX-fur1::LEU2*	This work
Y294_*CEL3A_SNC2*	*ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-his3/HIS3-PGK1p-SNC2-PGK1t-kanMX-fur1::LEU2*	This work
Y294_*CEL3A_SSO1*	*ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-his3/HIS3-PGK1p-SSO1-PGK1t-kanMX-fur1::LEU2*	This work
Y294_*CEL3A_SSO2*	*ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-his3/HIS3-PGK1p-SSO2-PGK1t-kanMX-fur1::LEU2*	This work
Y294_*CEL3A_SEC9*	*ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-his3/HIS3-PGK1p-SEC9-PGK1t-kanMX-fur1::LEU2*	This work
Y294_*CEL3A_SNC1_SSO1*	*ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-his3/HIS3-PGK1p-SNC1-PGK1t-kanMX-PGK1p-SSO1-PGK1t-Shble-fur1::LEU2*	This work
Y294_*CEL3A_SNC1_SSO2*	*ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-his3/HIS3-PGK1p-SNC1-PGK1t–kanMX-PGK1p-SSO2-PGK1t-Shble-fur1::LEU2*	This work
Y294_*CEL3A_SNC2_SSO2*	*ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-his3/HIS3-PGK1p-SNC2-PGK1t–kanMX-PGK1p-SSO2-PGK1t-Shble-fur1::LEU2*	This work
Y294_*CEL3A_SNC2_SSO1*	*ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-his3/HIS3-PGK1p-SNC2-PGK1t–kanMX-PGK1p-SSO1-PGK1t-Shble-fur1::LEU2*	This work
Y294_*CEL3A_SNC1_SSO1_SEC9*	*ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-his3/HIS3-PGK1p-SNC1-PGK1t-kanMX-PGK1p-SSO1-PGK1t-Shble-PGK1p-SEC9-PGK1t-hph-fur1::LEU2*	This work

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Table 3: Continued

Y294_*CEL3A_SNC1_SSO2_SEC9*	*ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-his3/HIS3-PGK1p-SNC1-PGK1t–kanMX-PGK1p-SSO2-PGK1t-Shble-PGK1p-SEC9-PGK1t-hph-fur1::LEU2*	This work
Y294_*CEL3A_SNC2_SSO2_SEC9*	*ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-his3/HIS3-PGK1p-SNC2-PGK1t–kanMX-PGK1p-SSO2-PGK1t-Shble-PGK1p-SEC9-PGK1t-hph-fur1::LEU2*	This work
Y294_*CEL3A_SNC2_SSO1_SEC9*	*ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-his3/HIS3-PGK1p-SNC2-PGK1t–kanMX-PGK1p-SSO1-PGK1t-Shble- PGK1p-SEC9-PGK1t-hph-fur1::LEU2*	This work
S. cerevisiae Y294 (*CEL7A* Parental)	*ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3-fur1::LEU2*	Ilmén *et al.* (2011)**
Y294_*CEL7A_SNC1*	*ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3-PGK1p-SNC1-PGK1t – kanMX-fur1::LEU2*	This work
Y294_*CEL7A_SNC2*	*ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3-PGK1p-SNC2-PGK1t – kanMX-fur1::LEU2*	This work
Y294_*CEL7A_SSO1*	*ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3-PGK1p-SSO1-PGK1t-kanMX-fur1::LEU2*	This work
Y294_*CEL7A_SSO2*	*ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3-PGK1p-SSO2-PGK1t-kanMX-fur1::LEU2*	This work
Y294_*CEL7A_SEC9*	*ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3-PGK1p-SEC9-PGK1t-kanMX-fur1::LEU2*	This work
Y294_*CEL7A_SNC1_SSO1*	*ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3- PGK1p-SNC1-PGK1t-kanMX-PGK1p-SSO1-PGK1t-Shble-fur1::LEU2*	This work
Y294_*CEL7A_SNC1_SSO2*	*ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3- PGK1p-SNC1-PGK1t–kanMX-PGK1p-SSO2-PGK1t-Shble-fur1::LEU2*	This work
Y294_*CEL7A_SNC2_SSO2*	*ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3- PGK1p-SNC2-PGK1t–kanMX-PGK1p-SSO2-PGK1t-Shble-fur1::LEU2*	This work
Y294_*CEL7A_SNC2_SSO1*	*ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3- PGK1p-SNC2-PGK1t–kanMX-PGK1p-SSO1-PGK1t-Shble-fur1::LEU2*	This work
Y294_*CEL7A_SNC1_SSO1_SEC9*	*ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3- PGK1p-SNC1-PGK1t-kanMX-PGK1p-SSO1-PGK1t-Shble- PGK1p-SEC9-PGK1t-hph-fur1::LEU2*	This work

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Table 3: Continued

Y294_*CEL7A_SNC1_SSO2_SEC9*	*ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3- PGK1p-SNC1-PGK1t–kanMX-PGK1p-SSO2-PGK1t-Shble- PGK1p-SEC9-PGK1t-hph-fur1::LEU2*	This work
Y294_*CEL7A_SNC2_SSO2_SEC9*	*ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3- PGK1p-SNC2-PGK1t–kanMX-PGK1p-SSO2-PGK1t-Shble- PGK1p-SEC9-PGK1t-hph-fur1::LEU2*	This work
Y294_*CEL7A_SNC2_SSO1_SEC9*	*ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3- PGK1p-SNC2-PGK1t–kanMX-PGK1p-SSO1-PGK1t-Shble- PGK1p-SEC9-PGK1t-hph-fur1::LEU2*	This work

* Accession nr for*Sf*-Cel3A: AEV40916.1

** Accession nr for *Te*-Cel7A: AAL89553

Enzyme assays

Yeast strains were inoculated in triplicate at an A_{600nm} of 1 into 20 ml YPD in 125 ml Erlenmeyer flasks and cultivated for 72 hours, for each of the two extracellular enzyme activity assays (*Sf*-Cel3A and *Te*-Cel7A). To evaluate the β-glucosidase secretion capabilities of the recombinant strains, enzyme assays (Den Haan *et al.,* 2007) were performed in triplicate at 24 hour intervals on the extracellular cell fractions of each of the *S. cerevisiae* Y294 (*CEL3A*) strains, overexpressing differential combinations of the exocytic SNARE genes. Assays were carried out using *p*NPG (*p*-nitrophenyl-α-D-glucopyranoside (Sigma – St. Louis, USA)) as substrate, with reaction times of 5 minutes at 50˚C. The extracellular cellobiohydrolase activity of the recombinant Y294 (*CEL7A*) strains was evaluated at 24-hour intervals according to an adapted method described by La Grange *et al.* (2001), using *p*NPC (*p*-nitrophenyl-β-D-cellobioside (Sigma – St. Louis, USA)) as substrate, with reactions carried out for 90-105 minutes at 50˚C. All spectrophotometric readings for both of the assays were taken using the Biorad xMark™ Microplate Spectrophotometer at A_{400nm} and a reference strain negative control (containing a vector with no cellulase encoding gene) and media-blanks were included to normalize activity readings.

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Growth analysis

Strains were inoculated in triplicate at a starting A_{600nm} of 1 into 20 ml YP (Yeast Extract Peptone - 1% yeast extract (Merck – Darmstadt, Germany), 2% peptone (Merck – Darmstadt, Germany)), medium supplemented with 2% glucose (Merck - Darmstadt, Germany), added after autoclaving, in 125 ml Erlenmeyer flasks. These flasks were incubated on a rotary shaker (200 rpm) at 30˚C for the duration of the analysis. Samples were diluted (1:10), after which A_{600} readings were taken using the Biorad xMark™ Microplate Spectrophotometer and the data normalized. Samples were taken every 3 hours for the first 32 hours, after which samples were taken at 4 hour intervals up and till 48 hours, with the final samples being taken after 56 hours, when growth had either ceased or strains had reached stationary phase.

SDS-PAGE, *N*-deglycosylation and densitometry

Strains were inoculated at an initial A_{600nm} of 1 into 20 ml double-strength buffered SC^{-ura} medium in 125 ml Erlenmeyer flasks and cultivated for 72 hours on a rotary shaker (200 rpm) at 30˚C. Extracellular protein fractions (18 µl) were analysed using a 10% SDS-PAGE gel, according to the method described by Laemmli (1970). The separated proteins were visualized using silver staining (Kroukamp *et al.,* 2013) whilst the deglycosylated protein samples were prepared using the Endo H (New England BioLabs – Hertfordshire, UK) enzyme as instructed by the manufacturer. Densitometric analysis was performed using the ImageJ software (http://rsbweb.nih.gov/ij/) on the silver stained gels, according to the manufacturer's instructions. Protein concentrations were determined using a standard curve attained from values using pure BSA standards (5-100 ng) from the BCA protein assay kit (Pierce – Rockford, USA).

Quantification of gene copy number using quantitative PCR

Real-time quantitative PCR was used to enumerate the respective antibiotic selection markers that had been used to facilitate gene integrations, allowing us to elucidate the copy number of each of the integrated SNARE genes of interest. Two reference genes, *ALG9* and *TFC1,* were selected to normalize the copy numbers of our genes of interest, as they are only represented by single copies in the *S. cerevisiae* genome (Teste *et al.,* 2009). All DNA concentration measurements were carried out using the ND-1000 Spectrophotometer (Thermo Scientific – Waltham, USA). Real-Time quantitative PCR was carried out using the KAPA™ HRM Fast PCR Kit on an Applied Biosystems StepOne Real-Time PCR System, whilst quantifications of gene copy number were carried out using the relative standard

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curve method (Applied Biosystems: Guide to Performing Quantitation of Gene Expression Using RT qPCR; 2008). The copy numbers of the overexpressed SNARE ORFs (in addition to the native copy) were determined relative to the *ALG9* and *TFC1* reference genes (**Table 4**).

Table 4: The quantification of additional SNARE ORFs integrated into the respective recombinant strains via delta integration. Rounded numbers are indicated in brackets. Standard deviations were < 5%.

STRAIN	Copy number				
	SNC1	***SNC2***	***SSO1***	***SSO2***	***SEC9***
CEL3A_*SNC1*	1.87 (2)	n/a	n/a	n/a	n/a
CEL3A_*SNC2*	n/a	0.89 (1)	n/a	n/a	n/a
CEL3A_*SSO1*	n/a	n/a	0.61 (1)	n/a	n/a
CEL3A_*SSO2*	n/a	n/a	n/a	0.94 (1)	n/a
CEL3A_*SEC9*	n/a	n/a	n/a	n/a	0.98 (1)
CEL3A_S1S1	2.09 (2)	n/a	1.02 (1)	n/a	n/a
CEL3A_S1S2	2.38 (2)	n/a	n/a	0.99 (1)	n/a
CEL3A_S2S2	n/a	1.05 (1)	n/a	1.04(1)	n/a

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Table 4: Continued

STRAIN	Copy number				
	SNC1	***SNC2***	***SSO1***	***SSO2***	***SEC9***
CEL3A_S2S1	n/a	1.47 (1)	0.94 (1)	n/a	n/a
CEL3A_S1S19	1.57 (2)	n/a	1.11 (1)	n/a	1.48 (1)
CEL3A_S1S29	1.85 (2)	n/a	n/a	1.35 (1)	1.12 (1)
CEL3A_S2S29	n/a	0.98 (1)	n/a	1.15 (1)	0.92 (1)
CEL3A_S2S19	n/a	0.99 (1)	1.18 (1)	n/a	1.09 (1)
CEL7A_*SNC1*	0.75 (1)	n/a	n/a	n/a	n/a
CEL7A_*SNC2*	n/a	1.01 (1)	n/a	n/a	n/a
CEL7A_*SSO1*	n/a	n/a	1.25 (1)	n/a	n/a
CEL7A_*SSO2*	n/a	n/a	n/a	5.05 (5)	n/a
CEL7A_*SEC9*	n/a	n/a	n/a	n/a	0.87 (1)
CEL7A_S1S1	1.18 (1)	n/a	0.87 (1)	n/a	n/a
CEL7A_S1S2	1.11 (1)	n/a	n/a	0.95 (1)	n/a
CEL7A_S2S2	n/a	0.95 (1)	n/a	0.79 (1)	n/a
CEL7A_S2S1	n/a	0.95 (1)	0.91 (1)	n/a	n/a
CEL7A_S1S19	1.35 (1)	n/a	1.05 (1)	n/a	1.05 (1)
CEL7A_S1S29	0.61 (1)	n/a	n/a	0.65 (1)	2.15 (2)
CEL7A_S2S29	n/a	0.96 (1)	n/a	0.88 (1)	0.76 (1)
CEL7A_S2S19	n/a	0.99 (1)	1.33 (1)	n/a	0.99 (1)

Evaluation of osmotic and ethanol tolerance

Strains were cultivated in 5 ml YPD tubes, inoculated from plate-grown cultures, on rotation (200 rpm) for 48 hours. These cultures were each diluted to an A_{600} of 0.5 and subsequent dilutions of 1:10 of the before-mentioned were also prepared. Both the initial dilution and the 1:10 serially diluted samples were then plated out using a cryo-replicator press (Duetz-System) on YPD agar plates containing 0.0 M, 0.4 M, 0.6 M or 0.8 M NaCl and incubated at 30˚C for 72 hours. The same general procedure was carried out using YPD agar plates containing 2%, 5% or 10% ethanol, with cultures being incubated for 72 hours at 30˚C.

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Results

Construction of recombinant strains and enzyme assays

A large ensemble of recombinant strains overexpressing the Golgi-to-plasma membrane SNAREs were created and screened for enhanced secretion of the *T. emersonii* Cel7A and *S. fibuligera* Cel3A. Preliminary screening was carried out on at least 20 colonies per recombinant strain, with the strain illustrating the highest extracellular enzyme activity selected for further study. All transformants were first confirmed using PCR prior to assays, ensuring that all recombinants had integrated at least one additional SNARE gene of interest per transformation. Colonies were inoculated and cultivated for 72 hours, after which they were normalized with regard to their A_{600} and evaluated for their secretory activity. This effectively helped us to assemble a range of recombinant strains that were, relative to the range of transformants assayed for overexpression, superior in their extracellular enzyme activity for *Sf*-Cel3A and *Te*-Cel7A. The total collection of strains is summarized in **Table 3**.

Quantitative-PCR investigation illustrated that the majority of selected transformations had only integrated a single additional gene copy (**Table 4**), with a few exceptions. The varying reaction efficiencies from triplicate measurements meant that slight deviations from absolute values were expected, although these were uncommon in the majority of strains tested. It is important to note that the selection process, selecting only the highest secreting transformants, could have excluded a number of strains that integrated a higher number of gene copies, which eventually had a derogatory effect on the secretion of the particular cellulolytic reporter protein.

The maximal increase in extracellular *Sf*-Cel3A activity, facilitated through single overexpression, was achieved following the overexpression of *SSO1,* yielding an improvement of 1.43-fold (43.8%) following the integration of a single additional gene copy. Furthermore, the overexpression of *SNC1* (20%) (two additional gene copies) and *SEC9* (22%) (one additional gene copy) also yielded positive results for this particular heterologous cellulase (**Fig. 2A**). In terms of improvements facilitated through simultaneous overexpressions, a maximal increase of 1.48-fold (48.8 %), relative to the parental strain, was achieved in a strain simultaneously overexpressing Snc1p, Sso1p and Sec9p (**Fig. 2B**), with each of these ORFs being overexpressed as a single additional gene copy (**Table 4**). The two strains overproducing Snc2p and Sso2p, respectively, illustrated a decrease in extracellular *Sf*-Cel3A activity, whilst quantitative qPCR (**Table 4**) revealed that each of these two strains only had one additional gene copy.

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A maximal increase in extracellular *Te*-Cel7A activity of approximately 1.71-fold (71%), relative to the parental strain, was achieved in the strain overexpressing the *SNC1* ORF, whilst a 1.46-Fold (46%) increase was achieved in a strain overexpressing the other *SNC2* v-SNARE (**Fig. 3A**). The strains overexpressing the latter two genes had integrated one and two additional gene copies, respectively (**Table 4**). Furthermore, an average increase in extracellular *Te*-Cel7A activity of around 33% was achieved in strains overproducing the t-SNARE components Sso1p, Sso2p and Sec9p, singularly, with these strains having integrated 1, 5 and 1 additional gene copies, respectively (**Table 4**). Improvements in extracellular *Te*-Cel7A activity were also achieved when overproduction of multiple exocytic SNARE subunits had been facilitated, yielding a maximal increase of approximately 1.52-fold (52%) in a strain overexpressing *SNC1* and *SSO2* simultaneously, both present as a single additional gene copy, with an increase of around 30 % prevalent for the rest of the simultaneously overexpressing strains (**Fig. 3B**).

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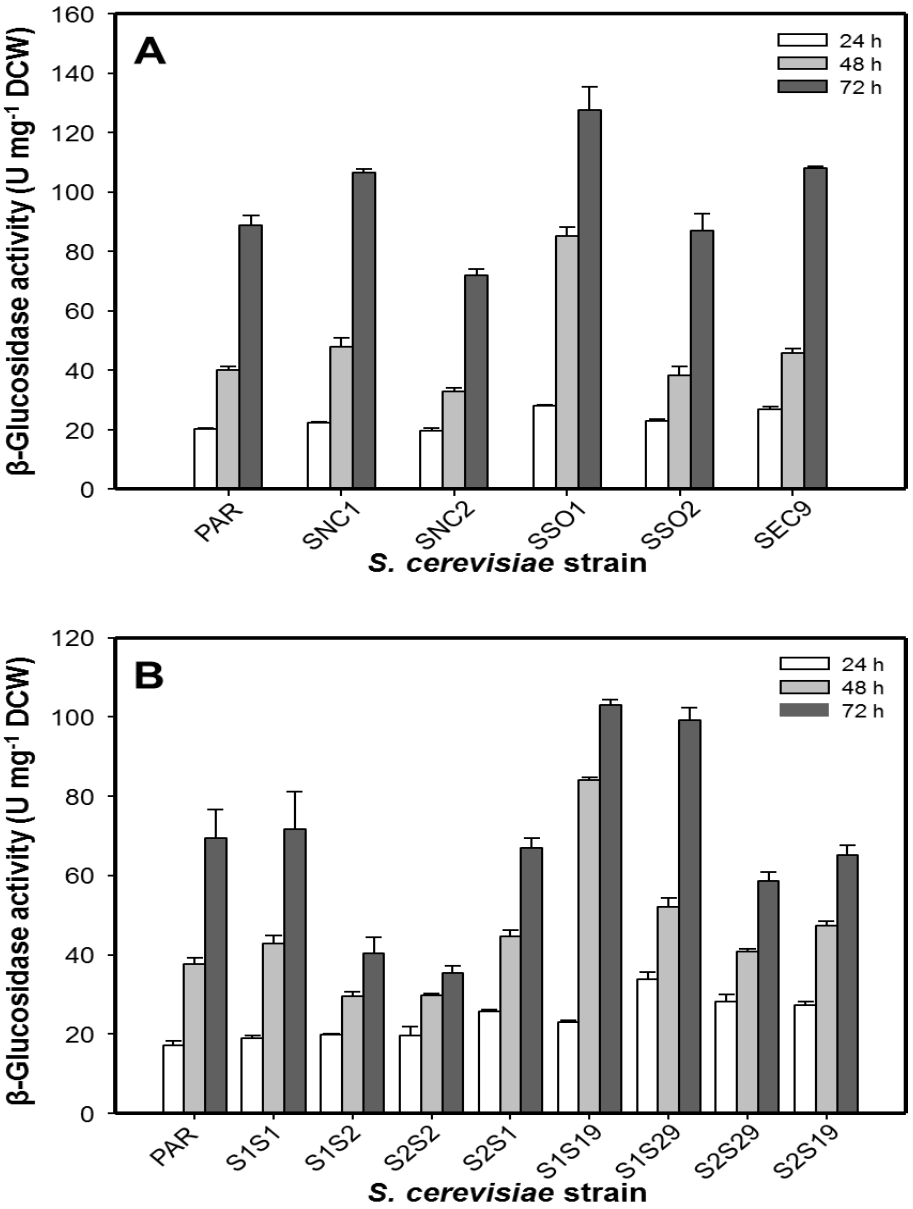

Fig. 2A & B:

Fig. 2: (A) The supernatant enzyme activities of the *S. cerevisiae* Y294 strains harbouring the ySFI episomal plasmid expressing the *Sf*-Cel3A, with each overexpressing one of the 5 respective exocytic SNARE proteins functioning at the plasma membrane interface, alongside the parental strain (PAR) at 24 hour intervals. All values represent mean values of assays done in triplicate with error bars indicating the standard deviation. **(B)** The supernatant enzyme activities of the *S. cerevisiae* Y294 strains harbouring the ySFI episomal plasmid expressing the *Sf*-Cel3A, with each overproducing differential combinations of the 5 respective SNARE proteins, alongside the parental strain (PAR). The first "S" in "S1S19" to "S2S29" indicates either *SNC1/SNC2,* depending on the additional numeral, whilst the second "S" represents either *SSO1/SSO2* using the same convention, with "9" indicating *SEC9*. All values represent mean values of assays done in triplicate with error bars indicating the standard deviation.

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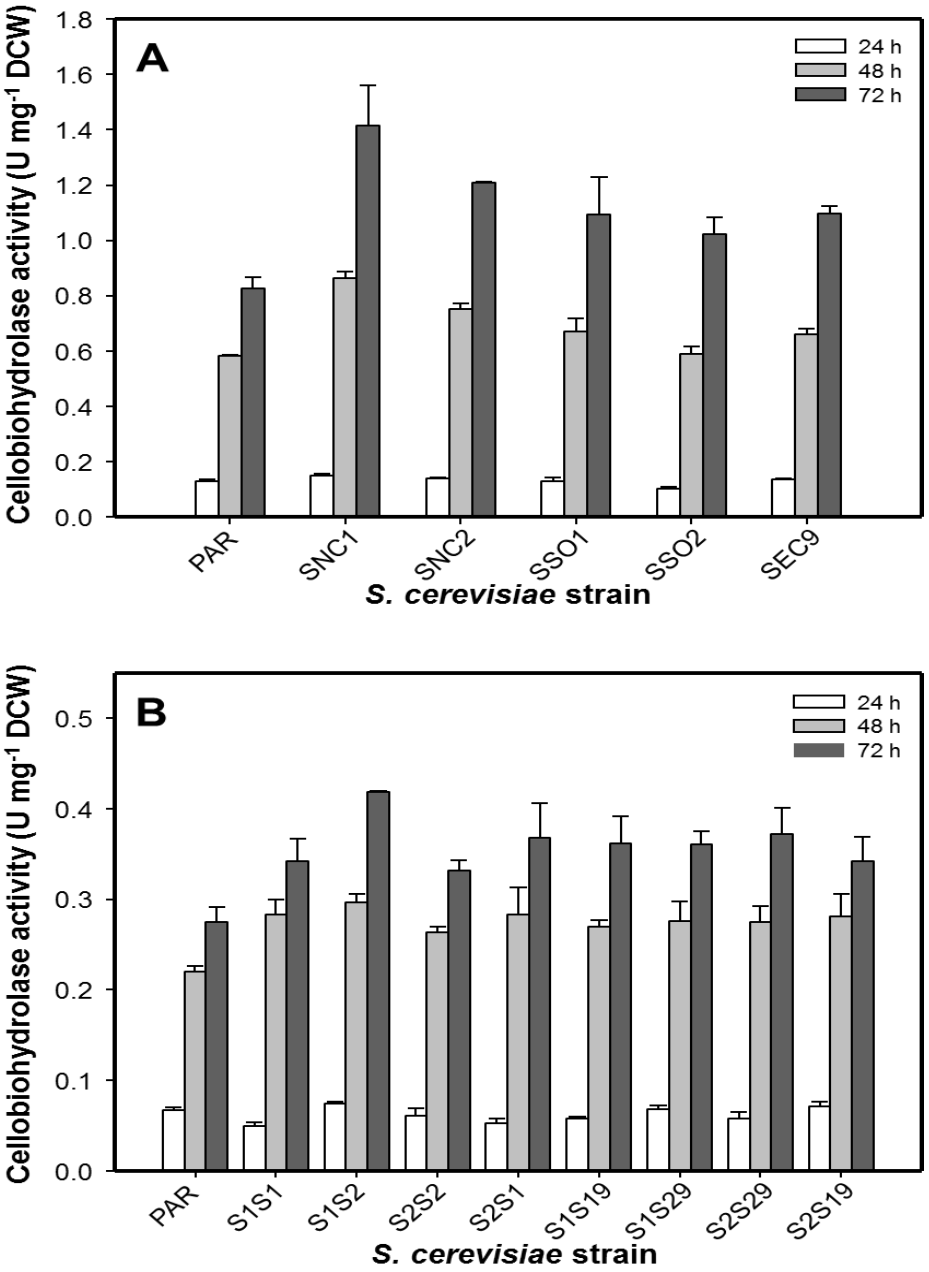

Fig. 3A & B:

Fig. 3: (A) The supernatant enzyme activities of the recombinant *S. cerevisiae* Y294 strains harbouring the pMI 529 episomal plasmid expressing the *Te*-Cel7A, with each overproducing one of the 5 respective SNARE proteins functioning at the plasma membrane interface, alongside the parental strain (PAR) at 24 hour intervals. All values represent mean values of assays done in triplicate with error bars indicating the standard deviation. **(B)** The supernatant enzyme activities of the recombinant *S. cerevisiae* Y294 strains harbouring the pMI529 episomal plasmid expressing the *Te*-Cel7A, with each overexpressing differential combinations of the 5 respective SNARE proteins, alongside the parental strain (PAR). The first "S" in "S1S19" to "S2S29" indicates either *SNC1/SNC2,* depending on the additional numeral, whilst the second "S" represents either *SSO1/SSO2*

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using the same convention, and "9" indicating *SEC9*. All values represent mean values of assays done in triplicate with error bars indicating the standard deviation.

SDS-PAGE analysis of secreted *Te*-Cel7A

A representative collection of strains were chosen to determine whether the increased extracellular activity could be shown as an increase in observed extracellular *Te*-Cel7A (~66kDa in deglycosylated form) protein on a 10% polyacrylamide gel. **Fig. 4** shows an increase in band intensity for the deglycosylated (D) samples of the strains overexpressing *SSO1*, *SNC2* and *SSO1* simultaneously (S2S1) and the strain simultaneously overexpressing *SNC2, SSO1* and *SEC9* (S2S19), compared to the parental (PAR) and reference strain (REF) (not expressing the heterologous protein). As all samples were normalized according to their respective optical densities, this confirmed that the amount of secreted *Te*-Cel7A successfully increased relative to the parental strain, which correlated well with the measured increases in extracellular enzyme activity illustrated (**Figs. 3A & B**). Densitometry analysis of the SDS-PAGE showed increases in the observed protein levels for the SNARE-overexpressing strains that concurred with the increased levels of CBH1 activity observed.

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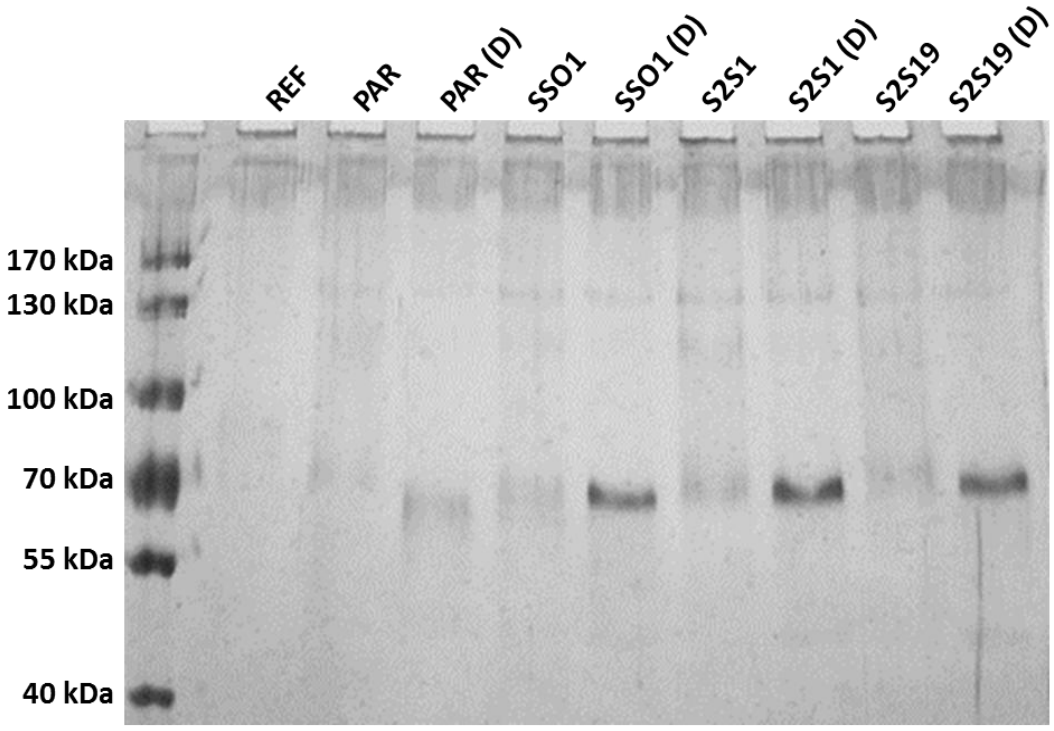

Fig. 4: Silver stained 10% SDS-PAGE gel illustrating the quantitative differences in *Te*-Cel7A secretory titers between the selected strains. The "D" indicates the deglycosylated samples. Other annotations used are as given for **Fig. 2 & Fig. 3**.

Growth analysis, osmotic- and ethanol tolerance of recombinant strains

The growth capabilities of a selection of strains, including the parental strains (Y294_*CEL7A* and Y294_*CEL3A*), the single SNARE-overexpression recombinants of the before-mentioned strains and a simultaneously overexpressing strain of each were investigated over a period of 56 hours (**Figs. 5A & B**). The two strains illustrating the greatest improvement in *Sf*-Cel3A secretion, overexpressing *SSO1* and simultaneously overexpressing *SNC1, SSO1* and *SEC9,* diminished in growth at around 29 hours (**Fig. 5A**). No significant differences in growth capability were detected for any of the SNARE-overproducing strains expressing the *Te*-Cel7A, relative to the parental strain (CBH1) (**Fig. 5B**).

The recombinant yeasts' tolerance to increasing levels of osmotic stress was determined, in order to establish whether the overexpression of SNARE proteins can led to derogatory effects on the yeast's basal growth capability. There was a slight decrease in osmotic tolerance in the Y294 (*CEL3A*) strains overproducing three of the SNARE subunits simultaneously (**Fig. 6A**). However, when these concentrations were increased to 1.2 M, none of the strains were able to grow (data not shown). The

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ethanol tolerance of the SNARE-overexpressing strains yielded similar results to that of the osmotic tolerance experiments (**Fig. 6B**), with the *Sf*-Cel3A harbouring strains overexpressing three of the exocytic SNARE subunits simultaneously, illustrating a clear decrease in growth competence at 5% ethanol. None of the strains were able to tolerate 10% ethanol (data not shown).

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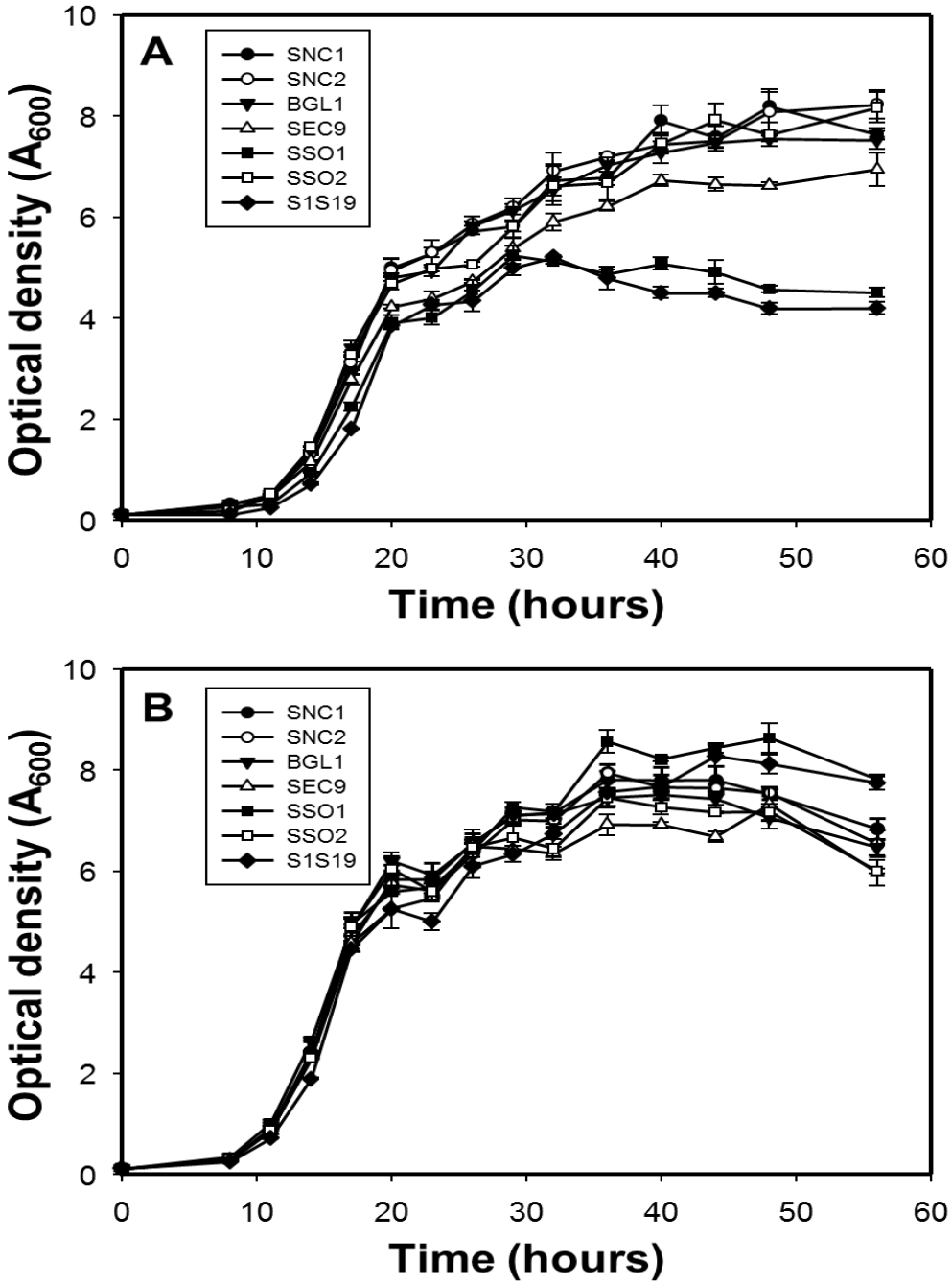

Fig. 5A & B:

Fig. 5: (A) Growth trends of the parental (BGL/Cel3A) and recombinant *S. cerevisiae* Y294 (*CEL3A*) strains, overexpressing Snc1p, Snc2p, Sso1p, Sso2p, Sec9p and the most superior collectively overexpressing strain (S1S19: overexpressing Snc1p, Sso1p and Sec9p) over a period of 56 hours. Values represent the mean of the triplicate cultures tested and the error bars represent the standard deviation. **(B)** Growth trends of the parental (CBH1/Cel7A) and recombinant *S. cerevisiae* Y294 (*CEL7A*) strains, overexpressing Snc1p, Snc2p, Sso1p, Sso2p, Sec9p and the most superior collectively overexpressing strain S1S29 (overproducing Snc1p, Sso2p and Sec9p) over a period of 56 hours. Values represent the mean of triplicate cultures tested and the error bars represent the standard deviation.

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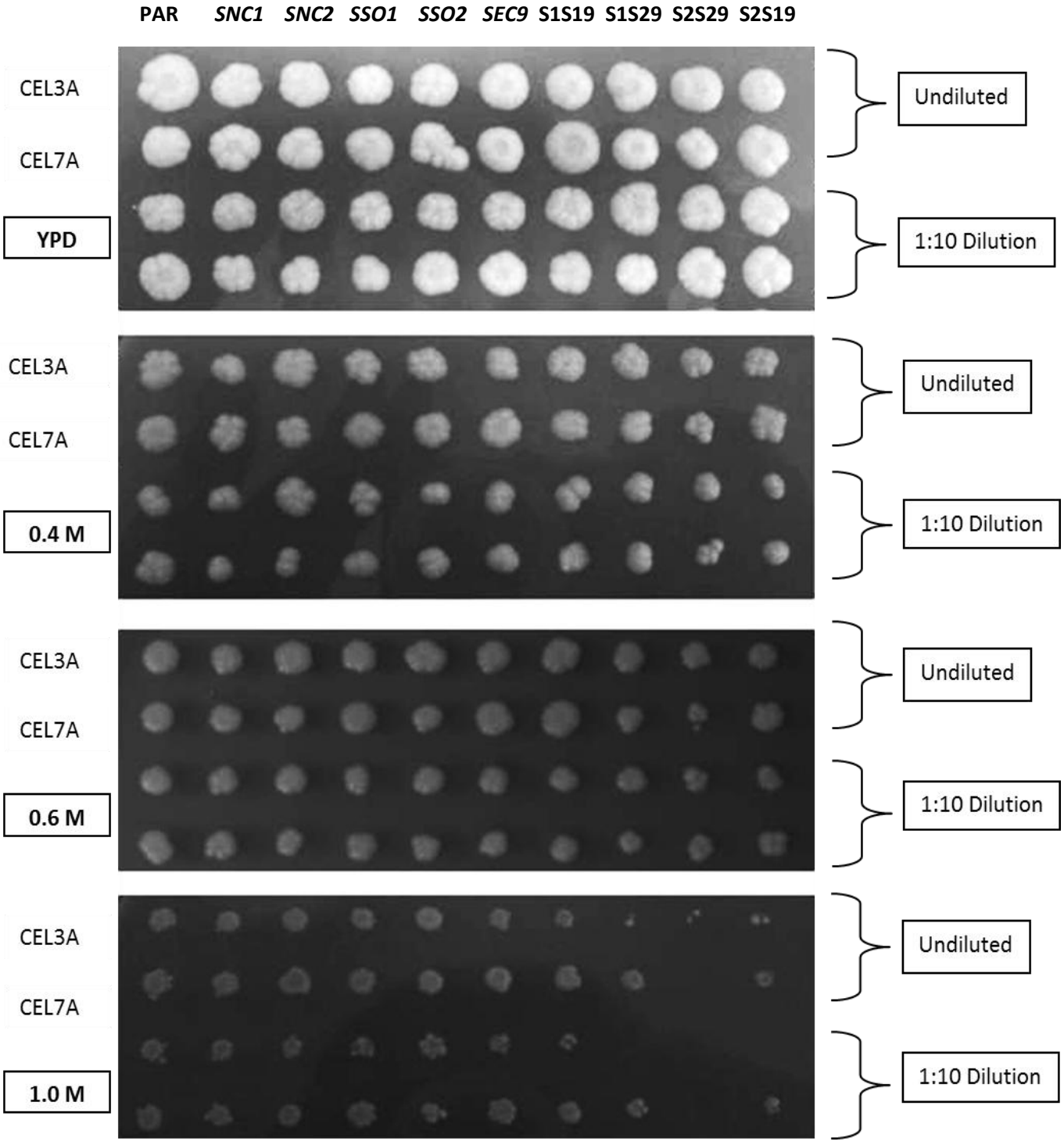

Fig. 6A:

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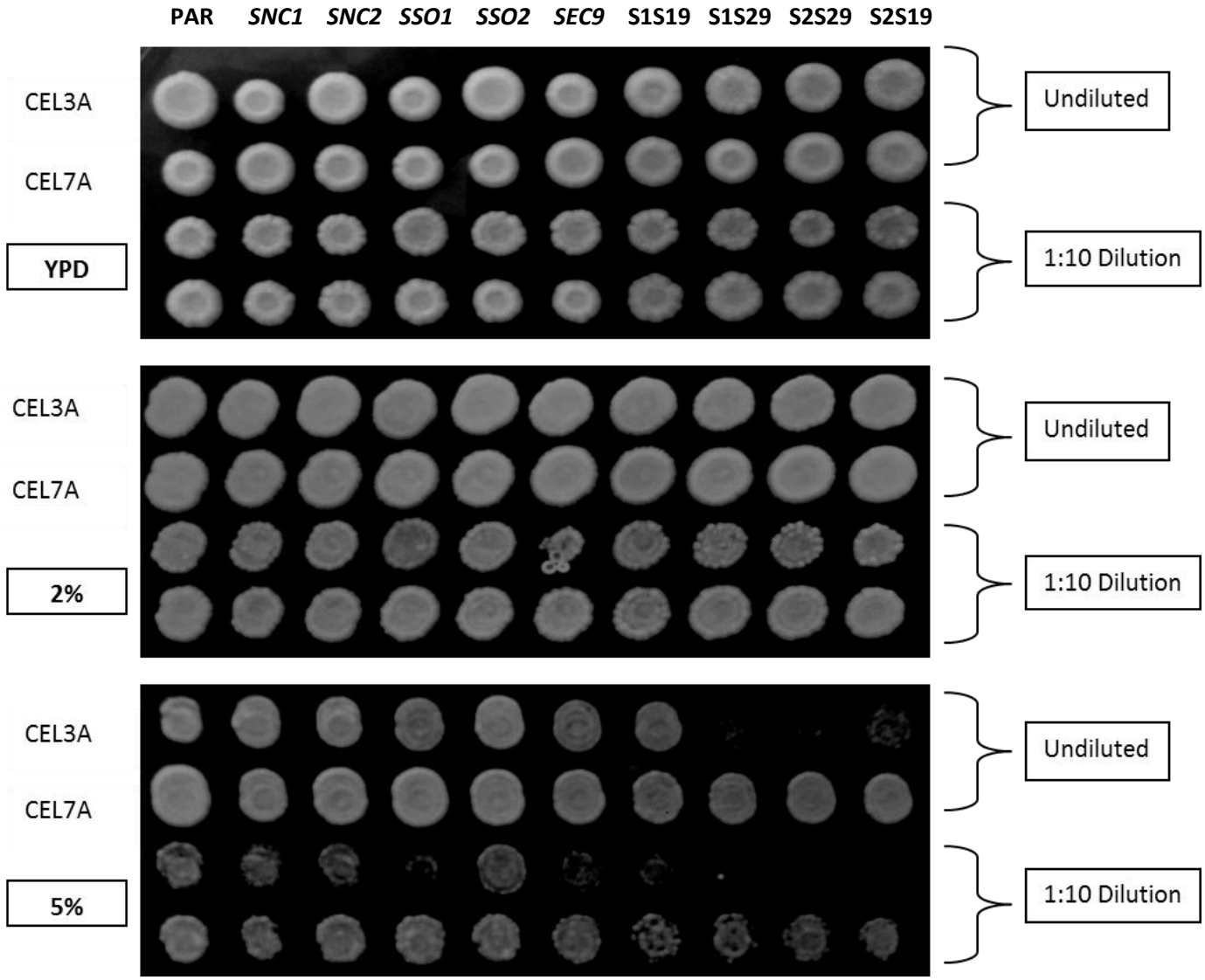

Fig. 6B:

Fig. 6: (A) A representation of the osmotic tolerance of the differential recombinant strains on YPD agar supplemented with increasing concentrations of NaCl (0.4M, 0.6M and 1M), with each set expressing a different cellulolytic reporter protein (*Sf*-Cel3A or *Te*-Cel7A). The recombinant strains are either singly or simultaneously expressing the differential components of the exocytic SNARE complex. **(B)** A representation of the ethanol tolerance of the differential recombinant strains on YPD agar supplemented with increasing concentrations of ethanol (2% and 5%), with each set expressing a different cellulolytic reporter protein (*Sf*-Cel3A or *Te*-Cel7A). The recombinant strains are either singly or simultaneously expressing the differential components of the exocytic SNARE complex.

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Discussion

In this study, attempts were made to increase the protein secretion capacity of *S. cerevisiae* through the single and simultaneous overexpression of the exocytic SNARE genes. The specific SNARE overexpressions that were facilitated had a more pronounced effect on the secretion of *Te*-Cel7A, with the scope for improvement for this enzyme being proportionately larger than for that of *Sf*-Cel3A, as the expression of cellobiohydrolases in *S. cerevisiae* has consistently proved problematic (Den Haan *et al.,* 2013). Improvements were distinctly protein-specific, with clear distinctions in secretory improvements for *Sf*-Cel3A and *Te*-Cel7A. Overexpression of *SSO1* had the most pronounced effect on the secretion of *Sf*-Cel3A, whilst *SNC1* yielded the most notable increases in *Te*-Cel7A secretion. Increases in extracellular protein activity also correlated with increases in band intensities, when recombinant extracellular protein samples were analyzed using SDS-PAGE and densitometry, confirming that an increase in the secretory reaction had been facilitated.

The simultaneously overexpressing strains, secreting either *Sf*-Cel3A or *Te*-Cel7A, generally also illustrated the most positive results whenever the before-mentioned SNAREs (*SSO1* and *SNC1*) were included in the repertoire of overproduced proteins, suggesting that of the two pairs of ohnologs investigated, these had the most marked effect on protein secretion. Some of the strains expressing *Sf*-Cel3A illustrated a decrease in their secretory capabilities, a phenomenon that isn't unique in SNARE overexpression studies in *S. cerevisiae* (Weinberger *et al.,* 2005*).* This could potentially be attributed to the possible titration of some of the interacting subunits, as an increase in one might render an interacting protein inaccessible to perform its function or prevent it from being recycled for recurring fusion reactions, which could lead to a decrease in efficiency of the overall secretory reaction. Most SNARE proteins are apparently present in excess and can concentrate as clusters, in doing so constituting a spare pool that may not be readily available for protein interactions (Di Sansebastiano, 2013), particularly at overexpressed levels.

Saccharomyces cerevisiae gene pairs (ohnologs) have been attributed to genome duplications (Byrne & Wolfe, 2005) and there seem to be differential effects on protein secretion brought about by overproduction of some of these SNARE gene pair components. The improvement in extracellular *Sf*-Cel3A activity varied between the Sso1/Sso2p paralogs, with an increase of 1.48-fold (48.3%) following Sso1p overproduction being contrasted by virtually no improvement following Sso2p overproduction, even though they were both present as a single additional gene copy (**Table 4**).

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Likewise, the overproduction of the Snc1/Snc2p paralogs maintained the same trend, with Snc1p overproduction leading to an increase of 20%, whilst its Snc2p paralog actually led to a decrease in extracellular *Sf*-Cel3A activity. The differences in secretory improvements for these paralogs were also mirrored in the strains producing the *Te-C*el7A, with Snc1p (71%) having a more pronounced effect than Snc2p (46%) at overproduced titers, even though the latter gene had an additional integrated gene copy (**Table 4**). These results, along with additive differences when these paralogs are differentially expressed together with other SNARE subunits, suggests that there is a distinction with regard to the utilization of these proteins for the secretory reactions of different heterologous cellulases, or that the effect of these overexpressions varies between each of the paralogs in the pair. This is well illustrated by the decrease in *Sf*-Cel3A secretory titers attained when Snc2p or Sso2p were overproduced in combination with the other exocytic SNARE ORFs (**Fig. 2B**), with a diminished effect being prevalent whenever Snc2p or Sso2p are added to the overproduced repertoire.

Our results also indicated that an increase in the copy number of a specific SNARE gene being overexpressed did not necessarily equate to a proportionate increase in extracellular protein concentration, as some of the most improved strains had only one additional gene copy (**Table 4**). This suggests that a fine balance is required with regard to secretory improvement and SNARE gene copy number and that the position of genomic integration could be another factor ultimately influencing the secretory phenotype. According to the data we have obtained, optimal improvements in secretion can often be brought about through the single integration of a particular exocytic SNARE gene. It is possible that strains that had potentially integrated more copies of the respective SNARE ORFs were not selected for further study during our initial strain construction and screening. This means that the SNARE-overexpressing strains that illustrated the highest extracellular cellulase activity (and subsequently selected for further study) would already have been partially optimized with regard to gene copy number.

The expression of heterologous proteins in *S. cerevisiae* can impose metabolic burdens on the cell and the associated decrease in specific growth rate of the yeast culture could therefore render the overall biotechnological process non-beneficial (Ostergaard *et al.,* 2000, Van Rensburg *et al.,* 2012). The majority of overexpressions had no significant deleterious effects on the growth capability of the yeast. However, the two most improved *Sf*-Cel3A expressing strains, overexpressing the *SSO1* t-SNARE component and the strain overexpressing the *SNC1, SSO1* and *SEC9* components simultaneously, illustrated a notable decrease in growth rate relative to the parental strain. The

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diminished growth rate only became prevalent after the cultures had entered the diauxic shift after 20 hours, when the yeast switches from the metabolism of glucose via glycolysis to the aerobic utilization of ethanol (Galdieri *et al.,* 2010). This seems to suggest that, at the significantly elevated titers of *Sf*-Cel3A that are being secreted in the before-mentioned strains, their ability to tolerate or utilize ethanol may have been affected, an observation that correlates well with our inhibitor tolerance data (**Fig. 6A & B**).

There was a notable correlation between the biomass of a yeast culture and its ethanol yield (Nigam *et al.,* 1985). The simultaneously overexpressing strains expressing the *Sf*-Cel3A illustrated a clear decrease in growth capability at increased ethanol concentrations and at increased osmotic stress levels (**Figs. 6A & 6B**). Since the overproduced exocytic SNARE proteins are directly or indirectly interacting with the plasma membrane and, given their relative abundance without an associated increase in the accessory proteins (Sec17p and Sec18p) required to disassemble and reactivate them (Jüschke *et al.,* 2005), it may explain compromises in osmotic and ethanol tolerance. The higher secretory titers (up to 127 U mg^{-1}) and the increased levels of possible glycosylation sites that the *Sf*-Cel3A possesses, determined using the NetNGlyc 1.0 software (http://www.cbs.dtu.dk/services/NetNGlyc/), might place a large enough burden on the cell, particularly the plasma membrane, to effectively interfere with its ability to tolerate increased (~1 M) NaCl and increased (~5%) ethanol concentrations.

Our results suggest that a more pronounced effect on the inhibitor tolerance can be brought about by simultaneous overexpression of exocytic SNAREs depending on the particular heterologous cellulase being secreted. The interaction between intracellular networks of proteins and metabolites are intricate and often very complex (Yoshikawa *et al.,* 2011), making it difficult to predict the cellular response to genetic perturbations of its basal gene expression levels. Often, the cellular system will illustrate fragility with regard to genetic perturbations (Krantz *et al.,* 2009). For example, the adjustment of gene expression levels is often amplified throughout the entire system, which can lead to detectable growth defects, a phenomenon we've observed in this work with regard to osmotic and ethanol tolerance in certain strains simultaneously overexpressing exocytic SNARE genes.

The findings of Ruohonen *et al.* (1997), Gasser *et al.* (2007), Hou *et al.* (2012) and Xu *et al.* (2013), together with the secretory improvements we have illustrated in *S. cerevisiae* for the exocytic SNAREs at overexpressed levels, demonstrate that invigorating the Golgi-to-plasma membrane

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protein trafficking steps, with particular emphasis on the respective SNARE components, is a novel and feasible approach for improving the heterologous protein secretion capacity of *S. cerevisiae.* We have illustrated, for the first time, that specific exocytic SNARE proteins can differentially increase the secretory capacity of *S. cerevisiae* for different heterologous cellulases, whilst simultaneous overexpression of certain exocytic SNARE proteins can, in specific combinations, also increase the efficiency of protein secretion for different cellulolytic reporter proteins.

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Conflict of interest statement:

The authors declare that there are no conflicts of interest associated with the submission of this study.

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Chapter 4

Overexpression of native *Saccharomyces cerevisiae* ER-to-Golgi SNARE genes increased heterologous cellulase secretion

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Abstract:

SNAREs (soluble N-ethylmaleimide-sensitive factor attachment receptor proteins) are essential components of the yeast protein trafficking machinery and are required at the majority of membrane fusion events in the cell, where they facilitate SNARE-mediated fusion between the protein transport vesicles, the various membrane-enclosed organelles and, ultimately, the plasma membrane. We have demonstrated an increase in secretory titers for the *Talaromyces emersonii* Cel7A (a cellobiohydrolase) and the *Saccharomycopsis fibuligera* Cel3A (a β-glucosidase) expressed in *Saccharomyces cerevisiae* through single and co-overexpression of some of the ER-to-Golgi SNAREs (*BOS1, BET1, SEC22* and *SED5*). Overexpression of *SED5* yielded the biggest improvements for both of the cellulolytic reporter proteins tested, with maximum increases in extracellular enzyme activity of 22% for the *Sf*-Cel3A and 68% for the *Te*-Cel7A. Co-overexpression of the ER-to-Golgi SNAREs yielded proportionately smaller increases for the *Te*-Cel7A (46%), with the *Sf*-Cel3A yielding no improvement. Co-overexpression of the most promising exocytic SNARE components identified in literature for secretory enhancement of the cellulolytic proteins tested (*SSO1* for *Sf*-Cel3A and *SNC1* for *Te*-Cel7A) with the most effective ER-to-Golgi SNARE components identified in this study (*SED5* for both *Sf*-Cel3A and *Te*-Cel7A) yielded variable results, with *Sf*-Cel3A improved by 131% and *Te*-Cel7A yielding no improvement. This study has added further credence to the notion that SNARE proteins fulfil an essential role within a larger cascade of secretory machinery components that could contribute significantly to future improvements to *Saccharomyces cerevisiae* as protein production host.

Keywords: SNAREs, cellulases, secretion, yeast

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Introduction:

Yeasts have been utilized successfully for the large-scale heterologous production of intracellular and extracellular mammalian and plant proteins since the early 1980s (Romanos *et al.,* 1992; Romanos, 1995). *Saccharomyces cerevisiae* and yeasts in general provide a relatively efficient means to modify and secrete heterologous proteins in a eukaryotic host, whilst several species have been engineered specifically as heterologous protein production hosts (Gellissen *et al.,* 1995; Piontek *et al.,* 1998; Idiris *et al.,* 2010). The production of bioethanol from lignocellulosic substrates using recombinant yeast strains is currently not financially viable as an alternative fuel source, when compared to fossil fuels produced at large scale in technologically mature refineries (Stephen *et al.,* 2012; Den Haan *et al.,* 2015). Two of the major price components contributing to its lack of financial feasibility include (1) the high production cost of the commercial cellulases required to release sugars from the substrate; and (2) the high energy cost of lignocellulose pre-treatment at high temperatures. The cost of enzymatic saccharification is regarded as the third most expensive price component in the production of lignocellulosic bioethanol (Pu *et al.,* 2008; Aden & Foust, 2009; Stephen *et al.,* 2012; Isola, 2013).

Several strategies have been employed to increase the production capacity for heterologous proteins in *S. cerevisiae* including: (1) the engineering of molecular chaperones and foldases (Hackel *et al.,* 2006; Carla Fama *et al.,* 2007; Hou *et al.,* 2012); (2) engineering of the peptide leader sequence (Kjaerulff & Jensen, 2005); (3) optimization of the gene copy number (Ilmén *et al.,* 2011); (4) manipulation of promoter strength (Alper *et al.,* 2005); (5) engineering of the heterologous protein of interest (Huang & Shusta, 2005; Kim *et al.,* 2006; Den Haan *et al.,* 2013a); and (6) optimization of the expression conditions (Wedekind *et al.,* 2006). However, the majority of improvements have been found to be protein specific (Kroukamp *et al.,* 2013; Van Zyl *et al.,* 2014).

Soluble NSF (N-ethylmaleimide-sensitive factor) attachment receptor proteins (SNAREs) are a class of type II membrane proteins with a C-terminal segment that serves as the membrane anchor and a short (≈ 70 amino acid) α-helical SNARE-motif, which distinguish different SNAREs from each other (Hong & Lev, 2014). SNAREs are required at the majority of membrane fusion events during intracellular transport, facilitating protein trafficking between the various membrane-enclosed organelles and the plasma membrane, whilst simultaneously contributing to the specificity and fidelity thereof (Weber *et al.,* 1998; Götte & Fisher von Mollard, 1998; Grote *et al.,* 2000; Malsam *et al.,* 2008; Kloepper *et al.,* 2008; Hou *et al.,* 2012) (Fig. 1). SNAREs can be divided into two classes

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depending on the localization of their fusion activity, with different trafficking steps generally employing different v-/t-SNARE combinations (Weber *et al.,* 1998; Hu *et al.,* 2003). SNAREs present on protein transport vesicles are broadly referred to as v-SNAREs whilst their cognate partners, located on the particular target membranes to which these v-SNAREs bind, are known as t-SNAREs (Weber *et al.,* 1998; McNew *et al.,* 2000). V- and t-SNAREs are able to interact in *trans,* leading to the formation of functional (fusogenic), membrane-specific *trans*-SNARE complexes, or SNAREpins, which bridge the respective membranes, bringing them into close proximity and into an energetically favourable state for fusion (Melia *et al.,* 2002; Pobbati *et al.,* 2006; Malsam & Söllner, 2011).

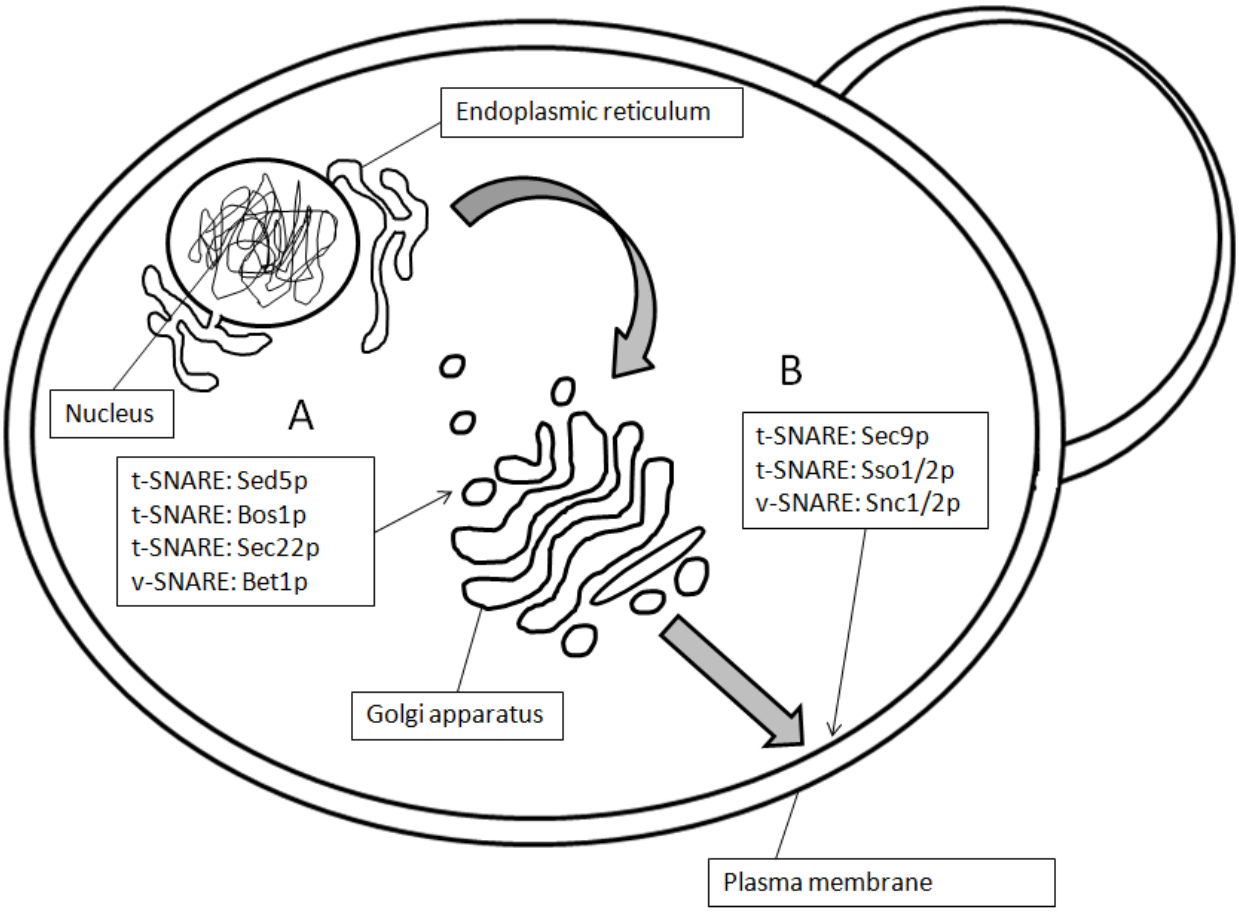

Fig. 1: A graphic illustration of the components of the anterograde secretory pathway with particular relevance to this study. **(A)** The collection of SNAREs facilitating fusion of the ER-derived secretory vesicles with the *cis*- Golgi. **(B)** The exocytic SNAREs facilitating fusion of the Golgi-derived secretory vesicles with the plasma membrane.

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Our previous findings (Van Zyl *et al.,* 2014) illustrated that the overexpression of exocytic SNAREs involved in the terminal step of the secretion pathway, namely fusion of the Golgi-derived secretory vesicles with the plasma-membrane, was able to increase secretion for two cellulolytic reporter proteins tested, namely *Talaromyces emersonii* Cel7A (*Te*-Cel7A) and *Saccharomycopsis fibuligera* Cel3A (*Sf*-Cel3A). With these results in mind, it was postulated that over-production of the SNARE components functioning earlier in the anterograde secretory pathway, facilitating fusion of the Endoplasmic Reticulum (ER)-derived secretory vesicles with the *cis*-face of the Golgi, could conceivably increase secretory titers for cellulolytic proteins even further. Although the conformational characteristics of the ER-to-Golgi SNARE complex have not been as well studied as its exocytic counterpart, it has nevertheless been described in sufficient detail so as to illuminate the components and their specific interactions leading to vesicle fusion at this particular membrane interface (Newman *et al.,* 1990; Dascher *et al.,* 1991; Hardwick & Pelham, 1992; Parlati *et al.,* 2000). Within the ER-to-Golgi subsection of the yeast secretion pathway, Sed5p, Sec22p, Bos1p and Bet1p fulfil roles of SNAREs facilitating the targeting and fusion of anterograde vesicles to the *cis*-Golgi (Sacher *et al.,* 1997; Ossipov *et al.,* 1999). The yeast ER-to-Golgi SNARE complex therefore consists of Bos1p, Sec22p and Sed5p, making up a functional t-SNARE sub-complex, and small quantities of the v-SNARE Bet1p (Søgaard *et al.,* 1994; Stone *et al.,* 1997; Parlati *et al.,* 2002). The t-SNARE Sed5p combines cooperatively with the two light chains, Bos1p and Sec22p, to form a functional t-SNARE ternary complex that is able to receive transport vesicles from the ER, vesicles harbouring the v-SNARE Bet1p (Parlati *et al.,* 2002).

In this study, we investigated for the first time whether overexpression of the ER-to-Golgi SNARE components, facilitating vesicle fusion with the *cis*-Golgi, could increase heterologous protein secretion, with specific cellulolytic reporter proteins (*Sf*-Cel3A and *Te*-Cel7A) selected for expression in *S. cerevisiae*. The ER-to-Golgi t-SNARE components Bos1p, Sec22p and Sed5p, in addition to the v-SNARE Bet1p were constitutively over-produced, singly and collectively, in recombinant *S. cerevisiae* strains and their secretory abilities evaluated both quantitatively and qualitatively. In addition, we explored whether particular combinations of the most effective exocytic and ER-to-Golgi SNARE components could improve the secretory titers of these cellulolytic reporter proteins when co-overexpressed, whilst investigating the effects of these gene overexpressions on the basal growth capability of the yeast.

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Materials and Methods

Media and culturing conditions

S. cerevisiae strain Y294 (MATα; *his3Δ; leu2Δ*; *lys2Δ*; *ura*3*Δ*) (ATCC 201160) was utilized as background strain. Yeast cells were routinely cultivated at 30˚C in YPD medium (Yeast Extract Peptone Dextrose) (1% yeast extract (Merck – Darmstadt, Germany), 2% peptone (Merck – Darmstadt, Germany), 2% glucose (Merck – Darmstadt, Germany)). All *S. cerevisiae* transformants were selected on YPD agar supplemented with 100-200 µg mL^{-1} of the appropriate antibiotic (G418 disulphate (Melford Laboratories – Ipswich, United Kingdom), hygromycin B (Calbiochem – San Diego, USA), clonNAT (Werner BioAgents – Jena, Germany) and zeocin (Melford – Ipswich, United Kingdom)), whilst liquid cultures were cultivated on a rotary shaker (200 rpm) at 30˚C. For protein studies yeast strains were grown on 2 x SC^{-ura} (double strength synthetic complete) medium (3.4 g L^{-1} yeast nitrogen base (Difco - Sparks, USA) with all amino acids except uracil, 2% succinate (Sigma – St. Louis, USA), 1% ammonium sulfate (Merck - Darmstadt, Germany) and 2% glucose (Merck - Darmstadt, Germany), pH adjusted to pH 6.0 using 10 N sodium hydroxide). *Escherichia coli* DH5α was used for general cloning procedures and transformed strains were routinely cultivated in Luria Bertani (LB) broth (0.5% yeast extract (Merck - Darmstadt, Germany); 1% tryptone (Merck - Darmstadt, Germany); 1% NaCl (Merck - Darmstadt, Germany) supplemented with 100 µg mL^{-1} ampicillin (Roche – Johannesburg, South Africa) at 37˚C.

Plasmid and Strain construction

Standard DNA manipulation protocols were followed (Sambrook & Russel, 2001). Initial PCR products were amplified using the Phusion® High-Fidelity DNA Polymerase (Thermo Scientific - Waltham, USA) on an Applied BioSystems 2720 Thermocycler (Life Technologies – California, USA) as instructed by the manufacturer, using forward and reverse primers that include *Pac*I and *Asc*I restriction sites for subsequent directional cloning into the pBKD1 (McBride *et al.,* 2008), pBHD1 (Kroukamp *et al.,* 2013), pBCD1 and pBZD1 (McBride *et al.,* 2008) yeast expression vectors. The pBCD1 vector was constructed by replacing the *TEF1*p-*Kan*MX-*TEF1*t selectable marker gene of pBKD1 with the *PFK2*p-*NAT-HXT3*t gene cassette. These vectors harboured different antibiotic selection markers (*KanMX* for G418 disulphate resistance, *hph* for hygromycin B, *NAT* for cloNAT, and *Shble* for zeocin resistance, respectively) and the constitutive *PGK1* gene promoter and terminator sequences. Initial PCR

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products were first ligated into the pCloneJET 1.2 commercial vector (Fermentas – Sankt Leon-Rot, Germany) as instructed by the manufacturer, which includes the *bla* gene for ampicillin selection. PCR products/DNA fragments were routinely separated on 1% (w/v) agarose (Lonza – Rockland, USA) gels and fragments of appropriate sizes isolated using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research – California, USA). Sequence verification was carried out using the dideoxy chain termination method with an ABI PRISM™ 3100 genetic analyser (Applied Biosystems – Waltham, USA) (Central Analytical Facility, Stellenbosch University). Since the *BET1* and *BOS1* ORFs contained relatively small introns, primers were designed to successfully isolate and clone only the exons. The total collection of PCR primers used in the study is summarized in **Table 1**.

Table 1: All PCR and qPCR primers utilized in this study.

Gene	Restriction sites	Primer sequence
PGK1-L	*Hind*III, *Eco*RI, *Mun*I	5'-GTCAAGCTTGAATTCAATTGGGGCCCTCCCTCC-3'
ALG9-L		5'-TGCATTTGCTGTGATTGTCA-3'
ALG9-R		5'-GCCAGATTCCTCACTTGCAT-3'
TFC1-L		5'-ACACTCCAGGCGGTATTGAC -3'
TFC1-R		5'-CTTCTGCAATGTTTGGCTCA-3'
GEN1-L		5'-CCGCGATTAAATTCCAACAT-3'
GEN1-R		5'-CGATAGATTGTCGCACCTGA-3'
ZEO2-L		5'-GACGCGTGTACGCATGTAAC-3'
ZEO2-R		5'-TTGCAAATTAAAGCCTTCGAG-3'
HYG2-L		5'-ATTTCGGCTCCAACAATGTC-3'
HYG2-R		5'-GATGTTGGCGACCTCGTATT-3'
CLON1-L		5'-TGACCACTCTTGACGACACG-3'
CLON1-R		5'-CCCATCCAGTGCCTCGATG-3'
CBH1-L		5'-CTGACGTCGAATCCCAATCT-3'
CBH1-R		5'-CACCTGGAGGGTTAGAAGCA-3'
BGL1-L		5'-TTTGGTAAAGCGAACCCATC-3'
BGL1-R		5'-AGGTTCACCACTCGATGGAC-3'
BET1-L	*Pac*I	5'-GATCTTAATTAAATGAGTTCAAGATTTGCAGGGGGAAACG-3'
BET1-R	*Asc*I	5'-GATCGGCGCGCCTTATGTAATCCATACCCAAAAAAATAG-3'
SEC22-L	*Pac*I	5'-GATCTTAATTAAATGATAAAGTCAACACTAATCTACAG-3'
SEC22-R	*Asc*I	5'-GATCGGCGCGCCCTATTTGAGGAAGATCCACC-3'
BOS1-L	*Pac*I	5'-GATCTTAATTAAATGAACGCTCTTTACAACC-3'
BOS1-R	*Asc*I	5'-GATCGGCGCGCCCTATCTTAACCATTTCAACAC-3'
SED5-L	*Pac*I	5'-GATCTTAATTAAATGAACATAAAGGATAGAACTTCAG-3'
SED5-R	*Asc*I	5'-GATCGGCGCGCCTTAATTGACTAAAACCCAAATAAC-3'

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Plasmid isolations were carried out using the cetyltrimethylammonium bromide (CTAB) method (Sambrook & Russel, 2001). All plasmids constructed and utilized in this study are summarized in **Table 2**. Yeast transformations were carried out using the LiOAc/DMSO-method (Hill *et al.,* 1991). Two *S. cerevisiae* Y294 strains were utilized as parental strains for the study, one expressing the *S. fibuligera CEL3A* (*Sf*-Cel3A) (Kroukamp *et al.,* 2013; Genbank AEV40916.1) on the ySFI episomal plasmid (Van Rooyen *et al.,* 2005) and the other expressing the *T. emersonii CEL7A-cCBM* (*Te*-Cel7A) (Ilmén *et al.,* 2011; Genbank AAL89553) on the pMI529 episomal vector (Ilmén *et al.,* 2011). Overexpression of the respective ORFs in the latter two parental strains was facilitated by integrating the expression cassettes through homologous recombination with native delta sequences distributed throughout the yeast genome (Lee & Da Silva, 1997). Genomic DNA extractions were carried out using the method described by Hoffman & Winston, (1987) and transformants were cultivated on selective YPD agar containing 100-200 µg mL^{-1} of the appropriate antibiotic (G418 disulphate (Melford Laboratories – Ipswich, United Kingdom), hygromycin B (Calbiochem – San Diego, USA) or zeocin (Melford – Ipswich, United Kingdom)). All transformants were confirmed using PCR amplifications carried out using a *PGK1* promoter-specific forward primer and a gene-specific reverse primer (**Table 1**). All strains constructed for this study are described in **Table 3.**

Table 2: Plasmids utilized in this study

Plasmid	Relevant genotype	Reference/source
pBKD1	*bla δ*-site $PGK1_P$-$PGK1_T$ *kanMX δ*-site	McBride *et al.,* (2008)
pBKD1-*BOS1*	*bla δ*-site $PGK1_P$-*BOS1*-$PGK1_T$ *kanMX δ*-site	This work
pBKD1-*BET1*	*bla δ*-site $PGK1_P$-*BET1*-$PGK1_T$ *kanMX δ*-site	This work
pBKD1-*SEC22*	*bla δ*-site $PGK1_P$-*SEC22*-$PGK1_T$ *kanMX δ*-site	This work
pBKD1-*SED5*	*bla δ*-site $PGK1_P$-*SED5*-$PGK1_T$ *kanMX δ*-site	This work
pBKD1-*SNC1*	*bla δ*-site $PGK1_P$-*SNC1*-$PGK1_T$ *kanMX δ*-site	Van Zyl *et al.,* (2014)
pBKD1-*SSO1*	*bla δ*-site $PGK1_P$-*SSO1*-$PGK1_T$ *kanMX δ*-site	Van Zyl *et al.,* (2014)
pBZD1	*bla δ*-site $PGK1_P$-$PGK1_T$ *Shble δ*-site	McBride *et al.,* (2008)
pBZD-*SEC22*	*bla δ*-site $PGK1_P$- *SEC22*-$PGK1_T$ *Shble δ*-site	This work
pBHD1	*bla δ*-site $PGK1_P$-$PGK1_T$ *hph δ*-site	Kroukamp *et al.,* (2013)
pBHD1-*BET1*	*bla δ*-site $PGK1_P$-*BET1*-$PGK1_T$ *hph δ*-site	This work
pBCD1	*bla δ*-site $PGK1_P$-$PGK1_T$ *NAT δ*-site	This work
pBCD1-*SED5*	*bla δ*-site $PGK1_P$-*SED5*-$PGK1_T$ *NAT δ*-site	This work

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Table 3: The total collection of strains utilized in this study.

Yeast strain	Relevant genotype	reference
S. cerevisiae Y294	MATα *his3Δ leu2Δ lys2Δ ura3Δ*	ATCC 201160
S. cerevisiae Y294 (*CEL3A* Parental)	*ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-fur1::LEU2*	Den Haan *et al.* (2007) *
Y294_*CEL3A_BET1* (Overexpressed)	*ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-his3/HIS3-PGK1p-BET1-PGK1t-kanMX-fur1::LEU2*	This work
Y294_*CEL3A_BOS1*	*ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-his3/HIS3-PGK1p-BOS1-PGK1t-kanMX-fur1::LEU2*	This work
Y294_*CEL3A_SEC22*	*ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-his3/HIS3-PGK1p-SEC22-PGK1t-kanMX-fur1::LEU2*	This work
Y294_*CEL3A_SED5*	*ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-his3/HIS3-PGK1p-SED5-PGK1t-kanMX-fur1::LEU2*	This work
Y294_*CEL3A_BOS1_BET1*	*ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-his3/HIS3-PGK1p-BOS1-PGK1t-kanMX-PGK1p-BET1-PGK1t-hph-fur1::LEU2*	This work
Y294_*CEL3A_ BOS1_BET1_SEC22*	*ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-his3/HIS3-PGK1p-BOS1-PGK1t-kanMX-PGK1p-BET1-PGK1t-hph-PGK1p-SEC22-PGK1t-Shble-fur1::LEU2*	This work
Y294_*CEL3A_SSO1_SED5*	*ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-his3/HIS3-PGK1p-SSO1-PGK1t-kanMX-PGK1p-SED5-PGK1t-NAT-fur1::LEU2*	This work
S. cerevisiae Y294 (*CEL7A* Parental)	*ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3-fur1::LEU2*	Ilmén *et al.* (2011) **
Y294_*CEL7A_BOS1*	*ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3- PGK1p-BOS1-PGK1t-kanMX-fur1::LEU2*	This work
Y294_*CEL7A_BET1*	*ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3-PGK1p-BET1-PGK1t-kanMX-fur1::LEU2*	This work

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Table 3: Continued

Y294_*CEL7A_SEC22*	*ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3-PGK1p-SEC22-PGK1t-kanMX-fur1::LEU2*	This work
Y294_*CEL7A_SED5*	*ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3-PGK1p-SED5-PGK1t-kanMX-fur1::LEU2*	This work
Y294_*CEL7A_BOS1_BET1*	*ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3-PGK1p-BOS1-PGK1t- kanMX-PGK1p-BET1-PGK1t-hph-fur1::LEU2*	This work
Y294_*CEL7A_BOS1_BET1_SEC22*	*ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3-PGK1p-BOS1-PGK1t- kanMX-PGK1p-BET1-PGK1t-hph-PGK1p-SEC22-PGK1t-Shble-fur1::LEU2*	This work
Y294_*CEL7A_BOS1_ BET1_SEC22_SED5*	*ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3- PGK1p-BOS1-PGK1t- kanMX-PGK1p-BET1-PGK1t-hph PGK1p-SEC22-PGK1t-Shble-PGK1p-SED5-PGK1t-NAT-fur1::LEU2*	This work
Y294_*CEL7A_ BET1_SEC22*	*ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3- PGK1p-BET1-PGK1t-kanMX-PGK1p-SEC22-PGK1t-Shble-fur1::LEU2*	This work
Y294_*CEL7A_SED5_SEC22*	*ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3- PGK1p-SED5-PGK1t-KanMX-PGK1p-SEC22-PGK1t-Shble-fur1::LEU2*	This work
Y294_*CEL7A_ SED5_SEC22_BET1*	*ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3- PGK1p-SED5-PGK1t-KanMX-PGK1p-SEC22-PGK1t-Shble-PGK1p-BET1-PGK1t-hph-fur1::LEU2*	This work
Y294_*CEL7A_SNC1_SED5*	*ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3- PGK1p-SNC1-PGK1t–kanMX-PGK1p-SED5-PGK1t-NAT-fur1::LEU2*	This work

* Accession nr for *Sf*-Cel3A: AEV40916.1

* * Accession nr for *Te*-Cel7A: AAL89553

Quantification of SNARE gene copy number and relative episomal expression

Real-time quantitative PCR was used to quantify the respective antibiotic selection markers that had been used to facilitate gene integrations, allowing us to elucidate the copy number of each of the integrated genes of interest. Two reference genes, *ALG9* and *TFC1,* were selected to normalize the copy numbers of our genes of interest, as they are only represented as single copies in the *S.*

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cerevisiae genome (Teste *et al.,* 2009). All DNA concentration measurements were carried out using the ND-1000 Spectrophotometer (Thermo Scientific - Waltham, USA). Real-time quantitative PCR was carried out using the KAPA^{TM} HRM Fast PCR Kit (Kapa Biosystems - Wilmington, USA) and the Applied Biosystems StepOne Real-Time PCR System (Applied Biosystems – Waltham, USA), whilst quantifications of gene copy number and relative episomal expression levels were carried out using the relative standard curve method (Applied Biosystems: Guide to Performing Quantitation of Gene Expression Using RT qPCR; 2008). All primers utilized for Real-time quantitative PCR are detailed in **Table 1**.

Enzyme assays

Yeast strains were inoculated in triplicate at an OD_{600nm} of 1.0 into 20 ml YPD in 125 ml Erlenmeyer flasks and cultivated for 72 hours for each of the two extracellular enzyme activity assays (*Sf*-Cel3A and *Te*-Cel7A). To evaluate the β-glucosidase secretion capabilities of the recombinant strains, enzyme assays (Den Haan *et al.,* 2007) were performed in triplicate at 24 hour intervals on the extracellular cell fractions of each of the *S. cerevisiae* Y294 (*CEL3A*) strains overexpressing differential combinations of the exocytic SNARE genes. Assays were carried out as described previously by Van Zyl et al. (2014) using *p*NPG (*p*-nitrophenyl-α-D-glucopyranoside (Sigma – St. Louis, USA)) as substrate. The extracellular cellobiohydrolase activity of the recombinant Y294 (*CEL7A*) strains was evaluated at 24 hour intervals according to an adapted method described by La Grange *et al.* (2001), using *p*NPC (*p*-nitrophenyl-β-D-cellobioside (Sigma – St. Louis, USA) as substrate, according to previously described methodology (Van Zyl *et al.,* 2014). All spectrophotometric readings for the enzymatic assays were taken using the Biorad xMark^{TM} Microplate Spectrophotometer (Bio-Rad Laboratories – California, USA) at $A_{400nm.}$ A reference strain (negative control containing a vector with no cellulase encoding gene) and media-blanks were included to normalize absorbance readings. Some variability was observed between identical strains in separate assays, which is ascribed to the use of separate batches of substrate over the study period.

SDS-PAGE, N-deglycosylation and densitometry

Strains were inoculated at an initial OD_{600nm} of 1.0 into 20 ml double-strength buffered SC^{-ura} medium in 125 ml Erlenmeyer flasks and cultivated for 72 hours on a rotary shaker (200 rpm) at 30°C. Extracellular protein fractions (20 µl) were analyzed using a 10 % SDS-PAGE gel, as described by

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Laemmli (1970). The separated proteins were visualized using silver staining (Kroukamp *et al.,* 2013) whilst the deglycosylated protein samples were prepared using the Endo H enzyme (New England BioLabs - Hertfordshire, UK) enzyme as instructed by the manufacturer. Densitometric analysis was performed on silver stained gels as previously described (Van Zyl *et al.,* 2014) using the ImageJ software (http://rsbweb.nih.gov/ij/).

Growth analysis

Strains were inoculated in triplicate at a starting OD_{600nm} of 0.05 into 20 ml YP (Yeast Extract Peptone - 1% yeast extract (Merck – Darmstadt, Germany), 2% peptone (Merck – Darmstadt, Germany)) medium supplemented with 2% glucose, added after autoclaving, in 125 ml Erlenmeyer flasks. These flasks were incubated on a rotary shaker (200 rpm) at 30˚C for the duration of the analysis. Samples were diluted (1:10), after which OD_{600nm} readings were taken using the Biorad xMark^{TM} Microplate Spectrophotometer and the data normalized with the use of media controls. The raw data utilized to construct these growth curves was then used to calculate the maximum specific growth rate (μ_{max} (h^{-1})) for each of the recombinant strains tested.

Evaluation of tolerance to stress parameters

Strains were cultivated in 5 mL YPD, inoculated from plate-grown cultures, on rotation (200 rpm) for 72 hours. These cultures were each diluted to an OD_{600nm} of 0.5 and subsequently serially diluted to 1:10 and 1:100. All the diluted samples were then plated out using the cryo-replicator press (Applikon Biotechnology – Delft, Netherlands) on YPD agar plates containing 0.8 M and 1.0 M NaCl (Merck - Darmstadt, Germany). The same general procedure was carried out using YPD agar plates containing either ethanol (4 % or 6 %) or the secretion stress indicator tunicamycin (Sigma - St. Louis, USA) (0.2 µg mL^{-1} or 0.5 µg mL^{-1}) with cultures incubated for 72 hours at 30˚C prior to analysis.

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Results:

Recombinant strain construction and enzyme assays

The integration of at least one additional SNARE gene per transformation in all recombinant yeast strains was first confirmed using targeted PCR before enzyme assays were performed. Preliminary screening to isolate transformants illustrating the highest extracellular activity, was carried out on at least 20 transformant colonies per recombinant strain, with the strain yielding the highest extracellular enzyme activity being selected for further study. Further details regarding the screening and selection of transformants are given in **Fig. S1** (**Addendum A**). Colonies were inoculated and cultivated for 72 hours, after which they were normalized with regard to their OD_{600nm} and evaluated for their secretory activity. This effectively allowed the assembly of recombinant strains that were (relative to the range of transformants assayed following overexpression), superior with regards to extracellular enzyme activity for *Sf*-Cel3A and *Te*-Cel7A. The total collection of strains constructed is summarized in **Table 3**.

The maximum increase in extracellular *Sf*-Cel3A activity facilitated through the single overexpression of the respective ER-to-Golgi SNAREs, was 22% through the overexpression of the *SED5* t-SNARE component, after 72 hours compared to the parental strain (**Fig. 2A**). All subsequently mentioned percentage increases refer to activity readings at 72 hours, relative to the parental strain expressing either *Sf*-Cel3A or *Te*-Cel7A. The rest of the SNAREs investigated yielded no improved secretory phenotypes for *Sf*-Cel3A, with Bet1p, Sec22p and Bos1p all yielding decreased extracellular enzyme activities when singly overexpressed (**Fig. 2A**). Simultaneous overexpression of the ER-to-Golgi SNAREs led to a decrease in extracellular *Sf*-Cel3A activity (**Fig. 2B**). The yeast was apparently unable to proliferate when all four SNARE candidates were overexpressed simultaneously, as we were unable to generate a yeast transformant containing all four gene cassettes. When *SED5* was overexpressed in conjunction with *SSO1,* the most promising exocytic SNARE protein for the improvement of *Sf*-Cel3A (Van Zyl *et al.,* 2014), the improved phenotype increased to approximately 130%, which surpassed the titers obtained when these proteins were overexpressed individually (increases of 22% for *SED5* and 49% for *SSO1*) (**Fig. 3A**). In an attempt to provide better resolution of these results for the cell-wall bound *Sf*-Cel3A, the total activity (cell and supernatant fraction) of these strains was determined. Improvements proved restricted as all three strains plateaued at around 50% (41%, 52% and 56%) higher activity relative to the parental Y294 strain (**Fig. 3B**).

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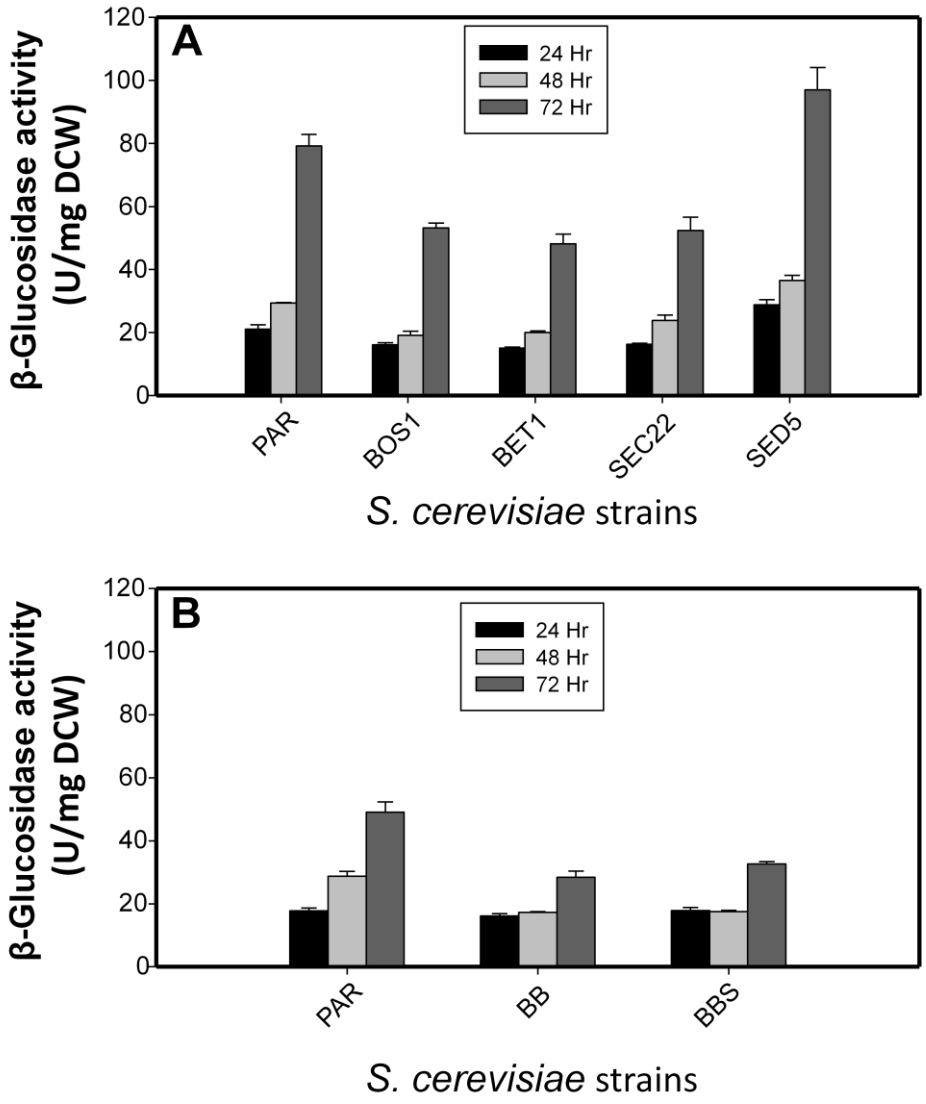

Fig. 2A & B:

Fig. 2: The supernatant enzyme activities of the recombinant *S. cerevisiae* Y294 strains harboring the ySFI episomal plasmid expressing the *Sf*-Cel3A, at 24 hour intervals. **A)** The respective SNARE genes individually overexpressed in each of the strains are indicated below (*BOS1, BET1, SEC22* and *SED5*), along with the parental strain (PAR) (not overproducing any of the SNARE genes) is also included. All values represent mean values of assays done in triplicate with error bars indicating the standard deviation. **B)** The co-overexpression of simultaneous SNARE genes relative to the parental strain. The first "B" indicates *BOS1,* the second "B" indicates *BET1,* the first "S" indicates *SEC22* whilst a second "S" indicates *SED5*. All values represent mean values of assays done in triplicate with error bars indicating the standard deviation. Appropriate reagent blank and reference strain controls were included for all of the assays performed. As reference stains (for either reporter gene) yielded no measurable activity these values were omitted from the graphs.

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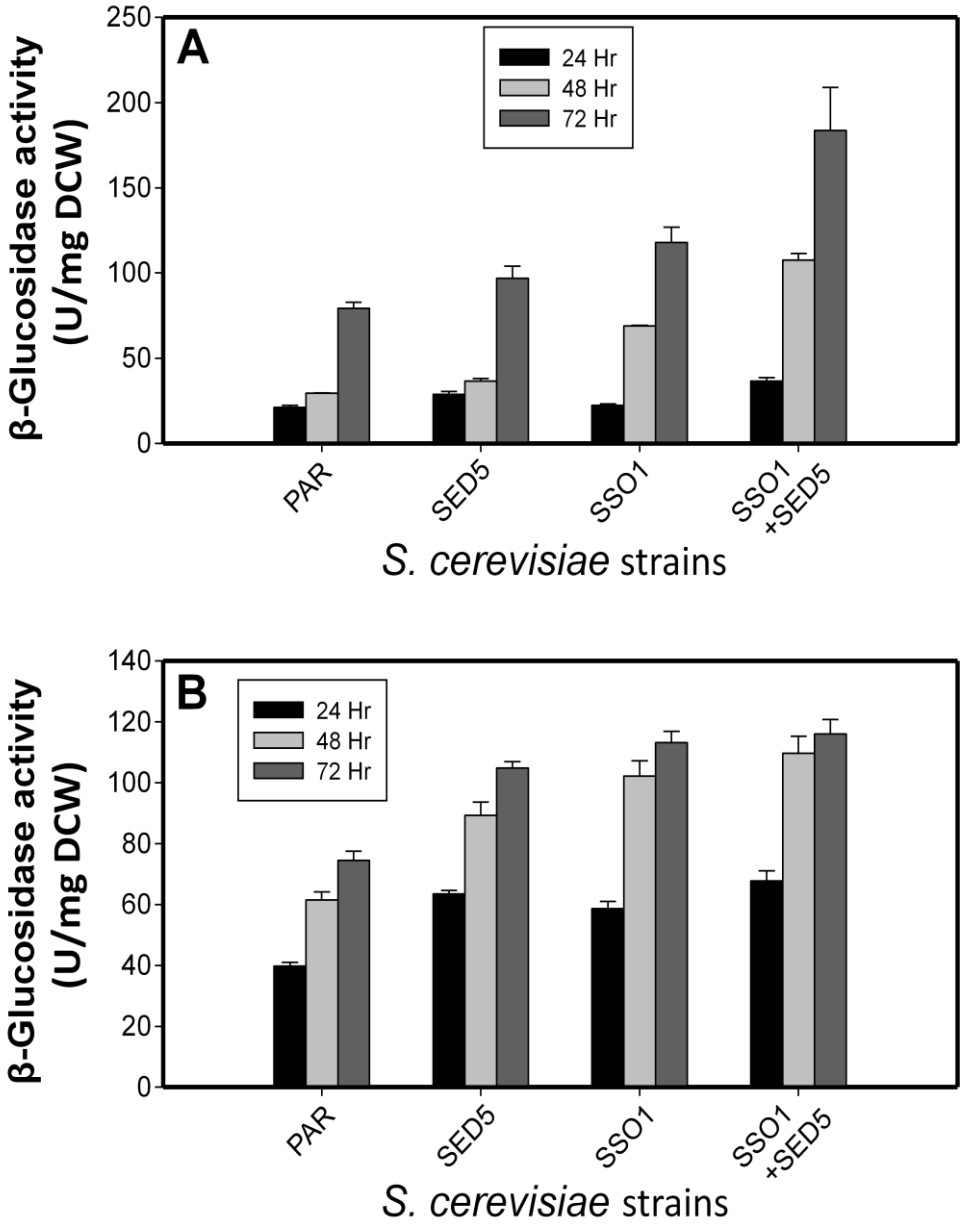

Fig. 3A & B:

Fig. 3: The supernatant enzyme activities of recombinant *S. cerevisiae* Y294 strains harboring the ySFI episomal plasmid, expressing the *Sf*-Cel3A, at 24 hour intervals. **A)** The respective SNARE genes overexpressed in each of the strains are indicated below (*SSO1*, *SED5* and a combinatorially expressing strain), along with the parental strain (PAR) (not over-producing any of the SNARE genes). All values represent mean values of assays done in triplicate with error bars indicating the standard deviation. **B)** The total enzyme activities (cell and supernatant fractions) of the above-mentioned *S. cerevisiae* Y294 strains, at 24 hour intervals. All values represent mean values of assays done in triplicate with error bars indicating the standard deviation.

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Single overexpression of the ER-to-Golgi SNAREs had a more significant phenotypic effect on extracellular *Te*-Cel7A activity, with overexpression of *SED5* again yielding the most significant improvement of 68%, whilst the overexpression of *BET1* and *SEC22* led to increases of 40% and 22%, respectively (**Fig. 4A**). Simultaneous overexpression of all four ER-to-Golgi SNAREs yielded an increase in extracellular activity of 46%, but as it became clear that *BOS1* overexpression negatively affected extracellular activities of both *Sf*-Cel3A and *Te*-Cel7A (**Figs. 2A & 4A**), an additional set of co-overexpressing strains harbouring the *Te*-Cel7A was constructed, with these additional combinations excluding *BOS1.* These results (**Fig. 4B**) illustrated that the co-overexpression of the ER-to-Golgi SNAREs resulted in a phenotype that improved activity only up to a certain point, with the strains overexpressing a combination of *SEC22* and *SED5*, and *BOS1*, *BET1* and *SEC22* yielding the maximum improvement of approximately 48%. We also co-overexpressed *SNC1,* the most effective exocytic SNARE identified in literature for *Te*-Cel7A improvements (Van Zyl *et al.,* 2014), with the top ER-to-Golgi SNARE candidate (*SED5*). The results, however, differ from the distinctive phenotypic increases achieved by overexpressing the individual SNARE genes for *Sf*-Cel3A, with no significant improvement detected when combining the two most effective SNARE components from these membrane interfaces (**Fig. 5**).

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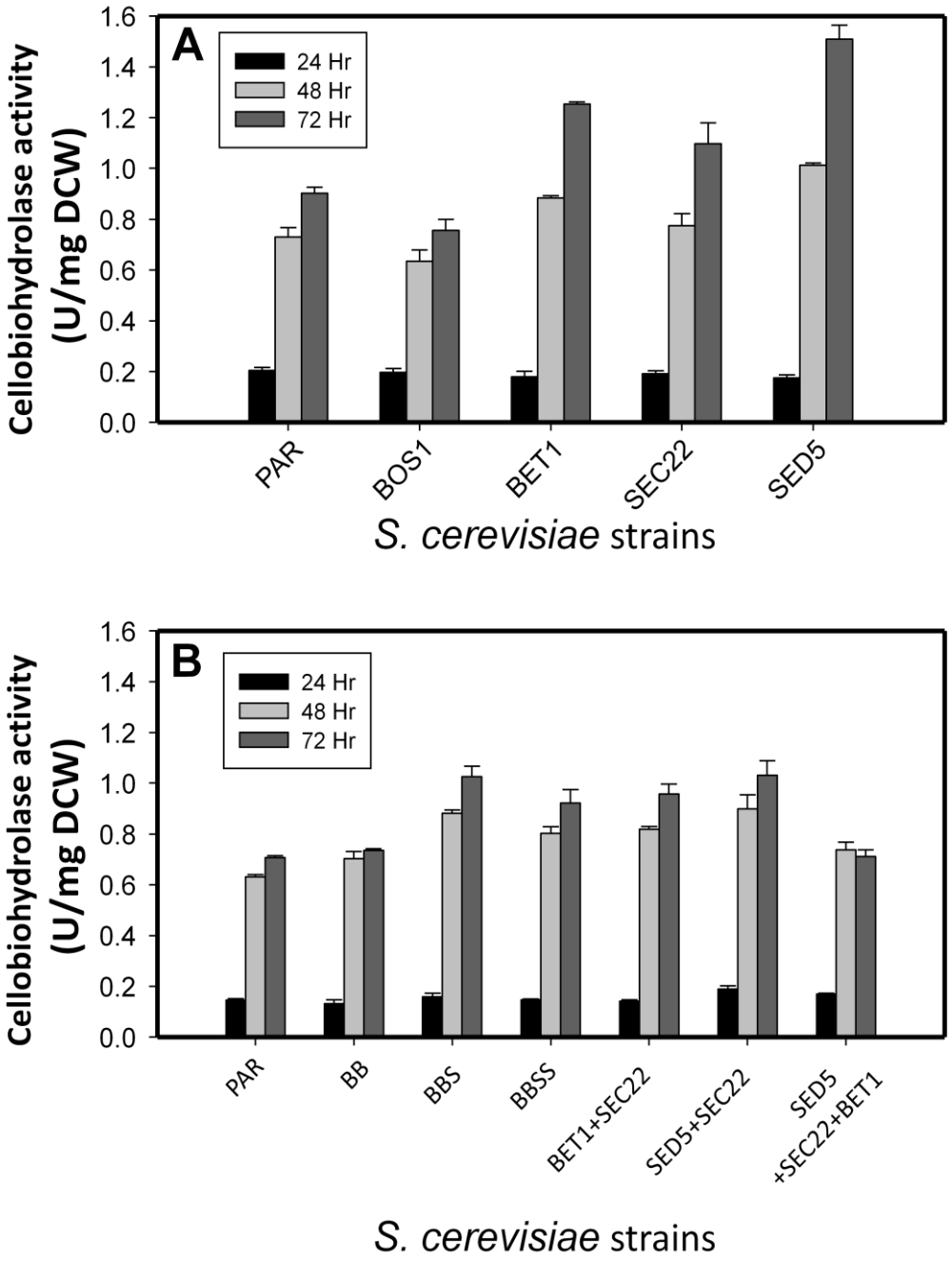

Fig. 4A & B:

Fig. 4: The supernatant enzyme activities of recombinant *S. cerevisiae* Y294 strains harboring the pMI529 episomal plasmid and expressing the *Te*-Cel7A at 24 hour intervals. **A)** The respective SNARE genes individually overexpressed in each of the strains are indicated below (*BOS1, BET1, SEC22* and *SED5*), along with the parental strain (PAR) (not overproducing any of the SNARE genes). **B)** The co-overexpression of simultaneous SNARE genes are represented and the parental strain also included with annotations as stipulated for **Fig. 2B**. All values represent mean values of assays done in triplicate with error bars indicating the standard deviation.

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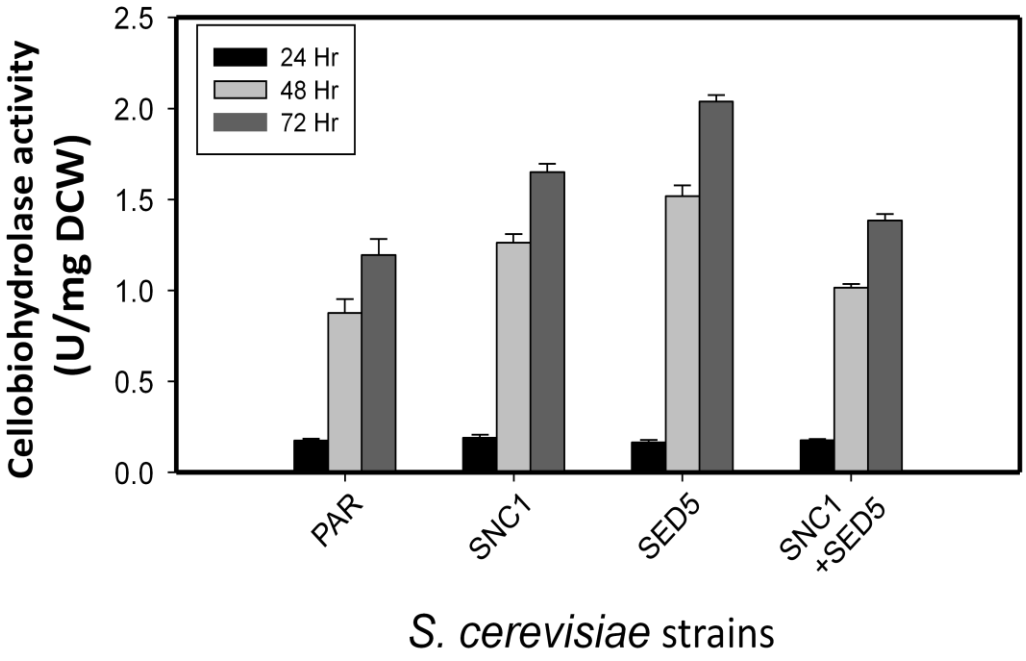

Fig. 5:

Fig. 5: The supernatant enzyme activities of recombinant *S. cerevisiae* Y294 strains harboring the pMI529 episomal plasmid and expressing the *Te*-Cel7A at 24 hour intervals. The SNARE genes being overexpressed (*SNC1*, *SED5* and a combinatorially overexpressing strain) along with the parental (PAR) strain (not overexpressing any SNAREs) are indicated. All values represent mean values of assays done in triplicate with error bars indicating the standard deviation.

The copy numbers of the overexpressed SNARE ORFs (in addition to the native copy) were determined relative to the *ALG9* and *TFC1* reference genes and the results are depicted in **Table 4**. Quantitative real-time PCR analysis of the entire repertoire of recombinant, SNARE-overexpressing strains revealed that all strains had only single additional integrated SNARE gene copies. Relative episomal expression levels, depicting the comparative differences between the parental and top three SNARE-overexpressing strains, for each of the heterologous proteins harboured on their respective episomal plasmids (ySFI and pMI529), were determined. The relative episomal expression levels for both the recombinant and parental strains (for each of the respective reporter proteins) remained consistent for the most promising strains selected (**Fig. 6A & B**), confirming that improvements in extracellular activity could be principally attributed to SNARE-overexpression as opposed to differences in basal episomal expression levels.

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Table 4: The quantified number of additional SNARE gene insertions for all recombinant strains constructed in this study. Rounded numbers are indicated in brackets.

Strain	Copy number					
	BOS1	***BET1***	***SEC22***	***SED5***	***SNC1***	***SSO1***
CEL3A_*BOS1*	0.92 (1)	n/a	n/a	n/a	n/a	n/a
CEL3A_*BET1*	n/a	0.93 (1)	n/a	n/a	n/a	n/a
CEL3A_*SEC22*	n/a	n/a	0.93 (1)	n/a	n/a	n/a
CEL3A_*SED5*	n/a	n/a	n/a	0.92 (1)	n/a	n/a
CEL3A_*BOS1_BET1*	0.92 (1)	0.98 (1)	n/a	n/a	n/a	n/a
CEL3A_*BOS1_BET1_SEC22*	0.91 (1)	1.00 (1)	0.98 (1)	n/a	n/a	n/a
CEL3A_*SSO1_SED5*	n/a	n/a	n/a	0.88 (1)	n/a	1.09 (1)
CEL7A_*BOS1*	1.04 (1)	n/a	n/a	n/a	n/a	n/a
CEL7A_*BET1*	n/a	0.95 (1)	n/a	n/a	n/a	n/a
CEL7A_*SEC22*	n/a	n/a	1.08 (1)	n/a	n/a	n/a
CEL7A_*SED5*	n/a	n/a	n/a	0.92 (1)	n/a	n/a
CEL7A_*BOS1_BET1*	0.70 (1)	0.88 (1)	n/a	n/a	n/a	n/a
CEL7A_*BOS1_BET1_SEC22*	0.83 (1)	0.93 (1)	1.01 (1)	n/a	n/a	n/a
CEL7A_*BOS1_BET1_SEC22_SED5*	1.06 (1)	1.03 (1)	1.02 (1)	0.89 (1)	n/a	n/a
CEL7A_*SED5_SEC22*	n/a	n/a	0.93 (1)	0.74 (1)	n/a	n/a
CEL7A_*BET1_SEC22*	n/a	1.00 (1)	1.01 (1)	n/a	n/a	n/a
CEL7A_*SED5_SEC22_BET1*	n/a	0.92 (1)	1.07 (1)	0.85 (1)	n/a	n/a
CEL7A_*SNC1_SED5*	n/a	n/a	n/a	1.11 (1)	1.03 (1)	n/a

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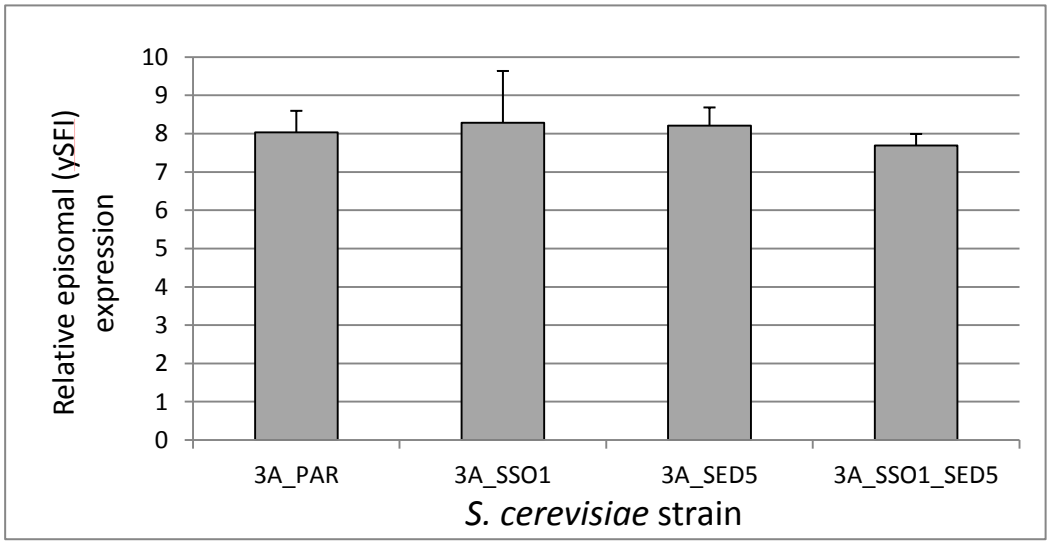

Fig. 6A: The relative episomal expression levels of a selection of the most improved strains harbouring the ySFI episomal plasmid, expressing the *Sf*-Cel3A (3A_PAR), and overexpressing different ER-to-Golgi SNAREs. All values represent the mean of triplicate reactions with error bars indicating the standard deviation.

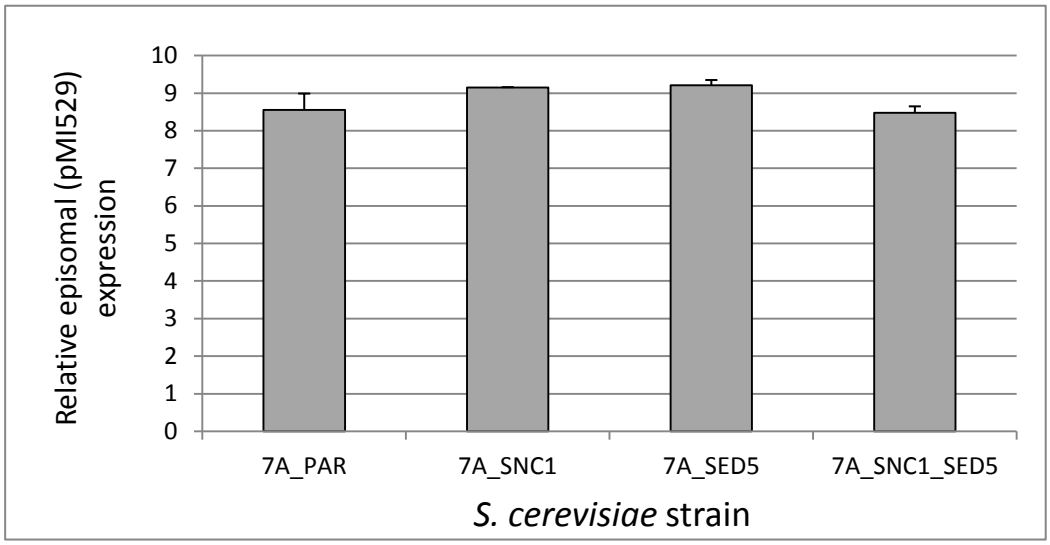

Fig. 6B: The relative episomal expression levels of a selection of the most improved strains harbouring the pMI529 episomal plasmid, expressing the *Te*-Cel7A (3A_PAR), and overexpressing different ER-to-Golgi SNAREs. All values represent the mean of triplicate reactions with error bars indicating the standard deviation.

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SDS-PAGE analysis of extracellular protein fractions

We have based the interpretation of our protein samples on our previous experience with these particular reporter proteins in terms of their activity, apparent sizes and glycosylation profiles (Kroukamp *et al.,* 2013; Den Haan *et al.,* 2013b; Van Zyl *et al.,* 2014). The SNARE-overexpressing strains illustrating some of the most improved phenotypes (*Sf*-Cel3A-*SED5, Sf*-Cel3A-*SSO1, Sf*-Cel3A-*S1S5, Te*-Cel7A-*SNC1* and *Te*-Cel7A-*SED5*) were selected for further analysis. The recombinant *Te*-Cel7A is always visible as a heterogeneous smear of roughly 70 to 170 kDa and, when N-deglycosylated, the protein migrates at approximately 65 kDa. Deglycosylated extracellular protein fractions of the selected strains expressing *Te*-Cel7A (indicated with a "D"), when compared to the parental (PAR) and reference (REF) strains (lacking the protein of interest), illustrated a clear increase in band intensity (at +/- 66 kDa) for the representative target protein on a 10% acrylamide gel (**Fig. 7A**). Kroukamp *et al.* (2013) illustrated that the *Sf*-Cel3A migrates at above 170 kDa, with the deglycosylated form present at approximately 100 kDa. The non-deglycosylated extracellular protein fractions of the *Sf*-Cel3A-expressing strains also illustrated a clear increase in band intensity (at +/- 170 kDa), particularly for the *Sf*-Cel3A-*S1S5* strain (**Fig. 7B**). These results confirmed that the increases in extracellular enzyme activity attained (**Figs. 2A, 3A and 4A**) correlated with an increase in the amount of detectable secreted protein. These strains had been normalized with regard to their optical densities to verify secretory titers and densitometric analysis of the specified protein bands confirmed the improvements in extracellular protein concentrations in ranges that concurred with observed activity increases.

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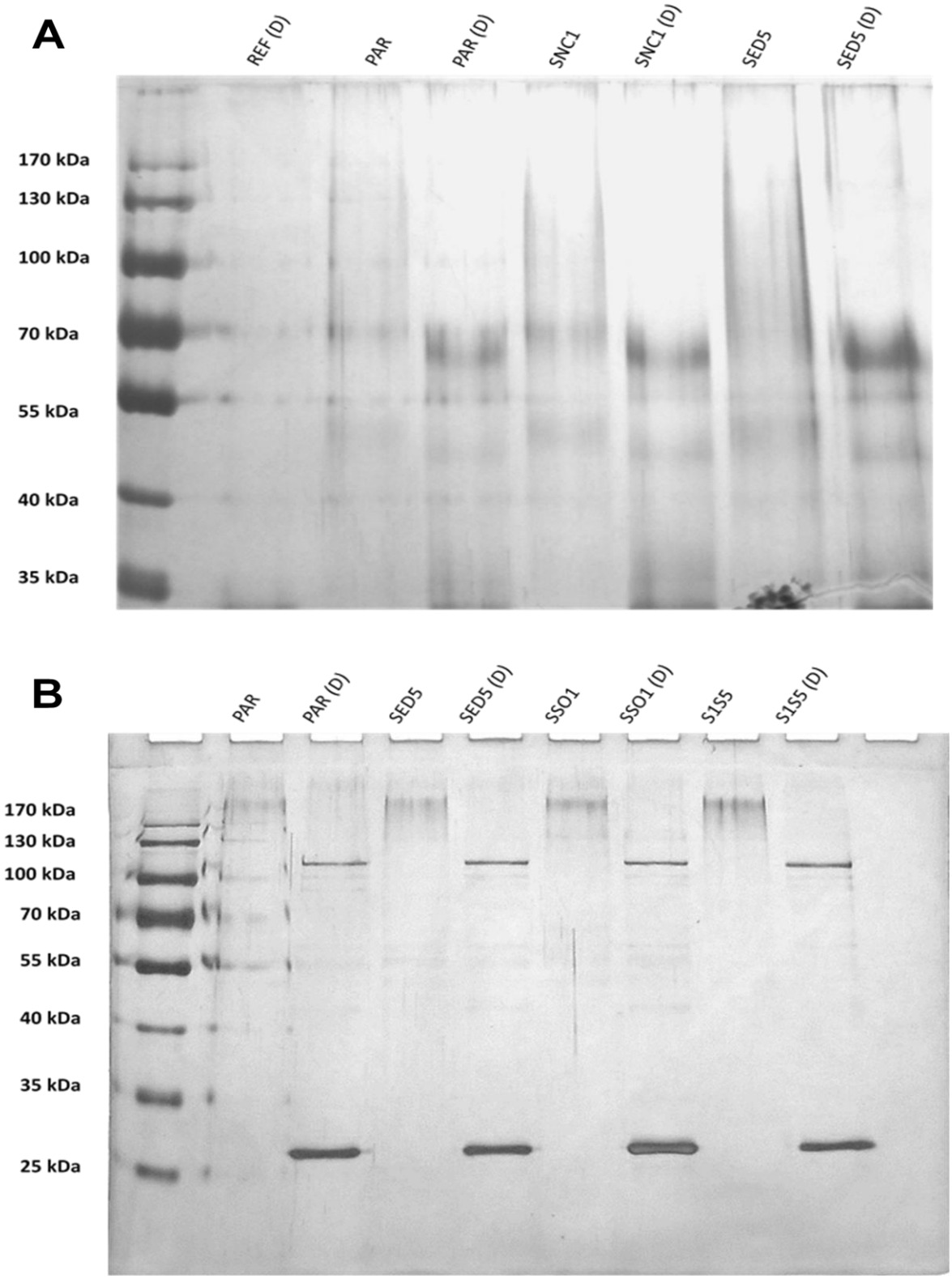

Fig. 7A & B:

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Fig. 7: A) A silver-stained 10% SDS-PAGE gel illustrating the quantitative differences in extracellular *Te*-Cel7A secretory titers between some of the most improved *S. cerevisiae* strains expressing this reporter protein - overexpressing *SNC1* and *SED5* along with the parental (PAR) strain and a reference (REF) strain (not producing the protein of interest). The "D" indicates deglycosylated samples. **B)** A silver-stained 10% SDS-PAGE gel illustrating the quantitative differences in extracellular *Sf*-Cel3A secretory titers between some of the most improved *S. cerevisiae* strains expressing this reporter protein - overexpressing *SED5, SSO1* and simultaneously expressing both of these genes (*S1S5*) along with the parental (PAR) strain.

Growth and inhibitor tolerance of the recombinant strains

The respective growth data sets for the strains expressing the *Sf*-Cel3A and *Te*-Cel7A are depicted in **Fig. 8A & B**, respectively. The maximum specific growth rates (μ_{max} (h^{-1})) and the final culture densities of these strains are summarised in **Table 5**. Investigation of the growth capabilities of the recombinant, SNARE-overexpressing strains illustrated a propensity amongst the most improved *Sf*-Cel3A producing strains (*Sf*-Cel3A-*SED5* and *Sf*-Cel3A-*S1S5*) for an extended lag phase (**Fig. 8A**) and a decreased maximum specific growth rate (**Table 5**), whilst the strain co-overexpressing three of these components (*Sf*-Cel3A-*BBS*) produced similar results. It is clear that basal growth capability of the most improved strain secreting this protein (*Sf*-Cel3A-*S1S5*) was significantly affected following the diauxic shift at approximately 24 hours, exhibiting a decrease in growth vigour until ultimately reaching an inferior terminal optical density to the rest of the investigated repertoire (**Table 5**). In contrast to the strains expressing the *Sf*-Cel3A, most of the *Te*-Cel7A producing strains displayed no signs of deleterious growth effects due to the respective SNARE-overexpressions (**Fig. 8B**). The most improved strain (*Te*-Cel7A-*SED5*) did illustrate a slightly extended lag phase, with a detectably lower maximum specific growth rate and lower culture densities following the diauxic shift at 24 hours, but reached the same end-point optical density as the rest of the strains.

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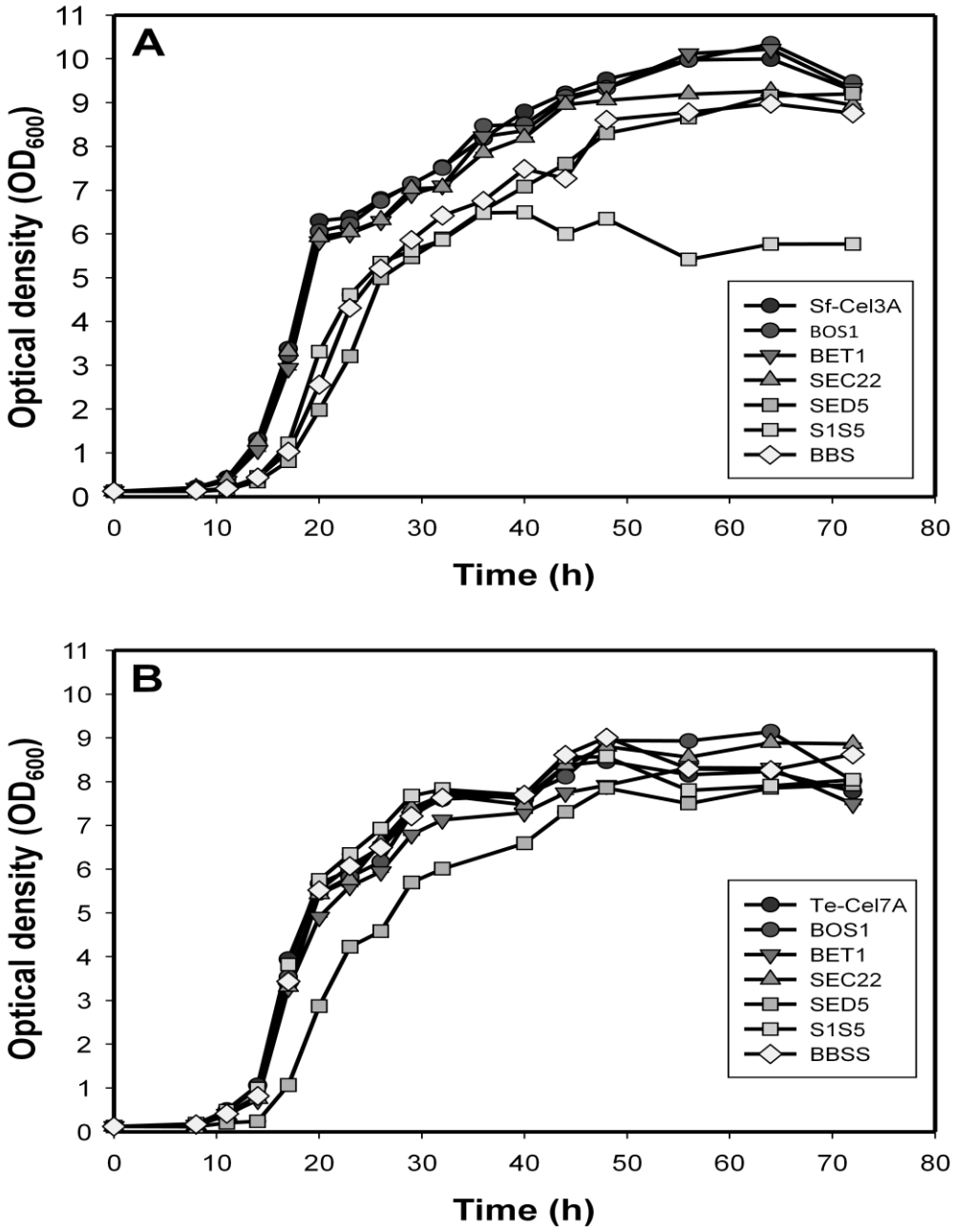

Fig. 8A & B:

Fig. 8: A) Growth trends for the parental (*Sf*-Cel3A) *S. cerevisiae* strain and all the singly overexpressing recombinant strains, along with the simultaneously overproducing (BBS – *BOS1, BET1* and *SEC22*) and combinatorially overexpressing (S1S5 – *SSO1* and *SED5*) strains. Strains were evaluated over a period of 72 hours, with values representing the mean of triplicate cultures tested and error bars indicating the standard deviation. **B)** Growth trends for the parental (*Te*-Cel7A) *S. cerevisiae* strain and all the singly overexpressing recombinant strains, along with the simultaneously over-producing (BBSS - *BOS1, BET1, SEC22* and *SED5*) and combinatorially overexpressing (S1S5 – *SNC1* and *SED5*) strains. Strains were evaluated over a period of 72 hours, with values representing the mean of triplicate cultures tested and error bars indicating the standard deviation.

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Table 5: A summary of the various maximum specific growth rates (μ_{max} (h^{-1})) for the selection of strains investigated. All experiments were performed in triplicate and standard deviations were below 5%.

ER-to-Golgi SNAREs			
Strain	**Heterologous protein**	**μ_{max} (h^{-1})**	**Final OD_{600}**
Parental (PMI529_*Te*-Cel7A)	*Te*-Cel7A	0.45	7.78
Cel7A_*BOS1*	*Te*-Cel7A	0.47	8.02
Cel7A_*BET1*	*Te*-Cel7A	0.45	7.49
Cel7A_*SEC22*	*Te*-Cel7A	0.47	8.86
Cel7A_*SED5*	*Te*-Cel7A	0.36	7.93
Cel7A_*SNC1_SED5*	*Te*-Cel7A	0.45	8.62
Cel7A_*BOS1_BET1_SEC22_SED5*	*Te*-Cel7A	0.46	8.05
Parental (ySFI_*Sf*-Cel3A)	*Sf*-Cel3A	0.45	9.28
Cel3A_*BOS1*	*Sf*-Cel3A	0.46	9.46
Cel3A_*BET1*	*Sf*-Cel3A	0.46	9.32
Cel3A_*SEC22*	*Sf*-Cel3A	0.46	8.94
Cel3A_*SED5*	*Sf*-Cel3A	0.37	9.20
Cel3A_*SSO1_SED5*	*Sf*-Cel3A	0.43	5.77
Cel3A_*BOS1_BET1_SEC22*	*Sf*-Cel3A	0.38	8.76

The overexpression of *SED5,* the most promising secretory-enhancing ER-to-Golgi SNARE component we've identified, led to detectable decreases in osmotic and ethanol tolerance for strains secreting both *Sf*-Cel3A and *Te*-Cel7A (**Fig. 9A & B**). Simultaneous SNARE-overexpression (*BOS1, BET1* and *SEC22*) also decreased osmotic and ethanol tolerance in the *Sf*-Cel3A expressing strain (**Fig. 9A**), whilst the most improved strain expressing this reporter protein (*Sf*Cel3A-*S1S5*) also illustrated a decreased tolerance to increased ethanol concentrations (**Fig. 9B**). Enhancements in extracellular *Sf*-Cel3A activity were associated with greater ER-stress as higher tunicamycin concentrations (0.2 µg mL^{-1}) led to a non-viable phenotype in all strains, whilst the *Te*-Cel7A expressing strains remained relatively unaffected – with the exception of the simultaneously overexpressing (*Te*-Cel7A-*BBSS*) and *SED5* overexpressing strains, which also illustrated sensitivity in this regard (**Fig. 9C**). Neither of the two sets of strains grew when tunicamycin concentrations were raised to 0.75 µg mL^{-1} (data not shown).

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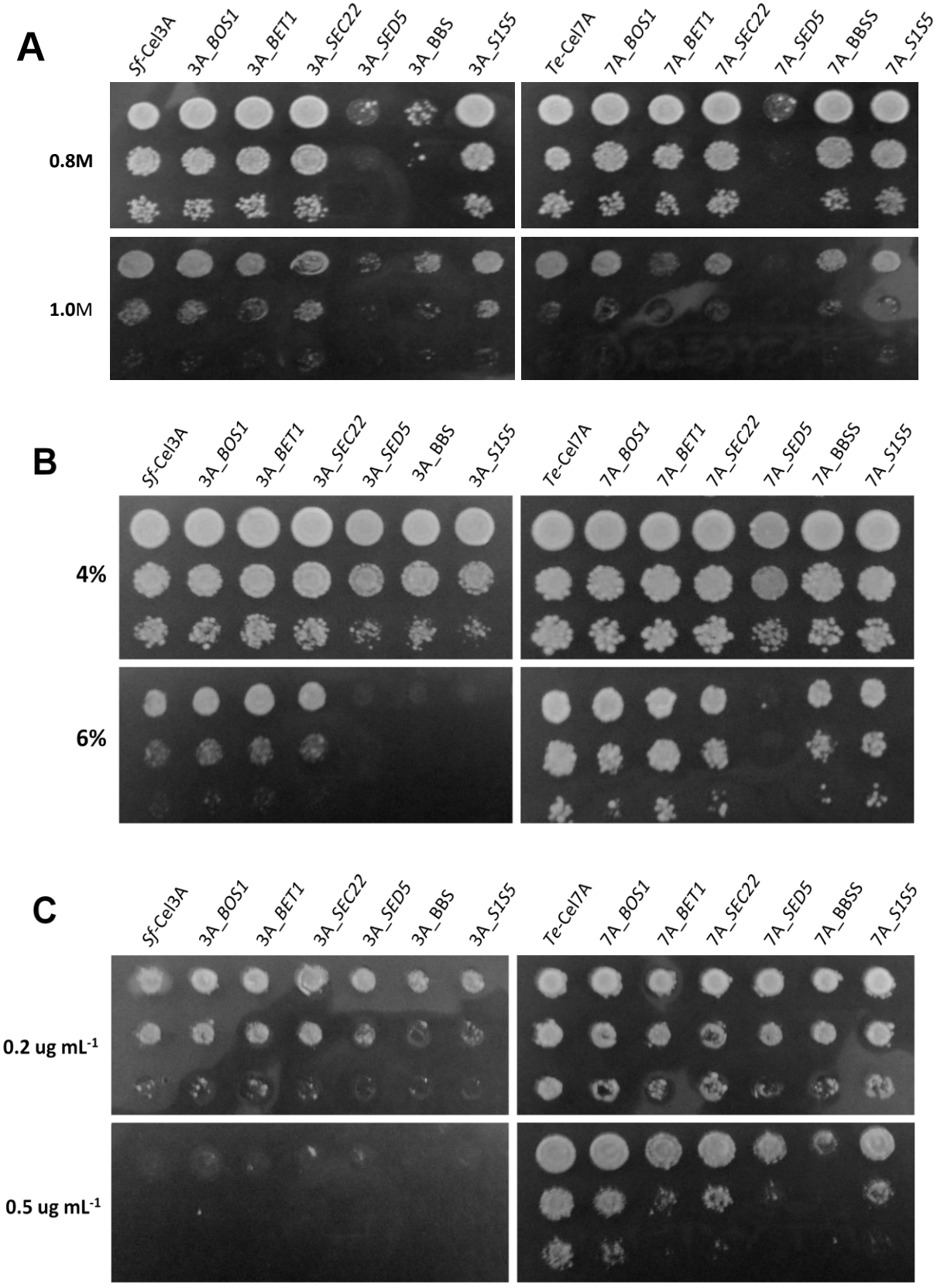

Fig. 9A, B & C:

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Fig. 9: A) A representation of the osmotic tolerance of the recombinant, SNARE-overexpressing *S. cerevisiae* strains, with each set expressing either *Sf*-Cel3A or *Te*-Cel7A. Overexpressed SNARE components are indicated, with the relevant NaCl concentrations within the growth media (0.8 M, 1.0 M) also illustrated. Annotations are as stipulated in **Fig. 2B**, with "S5" representing *SED5* and "S1" representing *SSO1* and *SNC1* for the *Sf*-Cel3A and *Te*-Cel7A-expressing strains, respectively. **B)** A representation of the ethanol tolerance of the strains as described above, in the presence of either 4% or 6% ethanol. **C)** A representation of the tolerance of the SNARE-overexpressing strains to increased ER-stress, induced by increasing concentrations of tunicamycin (0.2 µg mL^{-1} and 0.5 µg mL^{-1}) in the growth media.

Discussion

This study intended to further elucidate the potential role that SNARE proteins could play in increasing the currently attainable titers for heterologous cellulases in *S. cerevisiae,* with particular emphasis on the SNARE components facilitating fusion of the ER-derived secretory vesicles with the *cis*-Golgi. The challenge of high level cellobiohydrolase production (Van Zyl *et al.,* 2013), coupled with the critical role that β-glucosidases play in cellulase hydrolysis for bioethanol production (Xin *et al.,* 1993; Han & Chen, 2008; Singhania *et al.*, 2013) and the bottlenecks they pose to lignocellulosic fuels (Sørensen *et al.,* 2013), supported the selection of *Te*-Cel7A and *Sf*-Cel3A as reporter proteins.

Contrary to results reported concerning the exocytic SNAREs (Van Zyl *et al.,* 2014), a single ER-to-Golgi t-SNARE, Sed5p, was the most effective for the improvement of both *Sf*-Cel3A and *Te*-Cel7A extracellular enzyme activities, yielding improvements of 22% and 68%, respectively (**Figs. 2A & 4A**). This may indicate a more universally effective SNARE target for heterologous protein secretion enhancement than the rest of the investigated repertoire, or simply illustrate a cellular shortage that can be corrected when confronted with the production of heterologous proteins. Sed5p is relatively promiscuous in its SNARE-binding capacity, having been shown to form several different SNARE complexes *in vivo* (Tsui *et al.,* 2001) and being implicated not only in anterograde ER-to-Gogli transport, but in intra-Golgi and endosome-to-Golgi transport as well. This promiscuity, in conjunction with observations that Sed5p phosphorylation is essential to the maintenance of the Golgi structure and function, further illustrates the multifaceted nature of this SNARE component (Hardwick & Pelham, 1992; Wooding & Pelham, 1998; Weinberger *et al.,* 2005) and complicates elucidation of the mechanism leading to the enhanced secretion phenotype. The overproduction of Bos1p, Bet1p and Sec22p led to inferior phenotypes with regard to extracellular *Sf*-Cel3A activity

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(**Fig. 2A**), whilst the latter two components yielded less prominent increases (40% and 22%, respectively) for *Te*-Cel7A (**Fig. 4A**). Co-overexpression of the ER-to-Golgi SNAREs yielded variable results for extracellular *Te*-Cel7A activity, with the simultaneous overproduction of Bos1p, Bet1p and Sec22p yielding a maximal increase of 46% and the co-overproduction of Sec22p and Sed5p producing similar results (**Fig. 4B**). Simultaneous over-production of all ER-to-Golgi SNAREs except Sed5p (i.e. Bos1p, Bet1p and Sec22p) resulted in a phenotype exhibiting decreased extracellular *Sf*-Cel3A activity relative to the parental strain (**Fig. 2B**). However, attempts to simultaneously overexpress all four ER-to-Golgi SNAREs in a strain producing *Sf*-Cel3A were unsuccessful, further illustrating the greater cellular impact of this heterologous protein. Our results seem to reaffirm that the overexpression of specific SNARE components can contribute to a phenotypic response that results in improved extracellular enzyme activity for two fungal cellulases we've investigated, and that SNAREs may be an essential contributory element to these improvements.

The gene dosage for SNARE overexpression did not necessarily led to a proportionate phenotypic response as, following the selection of the highest secreting transformants per recombinant strain, all of the top candidate strains had only integrated a single additional SNARE gene copy (**Table 4**). It is important to note that as delta integration can result in gene integration into variably expressed areas of the genome, with specific areas prone to transcriptional silencing or hyper-activation, our single copy integrations cannot be considered absolute measurements of SNARE gene effects, though our screening methodology to select only top transformants attempted to assist in isolating the most improved transformants in this regard. Relative episomal expression levels between the top SNARE-overexpressing strains and their respective parental strains remained consistent, enabling us to predominantly attribute improvements to SNARE-overexpression as opposed to variance in basal episomal expression levels (**Fig. 6A & B**).

The combinatorial investigation of both the exocytic and ER-to-Golgi SNAREs partially confirmed that additive phenotypic improvements could be facilitated through the co-overexpression of candidate SNARE components from different SNARE complexes within the secretory pathway. A maximal increase in extracellular *Sf*-Cel3A activity of approximately 130% was achieved following the co-overexpression of Sed5p and Sso1p, identified as the most effective exocytic SNARE candidate for the improvement of this particular cellulolytic reporter protein (Van Zyl *et al.,* 2014) (**Fig. 3A**). However, given the marked decrease in growth capability (**Fig. 8A**) of this recombinant strain, coupled with a plateau in increased activity at around 56% when the total enzyme activity (cellular and extracellular fractions) for these strains were measured (**Fig. 3B**), it is possible that cell lysis later

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in batch cultivation resulted in a notable fraction of the partially cell wall-bound β-glucosidase being released into the extracellular medium, resulting in an overestimation of enzyme activity (Gurgu *et al.,* 2011). The latter is especially likely as visible cell debris was present in the batch cultured media after 72 hours (data not shown). Nevertheless, the increase in extracellular β-glucosidase activity associated with this particular strain was confirmed following SDS-PAGE analysis of the extracellular protein fractions (**Fig. 7B**). The co-overproduction of Snc1p, the most promising exocytic SNARE identified for the improvement of *Te*-Cel7A, with Sed5p yielded no further increase in extracellular activity of this reporter protein, further illustrating the protein-specific nature of these SNARE-related phenotypes and potentially indicating variability in gene expression at different gene integration loci.

The two most improved *Sf*-Cel3A secreting strains, overexpressing Sed5p (*Sf*-Cel3A_*SED5*) and co-over-producing Sso1p and Sed5p (*Sf*-Cel3A_*S1S5*), as well as the strain over-producing multiple SNARE components simultaneously (*Sf*-Cel3A_BBS), all illustrated a decreased maximum specific growth rate (**Table 5**), an extended lag phase and a lower terminal culture density (*Sf*-Cel3A_*S1S5*) (**Fig. 8A**). The strain simultaneously over-producing Sed5p and Sso1p (*SF*-Cel3A_*S1S5*) became severely affected following the diauxic shift at 24 hours, when the yeast shifts from the utilization of glucose via glycolysis to the aerobic utilization of intrinsically produced ethanol. This is a phase where the lack of nutrients and the build-up of toxic metabolites from oxidative metabolism become increasingly prevalent (Galdieri *et al.,* 2010). It is therefore plausible that as ethanol becomes the primary carbon source later in batch cultivation, this strain becomes unable to proliferate adequately, leading to a decrease in optical density and a deteriorated stationary/quiescent phase. This correlates well with the increased sensitivity to higher ethanol concentrations we've illustrated for this particular strain, in addition to osmotic and ER-stress sensitivities (**Figs. 9A, B & C**).

The most promising *Te*-Cel7A secreting strain, over-producing Sed5p, illustrated an extended lag phase (**Fig. 8B**), a lower maximum specific growth rate (**Table 5**) and slightly diminished culture densities following the diauxic shift at 24 hours, but it nevertheless reaches cell densities resembling that of the parental strain. The proposed susceptibility to ethanol stress highlighted by the decrease in growth rate following the diauxic shift (**Fig. 8B**) was confirmed as increased concentrations of both ethanol and sodium chloride significantly inhibited the growth of the *Te*-Cel7A-*SED5* strain (**Fig. 9A & B**).

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The expression of heterologous proteins in *S. cerevisiae,* including cellulases, can often impose a diverse range of metabolic burdens on the cell, which can potentially be exacerbated by phenotypic enhancements achieved through strain engineering (Ostergaard *et al.,* 2000; Van Rensburg *et al.,* 2012). Cells of *S. cerevisiae* are able to respond to osmotic stress, such as an increase in osmolarity of the growth medium, by enhancing its intracellular glycerol production as a compatible solute (Albertyn *et al.,* 1994; Nasser & El-Moghaz, 2010). It is therefore conceivable that incorrect intracellular production and distribution of this solute could lead to the perceived osmotic vulnerabilities we've been able to illustrate in the most improved strains in this study, though the underlying mechanism remains unknown. Gene ontology studies have indicated that the ethanol stress response in *S. cerevisiae* is significantly nullified by constraints on energy production, which leads to increased expression of genes involved in glycolysis and mitochondrial function and a decrease in gene expression related to energy-intensive, growth-associated processes (Stanley *et al.,* 2009).

The increased energy output for amino acid production to supply the increased demand for heterologous proteins in the recombinant strains could contribute significantly to the observed deficiencies in the ethanol stress response, though it is recognized that this response is polygenic (Hu *et al.,* 2007). Tunicamycin is a bacterial toxin inhibiting N-linked glycosylation of nascent polypeptides and can be used as a means for unfolded protein response (UPR) induction, effectively causing ER-stress in eukaryotic cells (Bull *et al.,* 2012). From our results, it is clear that the higher production rate and increased propensity for glycosylation for the larger *Sf*-Cel3A severely affected the growth capability of the yeast at higher tunicamycin concentrations, with total inhibition of growth at 0.5 µg mL^{-1} for all strains expressing this reporter protein. Conversely, upon increasing tunicamycin-induced ER stress for the strains expressing the *Te*-Cel7A, it was again the strain overexpressing the *SED5* component and the simultaneously overexpressing strain (*Te*-Cel7A-BBSS) - the two most improved strains in terms of secretory titers – that displayed increased sensitivity. It is therefore clear that an increase in ER-stress, coupled with the increased flux through the ER in the most improved strains, resulted in a phenotype with decreased vitality. The cell wall-associated nature of the larger *Sf*-Cel3A (Gurgu *et al.,* 2011) and the higher secretory titers (over ≈ 130 U mg^{-1}) of the recombinant protein may help to explain some of the growth impairments associated with the secretion of this cellulolytic protein, as well as the associated susceptibility to stress parameters as the same impairments in growth are not uniformly reflected in the strains secreting the *Te*-Cel7A.

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A minimum of five different types of transport vesicles emerge from the late Golgi compartment (Harsay & Bretscher, 1995). Based on vesicle density and molecular composition, two of these lead to the cell surface. As the mechanism for the preferential selection of these pathways for different heterologous proteins is unknown, this may contribute to the differential improvements we've described for the two cellulolytic reporter proteins. In addition, general polypeptide discrepancies such as protein size, levels of N-glycosylation and disulphide bridge formation likely also play significant roles in the observed variations.

For secreted proteins, there remains an additional hurdle to overcome as candidate proteins that are secreted from the cell membrane into the periplasmic space could easily be endocytosed before they have successfully diffused through the cell wall (Rodríguez-Limas *et al.,* 2015). This endocytic mechanism allows the cell to effectively assimilate and internalize extracellular material and molecules within sections of the plasma membrane and could provide a reasonable explanation for the limited increases in extracellular *Sf*-Cel3A activity attained, given the partially cell wall-bound nature of this heterologous protein (Gurgu *et al.,* 2011). In fact, it has been illustrated that *S. cerevisiae* can take up substantial amounts of proteins from the extracellular environment, often catabolising these, making this a potentially significant limiting factor for secreted protein concentrations (Huang *et al.,* 2008; Tyo *et al.,* 2014).

Systems and synthetic biology approaches have notably improved heterologous protein production in *S. cerevisiae* over recent years, helping to address issues associated with low yields and post-translational modifications (Rodríguez-Limas, 2013). Our current research adds credence to the notion that SNARE proteins form a key element within a larger cascade of interacting protein classes that, given efficient gene dosage, can contribute significantly to the future improvement of *S. cerevisiae* as commercial heterologous protein production host.

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Conflict of interest statement:

The authors declare that there are no conflicts of interest associated with the submission of this study.

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Chapter 5

The effects of SNARE gene overexpression on native protein secretion and the Unfolded Protein Response (UPR) in *Saccharomyces cerevisiae*

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Abstract:

The overexpression of specific components of the ER-to-Golgi and exocytic SNARE complexes increased heterologous cellulase secretion in *Saccharomyces cerevisiae* (Chapters 3 & 4). With this in mind, the most promising ER-to-Golgi and exocytic SNAREs were selected to determine whether their positive effects on heterologous cellulase secretion would enhance the secretion of native proteins in *S. cerevisiae*. The most effective exocytic SNAREs (Snc1p & Sso1p) identified in the previous chapters, illustrated a greater propensity for native protein secretion enhancement compared to the most effective ER-to-Golgi component (Sed5p). This suggests that heterologous proteins are more significantly limited at the ER-Golgi interface as opposed to the exocytic fusion reaction. The effects of overexpressing the most promising ER-to-Golgi SNARE component identified for the improvement of both *Saccharomycopsis fibuligera*-Cel3A and *Talaromyces emersonii*-Cel7A (Sed5p) on the unfolded protein response (UPR) were also elucidated. Co-overexpression of Sed5p and *Sf*-Cel3A led to a measurable decrease in the enhanced UPR induction associated with this heterologous cellulase. An enhanced UPR is generally associated with the build-up of misfolded and unfolded proteins in the ER; the improved secretory phenotype induced by Sed5p-overexpression, therefore decreased a UPR response that was upregulated by *Sf*-Cel3A production. Improved ER-to-Golgi and intra-Golgi transport may therefore have helped to ease secretory stress on the ER. This would allow folding homeostasis, wherein the cell must successfully balance its protein folding capacity with the flux of proteins through the secretion pathway, to revert closer to equilibrium.

Keywords: SNAREs, invertase, UPR, yeast, secretion

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Introduction:

Yeast are able to secrete hydrolytic enzymes that are able to act on more complex molecules in order to release nutrients which the cell can subsequently utilize (Koschwanez *et al.,* 2011). Enzyme secretion can therefore be considered a type of cooperation as the nutrients that the hydrolytic enzymes release can be utilized by cells other than the secreting members of the population. Yeast, including *S. cerevisiae*, secrete a number of hydrolytic enzymes into their extracellular environment, including acid phosphatase (Pho5p), phospholipase (Plb2p) and invertase (Suc2p) that are able to release nutrients from differential substrate molecules in the medium (Schmidt *et al.,* 1963; Dodyk *et al.,* 1964; Merkel *et al.,* 2005). Invertase, which has long been studied in yeast, hydrolyses the disaccharide sucrose into its glucose and fructose monosaccharide constituents and the release of these reducing sugars can be utilized to quantify extracellular invertase activity. In this study, we investigated the effects of overexpressing specific SNARE components, identified in the previous chapters as being promising for the improvement of heterologous cellulase secretion, on secretion of the native invertase.

The endoplasmic reticulum (ER) fulfils a plethora of functions in the cell, ranging from calcium storage and release, to the biosynthesis of membrane and secretory proteins (Back *et al.,* 2005). It is therefore not surprising that the ER is able to integrate many internal and external signals to coordinate a multitude of downstream processes, making it an ideal point of reference to investigate the holistic effects of protein secretion. In order to maintain a sensitive protein folding homeostasis in the ER, the cell must successfully balance its protein folding capacity with the flux of proteins through the secretion pathway (Montenegro-Montero *et al.,* 2015). When protein folding requirements exceed the ER's capabilities, unfolded proteins accumulate in this organelle, leading to ER-stress. When misfolded and folded proteins accumulate in the ER, resident transmembrane sensors trigger a conserved signalling pathway known as the unfolded protein response (UPR) (Kaufman, 1999; Mori, 2000; Patil & Walter, 2001; Kaufman & Clin, 2002; Montenegro-Montero *et al.,* 2015). The UPR leads to the activation of a major transcriptional program specifically aimed at not only increasing the folding capacity in the ER, but in some instances adjusting the secretory pathway itself (Travers *et al.,* 2000). The UPR has been implicated in decreasing ER loading through selective mRNA degradation, translational repression and potentially a universal reduction in protein synthesis (Harding *et al.,* 1999; Hollien & Weissman, 2006; Hollien *et al.,* 2009; Kimmig *et al.,* 2012). The afore-mentioned processes collectively function to relieve ER-stress and re-establish protein folding homeostasis in the ER (Ron & Walter, 2007).

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Ire1p is an ER-resident trans-membrane protein that has both kinase and endoribonuclease activity and detects the build-up of unfolded proteins in the ER (Cox *et al.,* 1993; Mori *et al.,* 1993; Bork & Sander, 1993). The expression of the UPR target genes is controlled by the bZIP transcription factor Hac1p (Cox & Walter, 1996; Mori *et al.,* 1996). The *HAC1* gene is expressed constitutively at basal levels, however, bZIP is not produced when unfolded proteins in the ER do no surpass a threshold due to an intron in the mRNA transcript. When Ire1p senses the build-up of unfolded proteins on its ER-luminal side, it oligomerizes, *trans*-autophosphorylates and undergoes conformational changes that led to the activation of its cytosolic RNase domain (Korennykh & Walter, 2012; Gardner *et al.,* 2013). This activated domain subsequently cleaves its only target, the *HAC1* mRNA, at two sites, releasing a 252 bp intron (Walter & Ron, 2011; Gardner *et al.,* 2013). The two resulting exons are then ligated by the tRNA ligase Trl1/Rgl1p, with the splicing reaction relieving the transcript of translational repression, producing a potent transcriptional activator (Gardner *et al.,* 2013). The levels of spliced *HAC1* (*HAC1*i) in the cell can therefore provide a quantitative measurement of UPR activation and ER stress, with higher levels of the spliced transcript indicating a more enhanced UPR.

As we have illustrated increases in secretion for two heterologous cellulases and the native invertase, we intended to elucidate the differential effects that the most effective ER-to-Golgi SNARE component (Sed5p) has on the induction of the UPR when overexpressed. In addition, we evaluated the UPR induction profiles of two cellulolytic reporter proteins, both in the presence and absence of SNARE-overexpression.

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Materials & Methods

Media and culturing conditions

Saccharomyces cerevisiae strain Y294 (*MATα*; *his3Δ*; *leu2Δ*; *lys2Δ*; *ura3Δ*) (ATCC 201160) was utilized as background strain. Yeast cells were routinely cultivated at 30˚C in YPD (Yeast Extract Peptone Dextrose) (1% yeast extract (Merck – Darmstadt, Germany), 2% peptone (Merck – Darmstadt, Germany), 2% glucose (Merck – Darmstadt, Germany)) medium. All *S. cerevisiae* transformants were selected on YPD agar supplemented with 200 µg mL^{-1} of G418 disulphate (Melford Laboratories – Ipswich, United Kingdom), whilst liquid cultures were cultivated on a rotary shaker (200 rpm) at 30˚C. *Escherichia coli* DH5α was used for general cloning procedures and strains were routinely cultivated in Luria Bertani (LB) broth (0.5% yeast extract (Merck - Darmstadt, Germany); 1% tryptone (Merck - Darmstadt, Germany); 1% NaCl (Merck - Darmstadt, Germany)) supplemented with 100 µg mL^{-1} ampicillin (Roche – Johannesburg, South Africa) at 37˚C.

Plasmid and strain construction

Standard DNA manipulation protocols were followed (Sambrook & Russel, 2001). Initial PCR products were amplified using the Phusion® High-Fidelity DNA Polymerase (Thermo Scientific - Waltham, USA) on an Applied BioSystems 2720 thermocycler, as instructed by the manufacturer, using forward and reverse primers that include *Pac*I and *Asc*I restriction sites for subsequent directional cloning into the pBKD1 yeast expression vector (McBride *et al.,* 2008), harbouring the *KanMX* selection marker for G418 disulphate resistance and the constitutive *PGK1* gene promoter and terminator sequences. Initial PCR products were first ligated into the pCloneJET 1.2 commercial vector (Fermentas – Sankt Leon-Rot, Germany) as instructed by the manufacturer, which includes the *bla* gene for ampicillin selection. PCR products/DNA fragments were routinely separated on 1% (w/v) agarose (Lonza – Rockland, USA) gels and fragments of appropriate sizes isolated using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, CA, USA).

Sequence verification was carried out using the dideoxy chain termination method with an ABI PRISM™ 3100 genetic analyser (Central Analytical Facility, Stellenbosch University). Plasmid isolations were carried out using the cetyltrimethylammonium bromide (CTAB) method (Sambrook and Russel, 2001). Yeast transformations were carried out using the LiOAc/DMSO-method (Hill et al. 1991) and gene overexpression facilitated via delta integration (Lee & Da Silva, 1997). The PCR

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primers and plasmids utilized in the study are summarized in **Table 1 & 2**, respectively, whilst the yeast strains are summarized in **Table 3**.

Table 1: PCR and qPCR primers utilized in this study.

Primer	Primer sequence
ALG9-L	5'-TGCATTTGCTGTGATTGTCA-3'
ALG9-R	5'-GCCAGATTCCTCACTTGCAT-3'
HAC1i-L	5'-GATCAGATCTACAACCGCCACT-3'
HAC1i-R	5'-GTACAGATCTCCCTCTTGCGATTGTC-3'
HAC1int-L	5'-GCGGGAAACAGTCTACCCTT-3'
HAC1int-R	5'-TGGGGCTAGTGTTCTTGTTCA-3'
HAC1all-L	5'-GATTTCCAGAGCACGAGGGG-3'
HAC1all-R	5'-TCGCAGGCTCCATTGTACAG-3'
SED5a-L	5'-CCCTGTAGAGATTGCCGAGC-3'
SED5a-R	5'-CACGTCGGTTTTCTTGAGCTG-3'
SNC1a-L	5'-CGTCATCTACTCCCTTTGACCC-3'
SNC1a-R	5'-GCTTGTAGTTCCGCAGTCCT-3'

Table 2: Plasmids utilized in this study.

Plasmid	Relevant genotype	Reference
pBKD1	*bla* δ-site $PGK1_P$-$PGK1_T$ *kanMX* δ-site	McBride *et al.* (2008)
pBKD1-*SED5*	*bla* δ-site $PGK1_P$-*SED5*-$PGK1_T$ *kanMX* δ-site	Van Zyl *et al.* (2015)
pBKD1-*SNC1*	*bla* δ-site $PGK1_P$-*SNC1*-$PGK1_T$ *kanMX* δ-site	Van Zyl *et al.* (2014)
pBKD1-*SSO1*	*bla* δ-site $PGK1_P$-*SSO1*-$PGK1_T$ *kanMX* δ-site	Van Zyl *et al.* (2014)
pBKD1-*HAC1i*	*bla* δ-site $PGK1_P$-*HAC1i*-$PGK1_T$ *kanMX*δ-site	This work

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Table 3: The yeast strains utilized in this study.

Yeast Strain	Relevant genotype	Reference
S. cerevisiae Y294	MATα *his3Δ leu2Δ lys2Δ ura3Δ*	ATCC 201160
Y294_*HAC1i*	MATα *his3Δ leu2Δ lys2Δ ura3Δ-PGK1p-HAC1i-PGK1t-kanMX*	This work
Y294_*SNC1*	MATα *his3Δ leu2Δ lys2Δ ura3Δ-PGK1p-SNC1-PGK1t-kanMX*	This work
Y294_*SSO1*	MATα *his3Δ leu2Δ lys2Δ ura3Δ-PGK1p-SSO1-PGK1t-kanMX*	This work
Y294_*SED5*	MATα *his3Δ leu2Δ lys2Δ ura3Δ-PGK1p-SED5-PGK1t-kanMX*	This work
S. cerevisiae Y294 (*CEL3A* Parental)	*ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-fur1::LEU2*	Den Haan *et al.* (2007) *
Y294_*CEL3A_SED5*	*ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-his3/HIS3-PGK1p-SED5-PGK1t-kanMX-fur1::LEU2*	Van Zyl *et al.* (2015)
S. cerevisiae Y294 (*CEL7A* Parental)	*ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3-fur1::LEU2*	Ilmén *et al.* (2011) **
Y294_*CEL7A_SED5*	*ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3-PGK1p-SED5-PGK1t-kanMX-fur1::LEU2*	Van Zyl *et al.* (2015)

* Accession nr for *Sf*-Cel3A: AEV40916.1

* * Accession nr for *Te*-Cel7A: AAL89553

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Quantification of extracellular invertase activity

Yeast strains of interest were cultivated in 5 mL YPD, inoculated from plate-grown cultures, on rotation (200 rpm) for 72 hours. These strains were then inoculated in triplicate at an A_{600nm} of 1 into 20 ml YP medium, with 2% galactose (Sigma-Aldrich – St. Louis, USA) added after autoclaving. Triplicate 3,5-Dinitrosalicylic acid (DNS) assays (adapted from Bailey *et al.,* 1992), to quantify extracellular invertase activity were performed, with one unit defined as the amount of enzyme required for the conversion of 1 µM substrate per minute. Assays were conducted in parallel on the respective strains at 24 hour intervals, starting at 48 hours, using 10% sucrose and appropriate media and reaction blanks.

Quantification of spliced HAC1 expression levels

Yeast strains of interest were cultivated in 5 mL YPD, inoculated from plate-grown cultures, on rotation (200 rpm) for 72 hours. These strains were inoculated at an A_{600nm} of 1 into 20 ml YPD in 125 ml Erlenmeyer flasks and cultivated for 17 hours, ensuring all strains had entered the logarithmic growth phase. Total RNA extractions were carried out using the Nucleospin®RNA Kit (Macherey-Nagel – Düren, Germany) and total RNA samples subsequently treated with the TURBO DNA-*free*™ kit (Ambion – Texas, USA) according to the manufacturer's instructions. The integrity of the RNA samples was determined and RIN-values obtained following analysis with a Bioanalyzer (Aligent – Santa Clara, USA) (Central Analytical Facility – Stellenbosch). The DNase-treated total RNA samples were used as template for first-strand cDNA synthesis using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific – Massachusets, USA) according to the manufacturer's instructions. Real-Time PCR was carried out using the KAPA^{TM} HRM Fast PCR Kit on an Applied Biosystems StepOne Real-Time PCR System. RT-qPCR was carried out using two sets of primers, one of which anneals within a permanently expressed area of the *HAC1* exon and the other within a section of the 252 bp intron, binding within both the exon and the intron, which would be progressively less expressed as *HAC1* splicing increases in relation to UPR induction. This strategy enabled relative expression-level quantification of *HAC1* splicing (*HAC1i*) and UPR induction using the variance between the two interrelated amplification products in relation to the *ALG9* reference gene. Transcriptional quantification was carried out using the relative standard curve method (Applied Biosystems: Guide to Performing Quantitation of Gene Expression Using RT qPCR; 2008). Wherever possible, samples were either kept on ice during use in a laminar flow cabinet (LabAire Systems – Minneapolis, USA) or

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stored at -80°C, whilst all efforts were continually made to ensure an RNase-free environment. All primers used in this study are included in **Table 1**.

Results:

The effects of SNARE overexpression on native invertase secretion

Transformants of the Y294 *S. cerevisiae* strain were confirmed using pairs of promoter-specific (*PGK1*$_p$) and gene-specific primers, with at least five transformants screened for increased extracellular invertase activity per transformation to identify the most promising SNARE-overexpressing (*SNC1*, *SSO1*, *SED5*) strain candidate. The concentration of reducing sugars (glucose) released from the sucrose substrate was determined, allowing for the determination of extracellular invertase activity, and the 48 and 72 hour readings are represented in **Fig. 1**. The overexpression of Snc1p, Sso1p and Sed5p, the most effective SNAREs identified to increase secretion of two heterologous cellulases (*Sf*-Cel3A and *Te*-Cel7A), led to increases in extracellular invertase activity after 72 hours of 32%, 53% and 14%, respectively, relative to the Y294 parental strain (**Fig. 1**). Both the Snc1p and Sso2p improvements were confirmed to be statistically significant as per the Student's T-test. Using our delta-integration strategy, each of these strains had integrated at least one additional SNARE gene copy, with our transformation and selection methodology generally resulting in single genome integrations (Van Zyl *et al.,* 2014; Van Zyl *et al.,* 2015).

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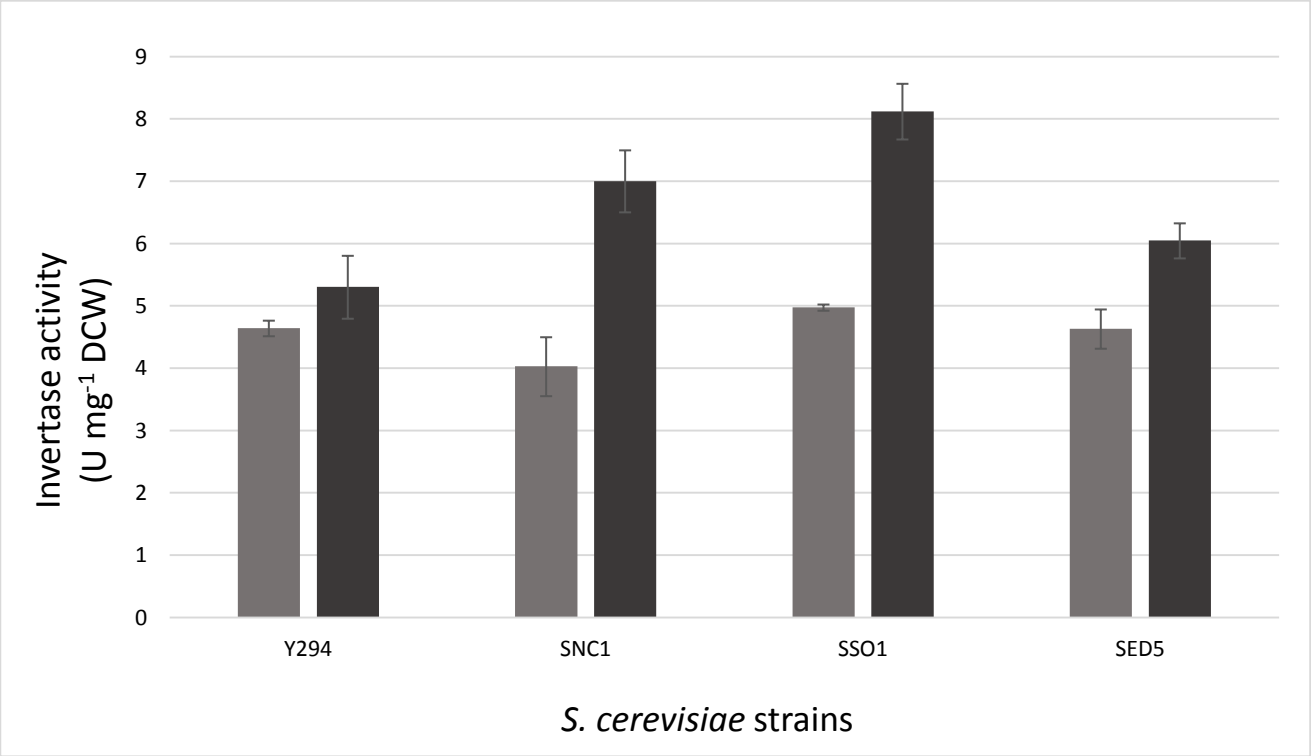

Fig. 1: Extracellular invertase activity of singly overexpressing *S. cerevisiae* Y294 strains (overexpressing *SNC1, SSO1* and *SED5*) and the Y294 parental strain at 48 and 72 hour intervals. All values represent mean values of assays done in triplicate with error bars indicating the standard deviation.

The effects of SNARE overexpression and heterologous cellulase production on UPR induction

The relative levels of spliced *HAC1i* expression were determined for a selection of the most promising strains constructed (Chapters 3 & 4). The strain collection (**Table 3**) included a positive control (*S. cerevisiae* Y294_*HAC1i*) harbouring at least one additional copy of the spliced *HAC1i* gene, integrated via homologous recombination into native delta sequences in the yeast genome, and a strain overexpressing *SED5* without any heterologously expressed cellulases (*S. cerevisiae* Y294_*SED5*). Also included were the strains harbouring the *Sf*-Cel3A and *Te*-Cel7A constructs with and without the overexpression of *SED5,* the ER-to-Golgi SNARE component that was most effective in improving secretion for both reporter proteins. This repertoire of strains allowed for the investigation into the effects of differential cellulolytic reporter proteins on UPR induction, whilst the effects of *SED5* overexpression in the presence and absence of the reporter proteins whose secretion it enhanced, can also be elucidated.

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The heterologous production of *Sf*-Cel3A led to enhanced levels of spliced *HAC1i* (**Fig. 2**). Therefore, this protein places a significant amount of stress on the ER compared to its *Te*-Cel7A counterpart. The overexpression of *SED5* evoked no enhanced UPR response relative to the basal levels in the Y294 reference strain, although a clear decrease in UPR activation was observed when overexpressed in conjunction with *Sf*-Cel3A, compared to only the heterologously expressed protein. Neither the *Te*-Cel7A, nor the *Te*-Cel7A-*SED5* strain led to changes in the relative levels of spliced *HAC1*i, illustrating a negligible UPR response that correlates well with results reported by Ilmén *et al.* (2011) for this particular heterologous protein.

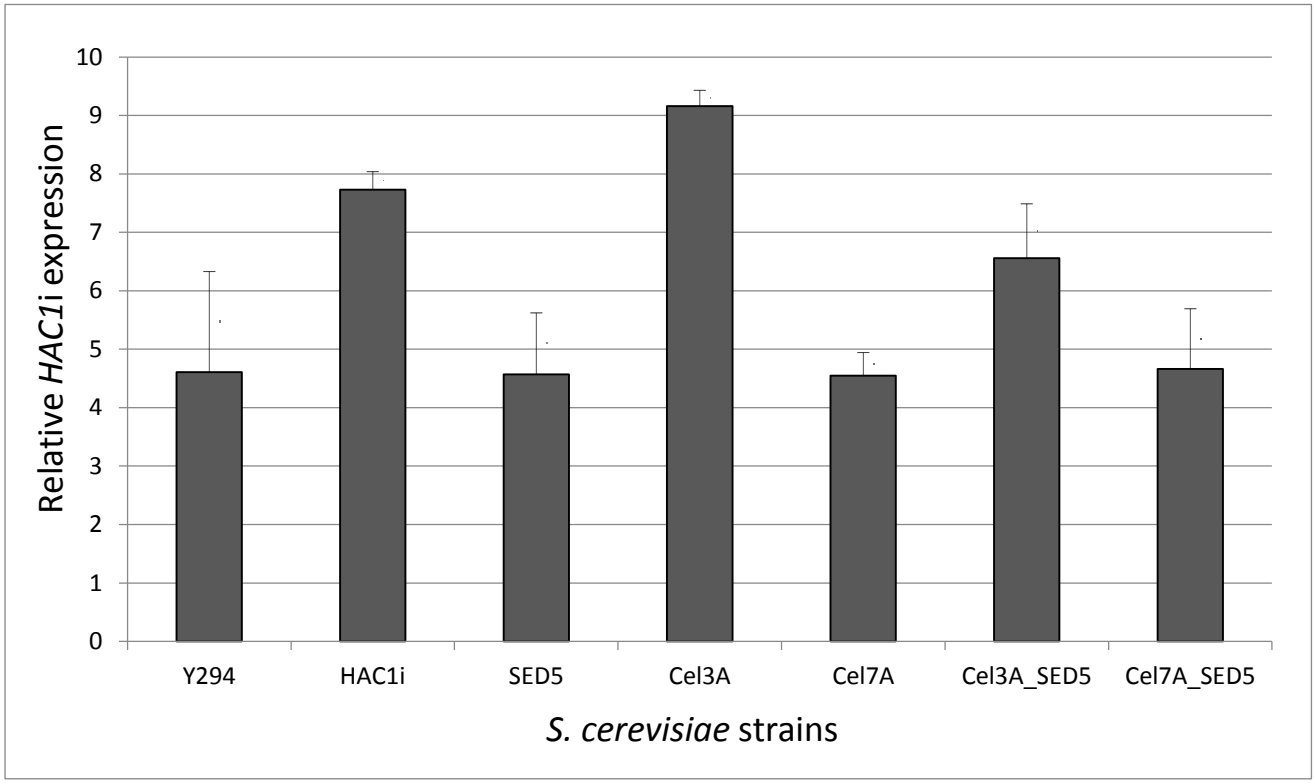

Fig. 2: Relative levels of spliced *HAC1* (*HAC1*i) for a selection of strains investigated. The *HAC1*i-overexpressing control strain is included, along with the Y294 strain overexpressing *SED5.* Strains expressing *Sf*-Cel3A or *Te*-Cel7A are compared with strains producing these proteins whilst simultaneously overexpressing *SED5* (Cel3A_SED5 and Cel7A_SED5). Error bars indicate the standard deviation from the mean value of triplicate samples.

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Discussion:

The overexpression of several SNARE components functioning between the ER and Golgi, and between the Golgi and plasma membrane led to noticeable improvements in secretion for both the heterologous *Sf*-Cel3A and the *Te*-Cel7A in *S. cerevisiae* (Van Zyl *et al.,* 2014; Van Zyl *et al.,* 2015). Two questions that these improvements therefore pose are: 1) whether the actual rate of protein production is increased as a whole; and 2) whether it is the relief of transport bottlenecks at particular points within the secretory pathway that are responsible for the improved secretory phenotype. Our attempts to further elucidate these questions were initiated by investigating the effects of overexpressing the most effective ER-to-Golgi (Sed5p) and exocytic (Sso1p and Snc1p) SNAREs on the secretion of the native *S. cerevisiae* invertase, in order to establish whether secretory improvements uniformly apply to natively produced proteins.

All the investigated SNAREs had a positive effect on the secretion of the native invertase with Snc1p, Sso1p and Sed5p leading to increases in extracellular activity of 32%, 53% and 14%, respectively. These SNARE components are therefore able to positively enhance the secretory reaction of both heterologous and natively produced proteins at both the *cis*-Golgi and plasma membrane interface. It is interesting to note that the most effective ER-to-Golgi component (Sed5p) produced a diminished effect on the native protein secretion phenotype compared to its exocytic counterparts (Snc1p and Sso1p). This reaffirms the notion that heterologous proteins are significantly limited with regards to their cumulative secretory reaction at an earlier point in the pathway, i.e. between the ER and the Golgi, as opposed to the final exocytic step. Considering the evolutionarily adapted secretory process relating to native proteins, it is perhaps not surprising that the folding and export of foreign, heterologous proteins become more problematic at both membrane interfaces.

The yeast t-SNARE Sed5p plays a major role in facilitating not only ER-to-Golgi protein traffic (in conjunction with Bos1p, Sec22p and Bet1p), but intra-Golgi (in conjunction with Gos1p, Ykt6p and Sft1p) transport as well (Parlati *et al.,* 2002). Furthermore, it was suggested Sed5p could be involved in Golgi-endosome protein traffic as well (Pelham *et al.,* 1999). Sed5p has also been show to form other SNARE complexes *in vitro* (Tsui *et al.,* 2001). The production of *Sf*-Cel3A has a more pronounced UPR induction profile to that of *Te*-Ce7A (**Fig. 2**), which correlates well with the simulated ER-stress profiles we have previously reported for both reporter proteins (Van Zyl *et al.,* 2015) and the reduced UPR profile for the *Te*-Cel7A reported in literature (Ilmén *et al.,* 2011). However, when Sed5p, which has been shown to improve secretory titers for *Sf*-Cel3A and *Te*-Cel7A,

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was overexpressed in conjunction with *Sf*-Cel3A, the UPR response is measurably reduced. The production of heterologous proteins in *S. cerevisiae* can saturate the secretory pathway, leading to the accumulation of both unfolded and misfolded proteins in the ER, disrupting the folding homeostasis in the ER and leading to progressive UPR activation (Shusta *et al*., 1998; Kaufman *et al*., 2002; Huang & Shusta, 2006). It is therefore postulated that the over-production of Sed5p effectively assists the cell in transporting a higher flux of a heterologous proteins from the ER to the Golgi. This could potentially help to relieve the build-up of unfolded and misfolded proteins in the ER-lumen when the cell is presented with a particularly problematic heterologous protein - in this case the ER-stress-inducing *Sf*-Cel3A. This would further underline the importance of the Sed5p t-SNARE within the overall cascade of secretory machinery components.

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Chapter 6:

General Discussion

The production of second generation bioethanol as an alternative fuel source from lignocellulosic substrates using recombinant yeast strains is not yet financially viable, when compared to fossil fuels produced at large scale in mature refineries (Stephen *et al.,* 2012). Two of the major price components contributing to its lack of financial feasibility include the high energy cost of lignocellulose pre-treatment at high temperatures and the high production cost of the commercial cellulases required to release fermentable sugars from the substrate. In fact, the cost of enzymatic saccharification is regarded as the third most expensive price component in the production of lignocellulosic bioethanol (Pu *et al.,* 2008; Aden & Foust, 2009; Stephen *et al.,* 2012; Isola, 2013). An increase in the attainable titers for heterologous cellulases could significantly increase the rate and efficiency of lignocellulose conversion to fermentable sugars, increasing the overall economics of the process. The current market for FDA-approved therapeutic proteins/antibodies (approximately $100 billion per annum according to BIOPHARMA®) means that the optimization of yeast secretion systems is widely desirable (Harford *et al.,* 1987; Schmidt, 2004; Rader, 2007; Idiris *et al.,* 2010; Hou *et al.,* 2012). An increase in the heterologous protein secretion capacity of *S. cerevisiae* would therefore benefit not only the renewable energy sector, but also the biopharmaceutical industry.

The production of heterologous proteins in *S. cerevisiae* is limited, with production titers often lower than 1% of theoretical estimates (Müller *et al.,* 1998; Liu *et al.,* 2014). Levels of secreted heterologous product also vary greatly for different heterologous proteins produced in *S. cerevisiae*, presumably as specific stages within the secretory pathway become inefficient and limiting (Idiris *et al.,* 2010). The inefficiency and limitations can generally be attributed to a particular quality of the heterologous polypeptide such as its hydrophobicity, glycosylation requirements and required levels of disulphide bond formation. Much focus is therefore placed on attempting to elucidate the specific bottlenecks within the secretory pathway that ultimately contribute to a decreased secretory phenotype for heterologous proteins in general. In this study, we attempted to elucidate whether the over-production of native *S. cerevisiae* ER-to-Golgi and exocytic SNARE proteins, components facilitating vesicular membrane fusion reactions at the *cis*-Golgi and plasma membrane, respectively, could increase secretory titers for two industrially significant fungal cellulases - *Sf*-Cel3A and *Te*-Cel7A, in

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addition to the native *S. cerevisiae* invertase. Further attempts were made to investigate some of the fundamental physiological effects of these genetic modifications, both in terms of growth capability and the cellular stress response, whilst attempting to elucidate the key SNARE components that could play determining roles in limiting or enhancing membrane fusion at the ER-Golgi and plasma membrane interfaces for different proteins.

SNARE overexpression differentially improved the secretory phenotype for heterologous and native proteins

Adequate SNARE gene dosage could, in many cases, improve the overall secretory phenotype that could be quantitatively and qualitatively measured. However, certain ER-to-Golgi and exocytic SNARE components (e.g. Bos1p) had negligible or even negative effects on secretion, which may be attributed to the variable levels of SNARE gene expression and variances in intracellular protein abundance. Ghaemmaghami *et al.* (2003) estimated that protein abundance in yeast can range from fewer than 50 to more than 10^6 molecules per cell. In this study, differences in improvements to the secretory phenotype were observed for both the Snc1/Snc2p and Sso1p/Sso2p paralogs, correlating well with suggestions in literature that these components are not only functionally distinct to some extent, but differ with regards to their preferential expression. One ER-to-Golgi t-SNARE component, Sed5p, was able to provide the most improved secretory phenotype for both heterologous cellulases (*Te*-Cel7A & *Sf*-Cel3A), with variable increases correlating well with the protein-specific nature of SNARE-related improvements. In addition, two exocytic components, the t-SNARE Sso1p and the v-SNARE Snc1p, yielded the most positive effects for *Sf*-Cel3A and *Te*-Cel7A, respectively.

Simultaneous overexpression of cognate SNARE components did not lead to proportionate additive secretory improvements, which could be attributed to the titration of mutual binding partners and regulatory components or, alternatively, the saturation of mutual transcription factors. The combinatorial overexpression of the two most effective ER-to-Golgi and exocytic SNAREs identified for the improvement of *Sf*-Cel3A production (Sed5p and Sso1p, respectively) led to significant improvements in the secretory phenotype, although cell lysis and the release of this largely membrane-bound cellulolytic reporter protein could partially explain the disproportionately enhanced extracellular activity. The afore-mentioned is especially likely as the total activity (cell and extracellular fractions) did not yield equally significant improvements, whilst visible cell debris in the batch cultured media became apparent when contrasted with the parental strain after 72 hours.

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Greater SNARE gene dosages did not lead to a proportionate secretory phenotype improvement as the majority of our most improved strains had only integrated single additional SNARE gene copies, whilst our screening methodology ensured only strains with significantly improved extracellular activity were selected for further analysis. Episomal variance between our most improved strains was negligible, meaning that improvements to the secretory phenotype could be principally attributed to SNARE-overexpression as opposed to differences in the basal levels of heterologous protein produced by the respective strains.

The positive effects that components of the SNARE complexes at both the *cis*-Golgi and plasma membrane had on secretion of *Sf*-Cel3A and *Te*-Cel7A indicate that limitations at these membrane interfaces begin to emerge when faced with the overexerting secretory demand of heterologous cellulases. Conversely, when considering the positive effects that the most effective exocytic SNAREs (Sso1p and Snc1p) had on secretion of the native invertase, compared to miniscule effects of the most effective ER-to-Golgi counterpart (Sed5p), it is clear that the yeast is more efficient in both the folding and transport of native proteins between the ER and the Golgi compared to the heterologous counterparts.

The physiological effects of SNARE gene overexpression

Secretory improvements relating to SNARE overexpression are not attained without cost as many of the improved secretory phenotypes were associated with at least a decrease in growth vigour. Selected strains illustrated a reduced cell vitality compared to the parental strains (*Sf*-Cel3A-*SSO1, Sf*-Cel3A-*SED5* and *Sf*-Cel3A-S1S5), or an increased vulnerability to ER-stress, with the latter particularly prevalent in the *Sf*-Cel3A-producing strains. These phenotypic responses, especially relating to the overexpression of the ER-to-Golgi t-SNARE Sed5p (which led to notable decreases in ethanol and osmotolerance) could at least in part be attributed to the additional energy cost of producing heterologous proteins at increased titers. In addition, the perturbation of intracellular traffic for specific solutes (e.g. glycerol) associated with maintaining intracellular homeostasis under stress-induced conditions due to ethanol and osmotic stress, could be an additional contributing factor though these mechanisms would need to be separately investigated. The mammalian Golgi collapses into the ER when placed under significant osmotic stress, a phenomenon attributed to imbalanced anterograde and retrograde protein transport between the ER and the Golgi (Lee & Linstedt, 1999). Since Sed5p has been suggested to be critical to the maintenance of overall Golgi structural

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distribution, aberrant distribution of this protein, potentially exacerbated by its overproduction, may explain some of the growth and stress susceptibilities we have identified.

The dynamic flux of Sed5p between the ER and the Golgi is essential to maintaining Golgi structure and function, especially relating to its phosphorylation state (Hardwick & Pelham, 1992; Weinberger *et al.,* 2005), which may explain why a disproportionate increase of novel Sed5p cannot be adequately processed by its regulatory components, leading to a sub-optimally functioning Golgi structure and a reduction in cell vitality. Furthermore, overexpression of *SED5* on multicopy vectors reduced the efficiency of ER-Golgi protein transport, in addition to increasing the number putative transport vesicles (Hardwick & Pelham, 1992). The afore-mentioned is especially relevant given that the maintenance of the steady state structure of the Golgi complex requires a delicate balance of inward and outward membranous traffic, whilst a significant increase in the levels of Sed5p, as described in the multicopy investigation, would lead to a predictably compromised ER-Golgi continuum (Hicks & Machamer, 2005). The Golgi of *S. cerevisiae* is unique in that it does not possess the classical stacked cisternae as is common in other yeast such as *P. pastoris,* but rather individual cisternae that are scattered throughout the cytoplasm (Franzusoff *et al.,* 1991; Preuss *et al.,* 1992; Nakano, 2008). This may indicate that the secretory phenotypes we have illustrated, in addition to the physiological side-effects, warrant further investigation in alternative production hosts with a more structured ER-Golgi continuum.

A higher energy cost and metabolic demand is associated with the ER folding and Golgi processing subsystems in the secretory pathway compared to other components (Feizi *et al.,* 2013). This may help to explain why the disproportionate overproduction of a component (Sed5p) that is paramount to the functioning of both of these subsystems, can have such a prominent effect on secretion compared to the exocytic SNARE counterparts when producing heterologous proteins. The derogatory growth effects of the entire ER-to-Golgi and exocytic SNARE repertoire we have investigated also became evident when simultaneous overexpression of all relevant components had been facilitated, illustrating that a metabolic burden did take effect. This is supported by the fact that all four ER-to-Golgi SNARE components could not be simultaneously overexpressed whilst retaining cell viability in a strain producing the more burdensome *Sf*-Cel3A. The mechanisms underlying both the increased susceptibility to ethanol and osmotic stresses would have been an interesting aspect to investigate, though the characterization of our collection of strains in relation to the completion of the project did not allow for this additional work to be included in the workflow of the study.

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The effects of SNARE overexpression on the UPR response

This increased stress placed on the early secretory machinery by heterologous proteins is exemplified by the significant unfolded protein response (UPR) associated with the heterologous production of *Sf*-Cel3A. This result correlated well with the increased vulnerability to ER-stress we have also attributed to the production of this protein (Van Zyl *et al.,* 2015), whilst a much reduced UPR associated with the production of *Te*-Cel7A correlates well with results reported in literature for this cellulolytic reporter protein (Ilmén *et al.,* 2011). An enhanced UPR is evoked by the build-up of unfolded and misfolded proteins in the ER (Kaufman, 1999) and it is therefore interesting to note that the UPR response can be diminished given an efficient Sed5p gene dosage, which leads to an increased secretory phenotype for *Sf*-Cel3A. The basal levels of UPR induction are not proportionately increased given SNARE (Sed5p) overexpression in isolation. This may indicate that a cellular shortage or demand for this SNARE component under heterologously producing conditions could at least in part be remedied given efficient gene dosage.

Sed5p has been illustrated to be relatively promiscuous in its binding capacity, being able to form at least nine other SNARE complexes *in vitro* (Tsui *et al.,* 2001) and having been implicated in ER-to-Golgi, intra-Golgi and endosome-Golgi fusion reactions (Weinberger *et al.,* 2005). With this in mind, it is postulated that Sed5p is able to alleviate some of the secretory stress associated with the build-up of misfolded and unfolded heterologous proteins in the ER through improved modulation of closely associated membrane fusion reactions and an improved flux of heterologous protein from the ER to the Golgi. This could be attributed to enhancing the anterograde fusion reactions with the *cis*-Golgi, in addition to intra-Golgi trafficking improvements which, if folding homeostasis is allowed to partially revert towards equilibrium as is required, would explain the decreased UPR response associated with the lowering of misfolded and folded protein levels in the ER. The increased secretory phenotype associated with both *Te*-Cel7A, *Sf*-Cel3A and the native invertase gives credence to an enhanced ER-to-Golgi protein transition upon Sed5p overexpression.

Concluding remarks

SNAREs are considered to be present in excess within the cell, potentially concentrating in clusters that constitute spare pools that may not be readily available for fusion-related interactions (Bethani *et al.,* 2009; Di Sansebastiano, 2013). It is therefore interesting to note the positive effects that SNARE overexpression is able to achieve at such limited gene dosages (Van Zyl *et al.,* 2014; Van Zyl *et*

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al., 2015). Considering the results we have obtained with a relatively modest engineering approach with regards to SNARE gene copy number and dosage, and bearing in mind the positive results reported in literature relating to the SM-proteins (Sec1p and Sly1p) facilitating the assembly reactions of both SNARE complexes we have investigated, we believe that the respective SNARE-related fusion reactions at the *cis*-Golgi and plasma membrane are noteworthy bottlenecks to the overall secretory process that, given an efficient SNARE gene dosage, can at least partially be alleviated. Additional investigations into the levels of SNARE RNA, in both singly and simultaneously overexpressing strains, would have added a further dimension to the study and these should be included in follow-up studies. Analysis of SNARE concentration increases via Western blots proved challenging as adequate antibodies were not readily available. In the few reports where quantitative differences in SNAREs are detected, these SNAREs are being expressed on multicopy plasmids, making it doubtful that our single integration strategy would have yielded quantifiable results (Petkovic *et al.,* 2014). However, if the relevant antibodies can be procured, they may in future help to shed light on the mechanisms that yielded the enhanced secretion phenotypes.

Future work should also investigate the plethora of regulatory components that collectively modulate SNARE assembly (SM, Rab proteins and tethering factors) and disassembly (α-SNAP (Sec18p) and NSF (Sec17p)) to determine whether further bottlenecks relating to vesicular fusion reactions in the secretory pathway can be relieved. Potential investigations may choose to combine the secretory-enhancing SM (Sec1p and Sly1p) and SNARE components (Sso1p, Snc1p and Sed5p) so far identified in literature. The handful of SNARE proteins involved in the release of the protein transport vesicles from the *trans*-Golgi, in addition to those facilitating intra-Golgi transport, also warrants further investigation. An expansion of candidate reporter proteins to include relevant pharmaceutical indicators such as Fab and single chain variable fragment antibodies (scFvs) (Frenzel *et al.,* 2013) could also prove fruitful. For example, over-expression of protein disulphide isomerase (*PDI*) increased secretory titers for five scFvs by 2-8-fold in *S. cerevisiae* (Shusta *et al.,* 1998), whilst over-expression of *PDI* increased secretion of *Pyrococcus furiosus* β-glucosidase by up to 3-fold (Powers et al. 2007) in the same host. Unfortunately, comparative studies simultaneously transcending the biomedical and industrial research spheres, with regards to their respective model reporter proteins, are significantly lacking.

Systems and synthetic biology approaches have notably improved heterologous protein production in *S. cerevisiae* over recent years, helping to address some of the issues associated with low yields and post-translational modifications (Rodríguez-Limas, 2013). Our current research adds credence to the

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notion that SNARE proteins form a key element within a larger cascade of interacting protein classes that, given efficient gene dosage, can contribute significantly to the future improvement of *S. cerevisiae* as commercial heterologous protein production host, within both a cellulolytic and biopharmaceutical context. Whilst ultimately falling short of producing the required titers for cellulolytic enzymes required for second-generation bioethanol production, these results may assist in developing engineering strategies focusing purely on more efficient eukaryotic protein production hosts such as *Pichia pastoris* that do not require fermentative capabilities. Furthermore, SNARE components seem to become limiting under heterologously producing conditions and constitute a notable element of the secretory bottlenecks at both the ER-Golgi continuum and the plasma membrane interface that at least in part contribute to a reduced secretory phenotype for heterologous proteins.

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Chapter 7

Conclusions

SNARE proteins make up an interesting component of the secretory cascade involved in the production, transport and delivery of extracellular proteins. Results obtained in this study indicate that whilst SNARE-overexpression does not complete the journey to ultimately reaching the secretory titers required to make the production of second generation bioethanol financially viable, they nevertheless hold great potential as contributory factors in this regard within both a cellulolytic and biopharmaceutical context for heterologous protein production.

From the data we have presented in this study, the following conclusions can be made:

- The overexpression of specific ER-to-Golgi and exocytic SNARE components led to notable improvements in heterologous cellulase and native invertase secretion.
- Improvements relating to differential SNARE overexpression were largely protein-specific.
- Simultaneous over-expression of cognate ER-to-Golgi and exocytic SNARE components did not lead to proportionate additive improvements to the secretion phenotypes obtained when these components were individually overexpressed.
- Simultaneous overexpression of cognate SNARE components led to a reduction in cell vitality and resistance to osmotic and ethanol stresses, especially for the exocytic SNAREs.
- The co-overexpression of components from different SNARE complexes, specifically Sed5p and Sso1p, led to drastic improvements in secretion, along with a significant reduction in cell viability exemplified by cellular lysis later in batch cultivation.
- The copy number of the overexpressed SNAREs did not have a significant impact on the secretory phenotype, with single copy integrations often leading to the most effective secretory phenotypes, though it must be acknowledged that the site of genomic integration can impact on the eventual SNARE expression efficiency.

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- Overproduction of components of the ER-to-Golgi and exocytic SNARE complexes (Sed5p, Sso1p and Snc1p) improved native invertase secretion.
- The Snc1p/Snc2p and Sso1p/Sso2p orthologs led to differing secretory phenotypes when overexpressed, confirming suggestions in literature that they are not only partially functionally distinct, but also preferentially expressed (Snc1p, Sso1p), leading to differential effects on the secretory phenotype.
- The Sed5p t-SNARE, given the specific gene dosage we have facilitated, was the most effective ER-to-Golgi component to increase secretory titers for both *Sf*-Cel3A and *Te*-Cel7A, whilst it was also able to improve native invertase secretion.
- Overproduction of the Sed5p t-SNARE, while improving the secretory phenotype, led to decreased ethanol, osmotic and secretory stress resistance, as well as diminished cellular growth vigor.
- The *Sf*-Cel3A had a more pronounced effect on the induction of the UPR, illustrating the problematic effects of this heterologous cellulase on the native ER folding machinery and highlighting a problematic area of the secretion pathway for other heterologous proteins.
- Overproduction of Sed5p reduced the levels of UPR induction in a *Sf*-Cel3A-producing strain, suggesting that improved membrane traffic between the ER and the Golgi could relieve the build-up of unfolded and misfolded proteins in the ER lumen that leads to increased levels of UPR activation.

The findings of Ruohonen *et al.* (1997), Gasser *et al.* (2007), Hou *et al.* (2012) and Xu *et al.* (2013), together with the secretory improvements we have illustrated in *S. cerevisiae* for the ER-to-Golgi and exocytic SNAREs at over-expressed levels (Van Zyl *et al.,* 2014; Van Zyl *et al.,* 2015, Chapter 5), demonstrate that invigorating these membrane trafficking steps, with particular emphasis on the respective SNARE components, is a novel and feasible approach to improving the heterologous protein secretion capacity of *S. cerevisiae.* Our results illustrate that over-expression of specific anterograde SNARE components was sufficient to overcome the proportionate lack of accessory proteins mediating their SNARE assembly reactions, whilst to some extent relieving secretory bottlenecks associated with specific heterologous cellulases.

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Addendum A

(Van Zyl *et al.,* 2015)

Supplementary data – Figure S1

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Screening Data:

Preliminary screening, to isolate transformants illustrating the highest extracellular activity, was carried out on at least 20 transformant colonies per strain constructed, with the strain yielding the highest extracellular enzyme activity being selected for further study. Our screening methodology, selecting only top transformants, therefore attempted to assist in isolating only the most improved transformants in order to overcome the expressional variability associated with different delta integration loci within the yeast genome.

Fig. S1.1 ***Sf*-Cel3A_*BOS1***

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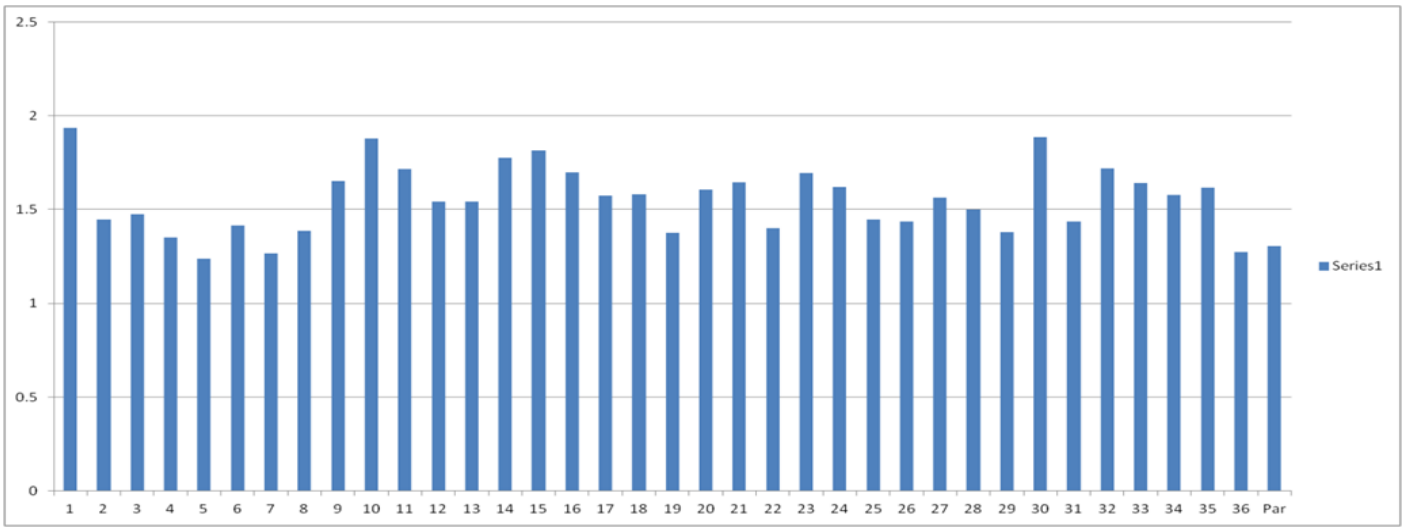

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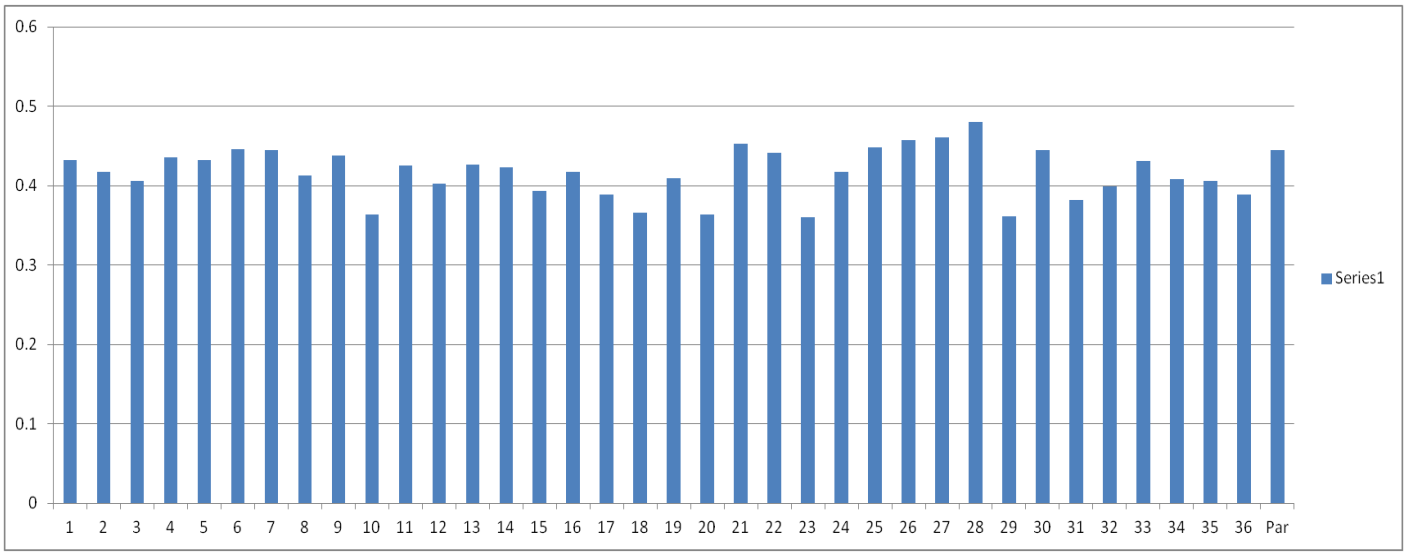

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Fig. S1.2 ***Sf*-Cel3A_*BET1***

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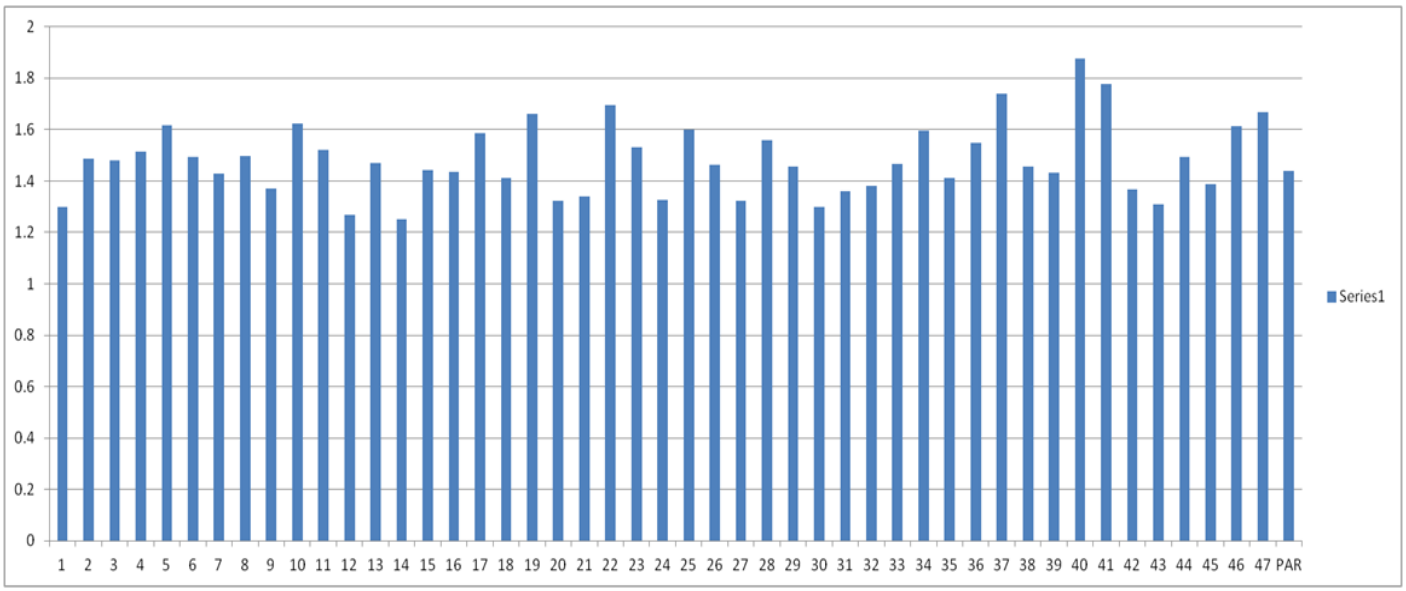

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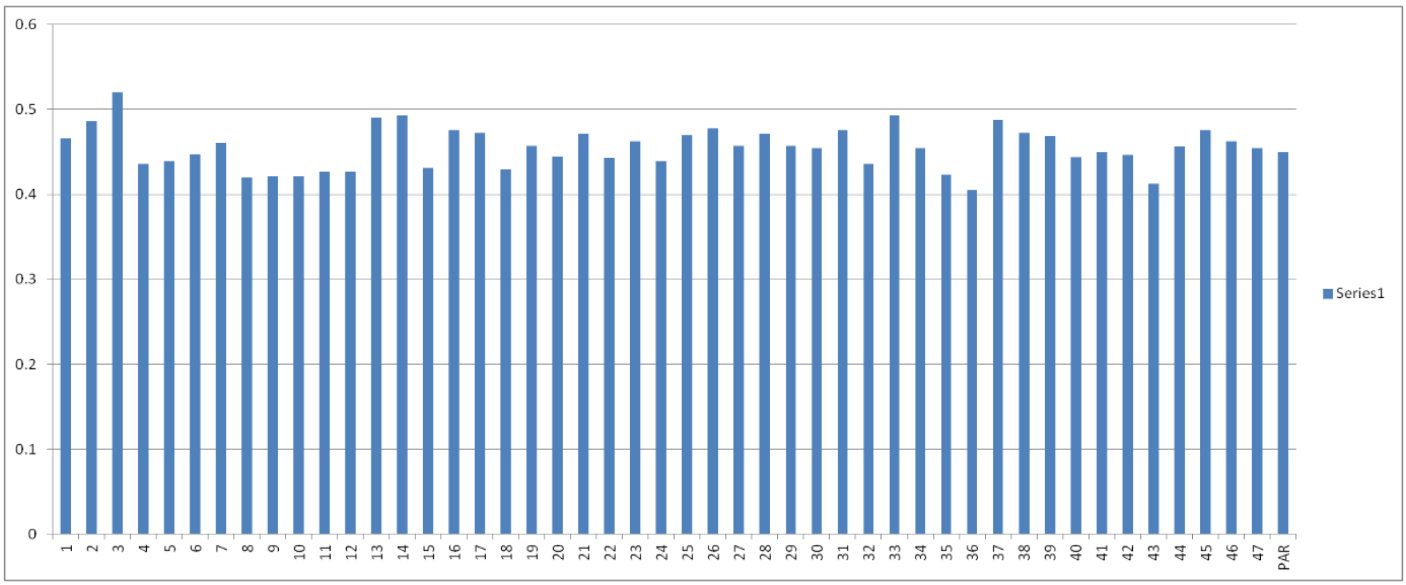

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Fig. S1.3 ***Sf*-Cel3A_*SEC22***

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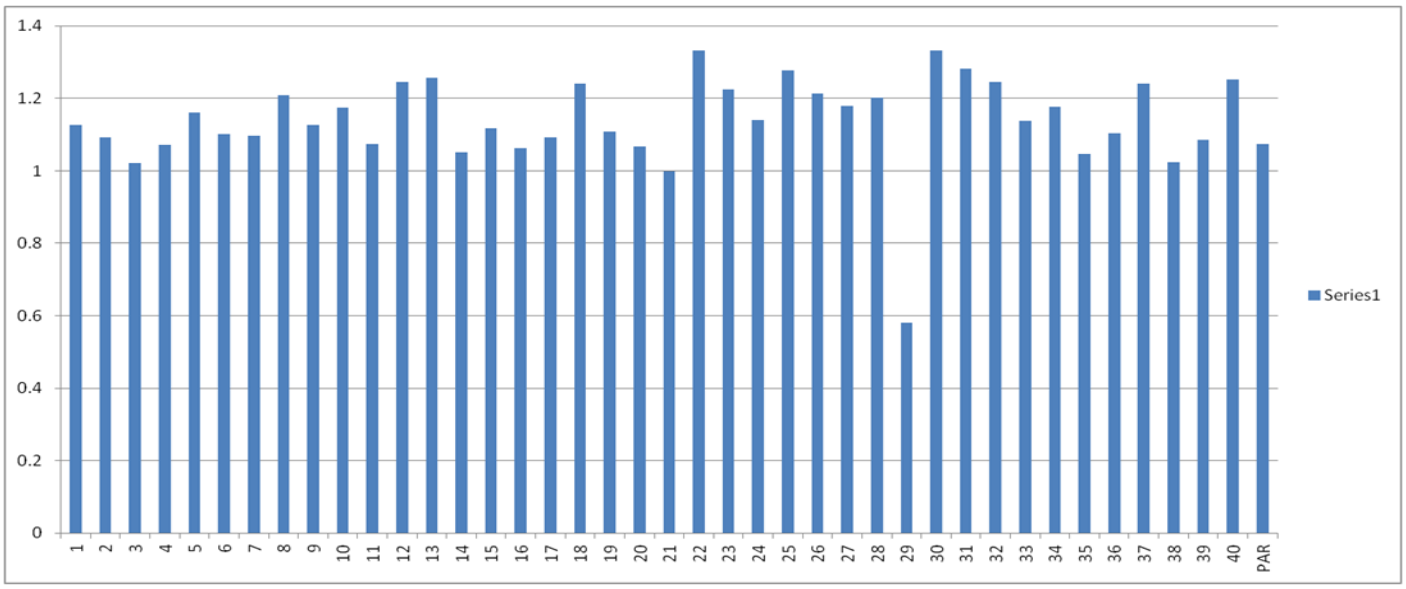

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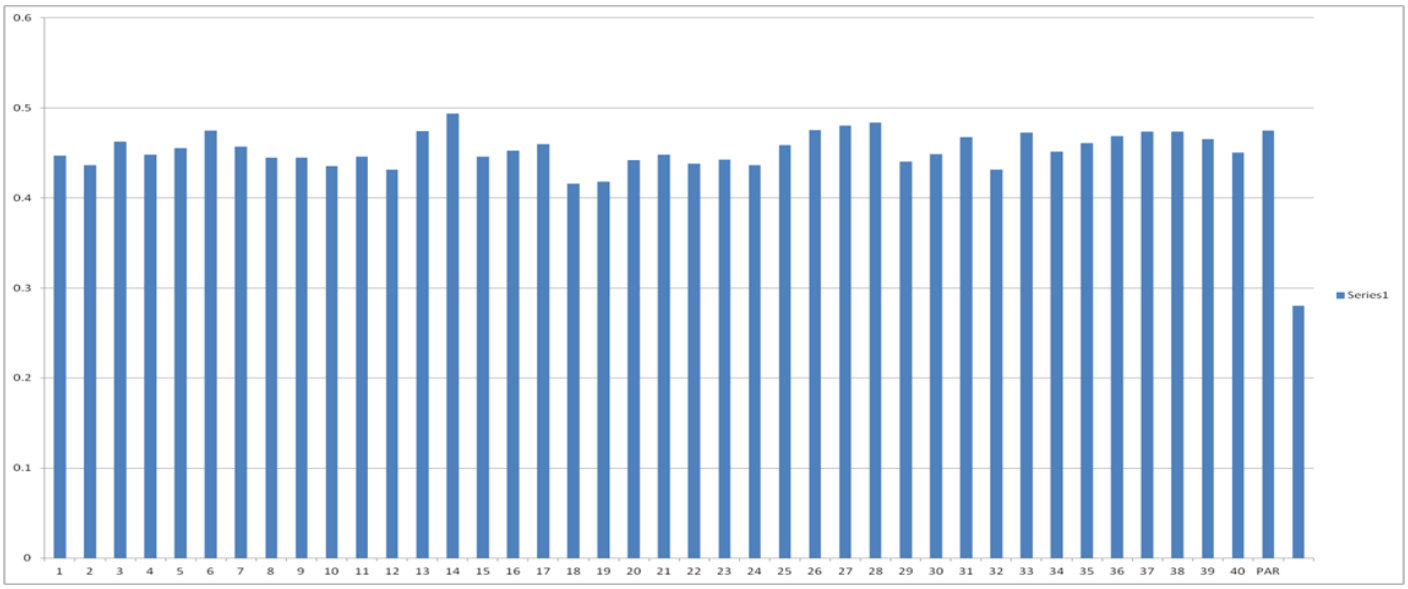

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Fig. S1.4 ***Sf*-Cel3A_*SED5***

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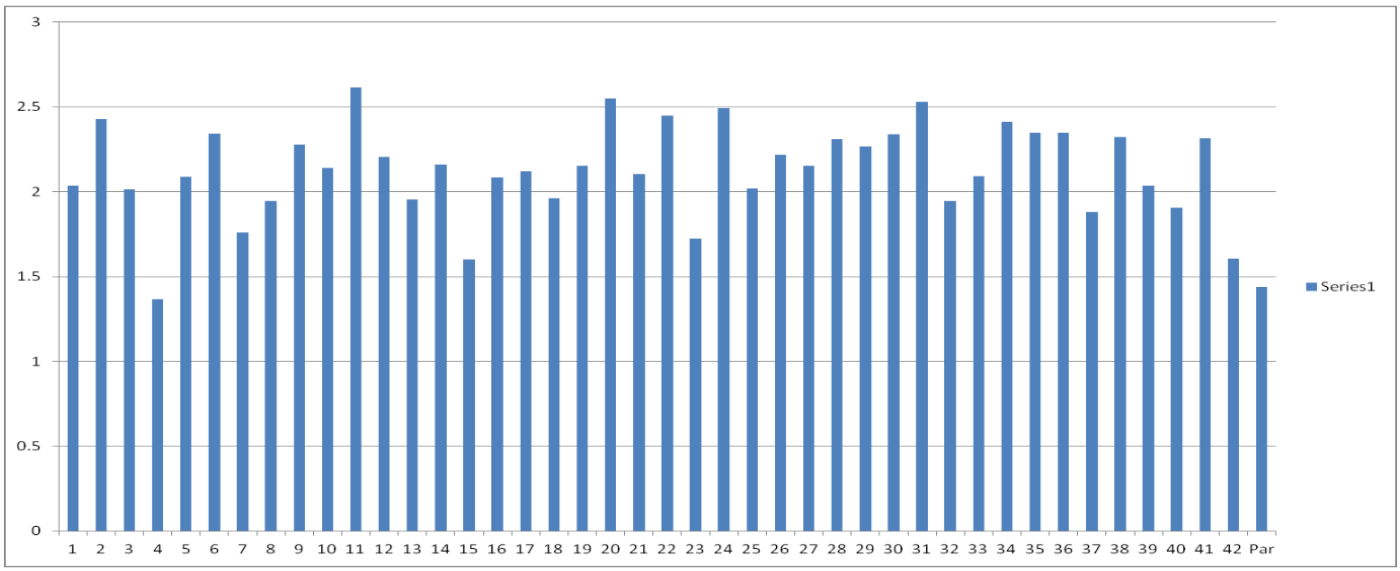

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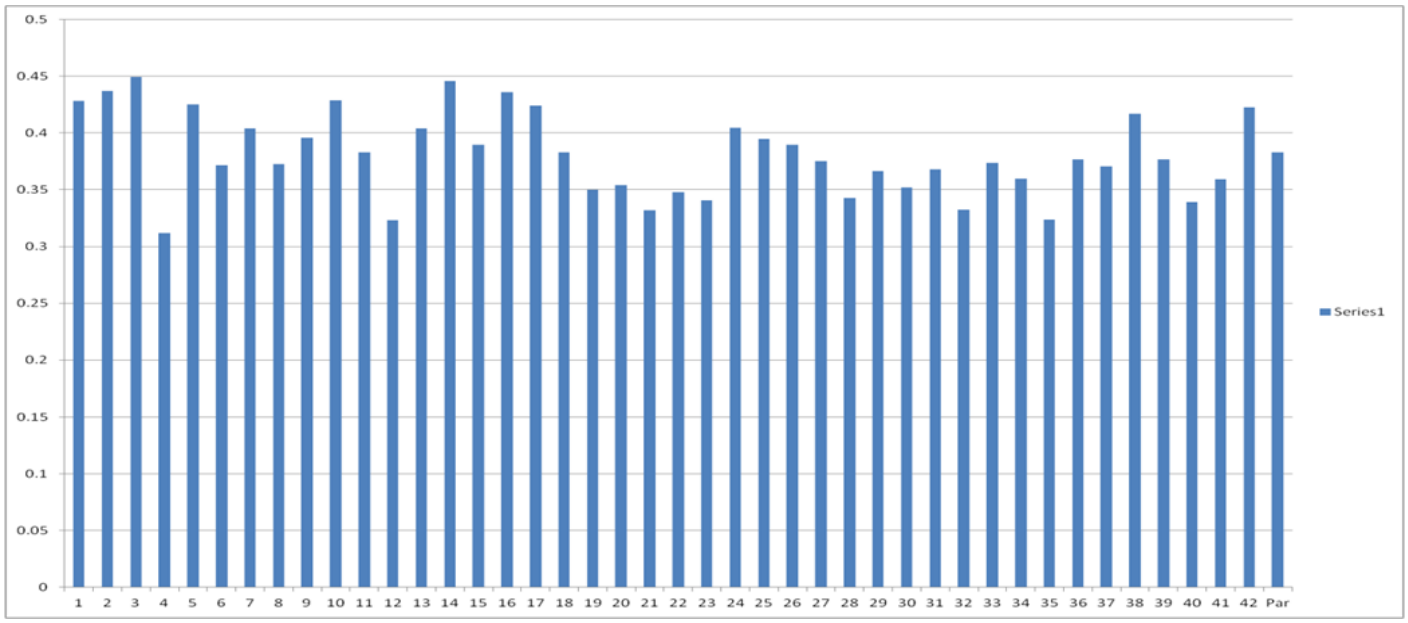

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Fig. S1.5 ***Sf*-Cel3A_*BOS1_BET1_SEC22***

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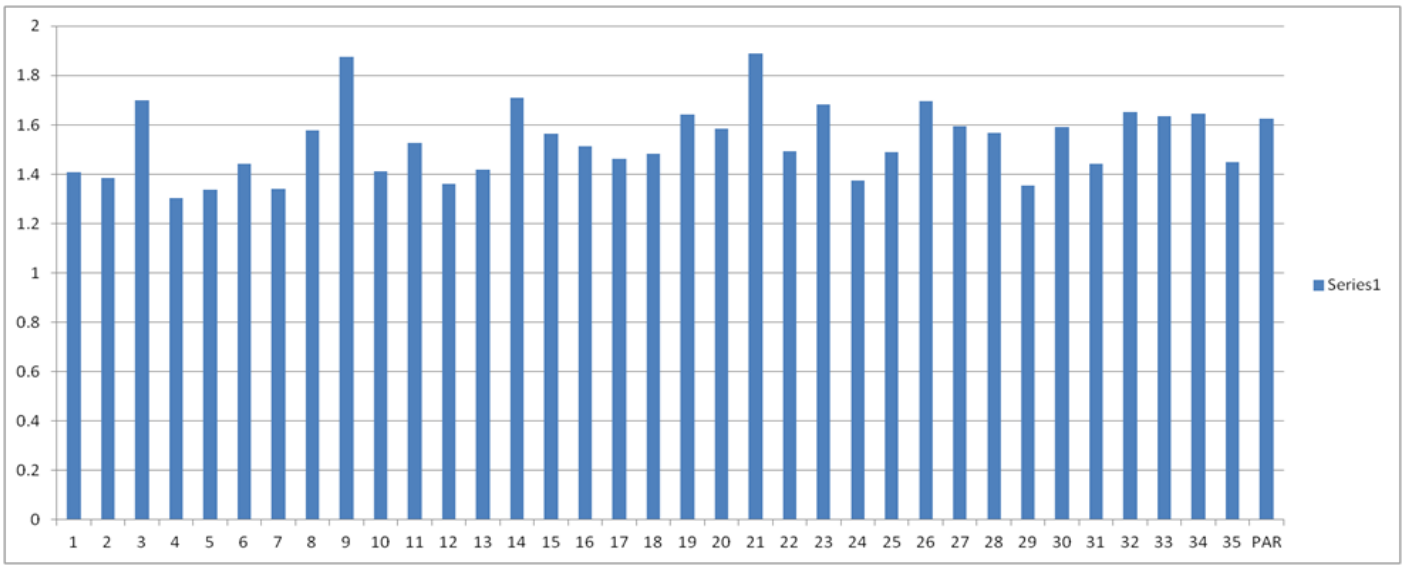

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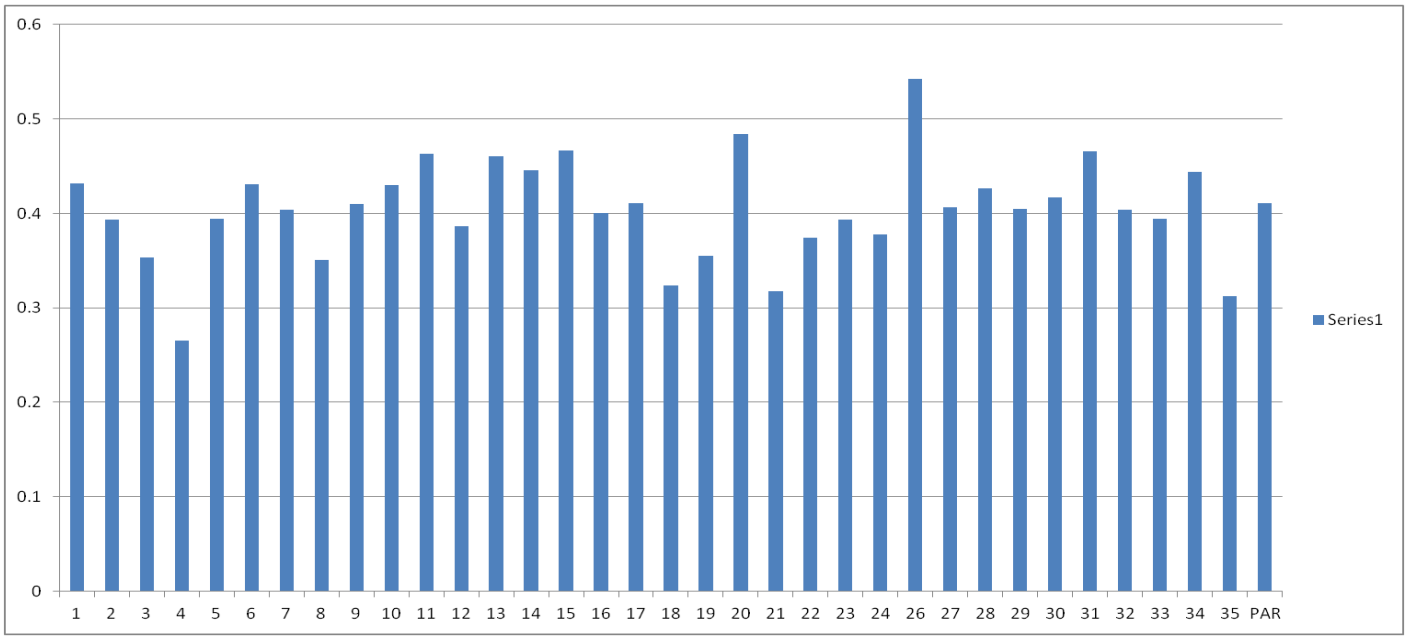

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Fig. S1.6 ***Sf*-Cel3A_*SSO1*_*SED5***

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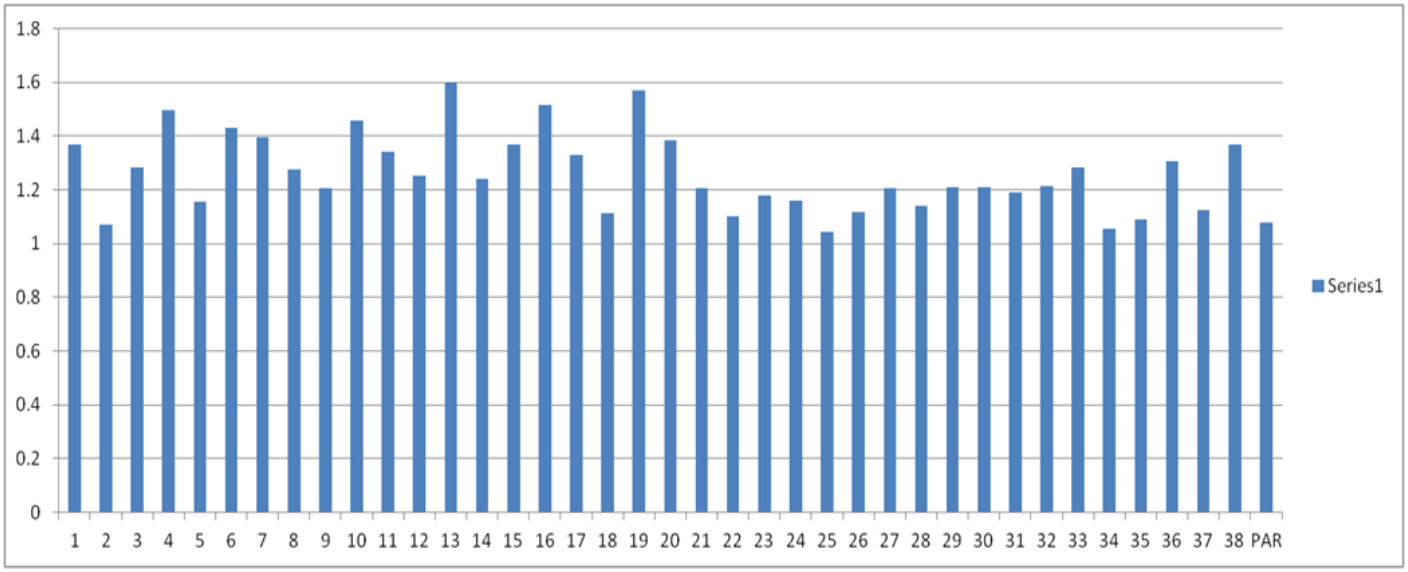

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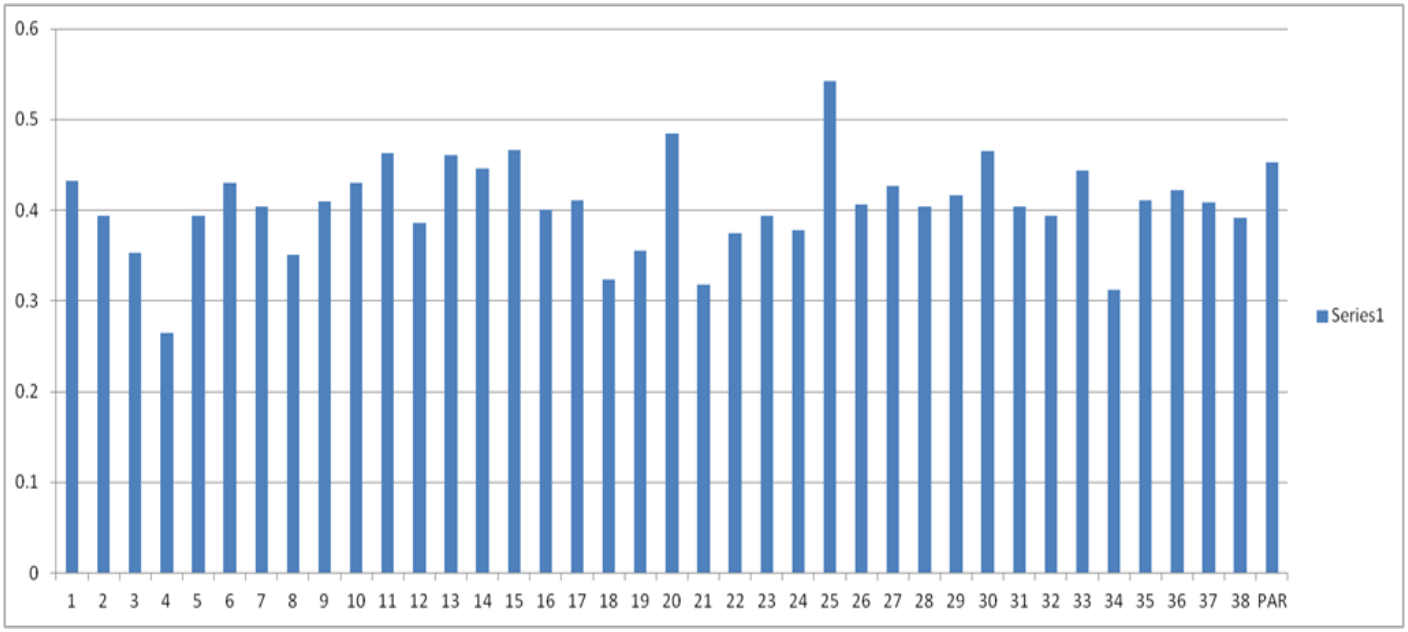

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Fig. S1.7 ***Te*-Cel7A_*BOS1***

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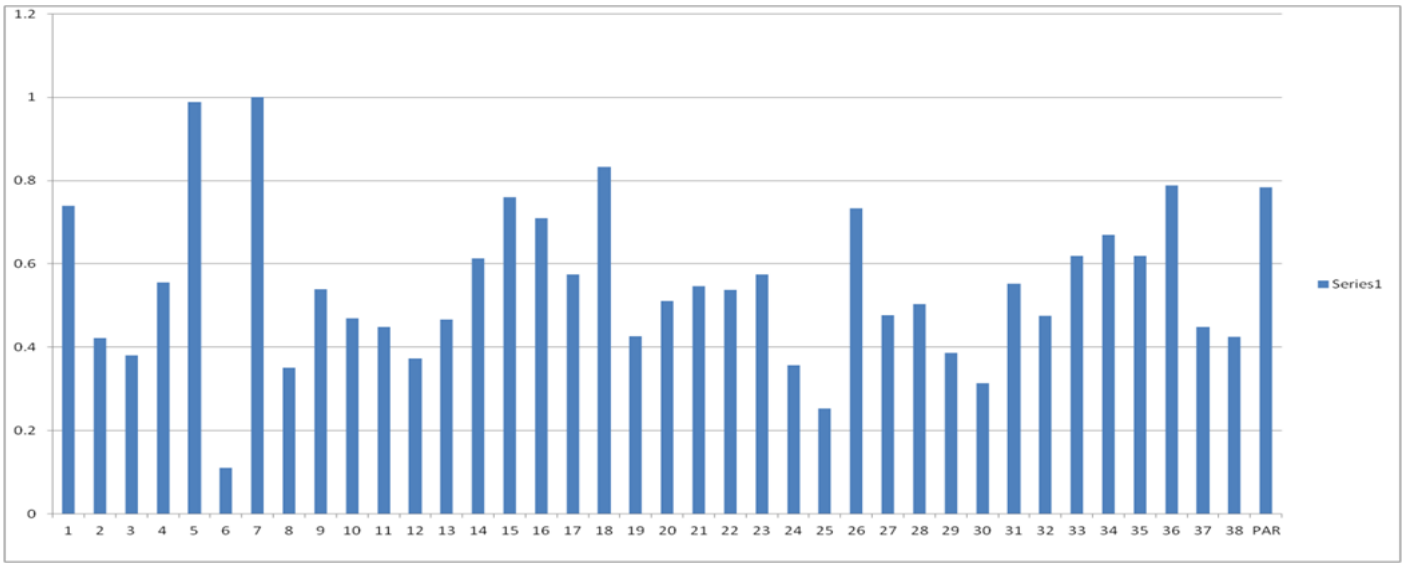

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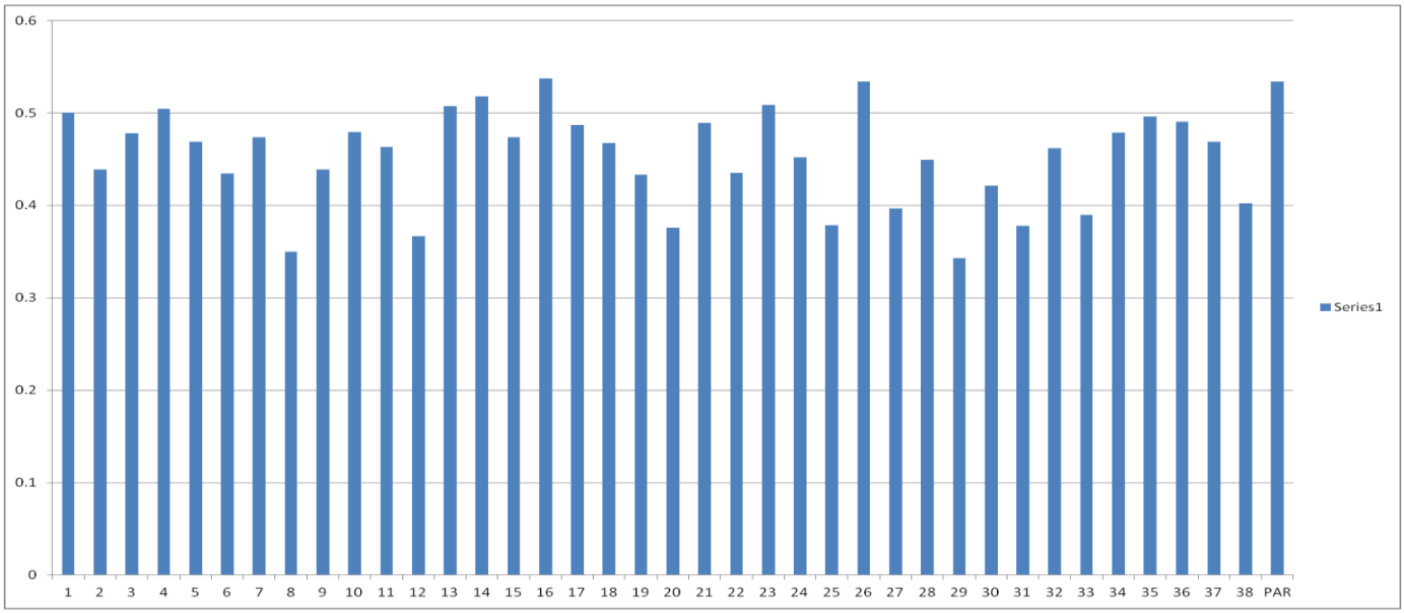

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Fig. S1.8 ***Te*-Cel7A_*BET1***

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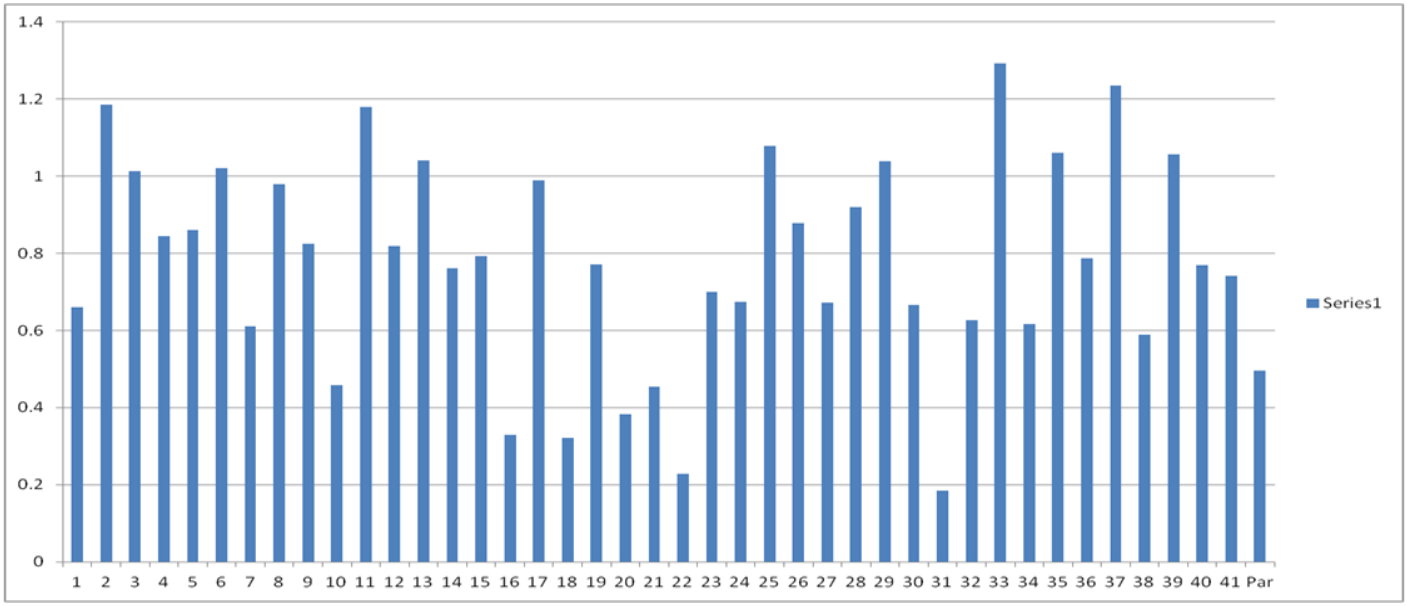

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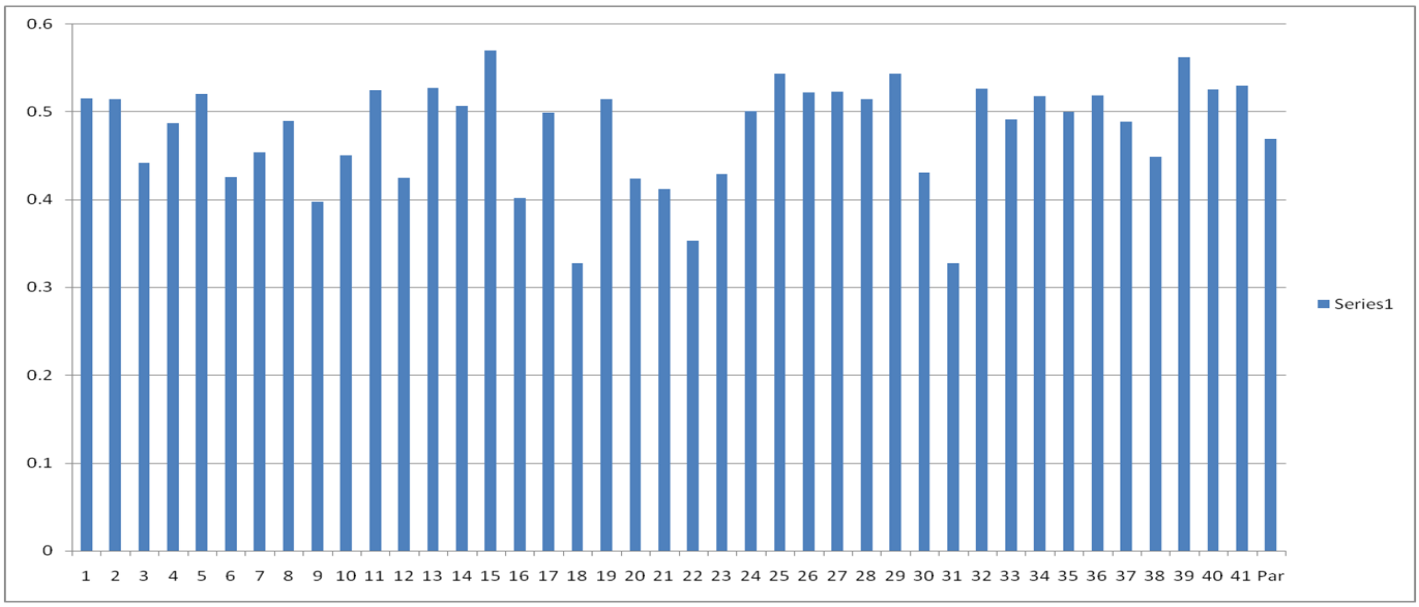

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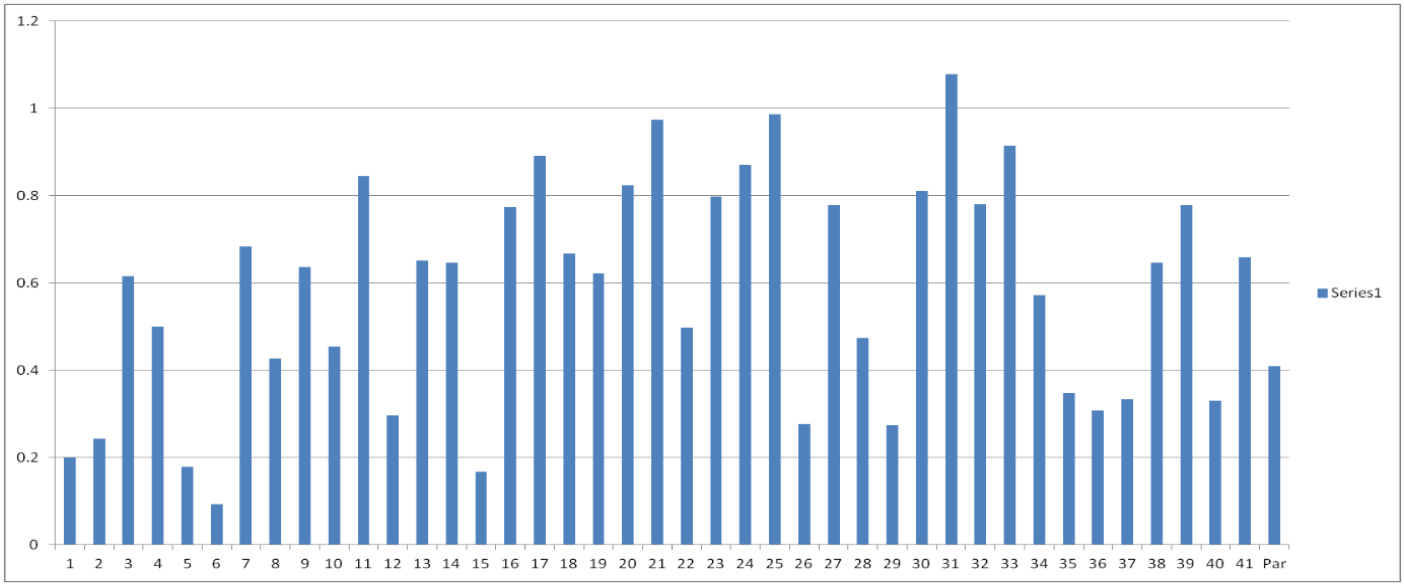
Fig. S1.9 ***Te*-Cel7A_*SEC22***

Activity (400nm) Selected: #31



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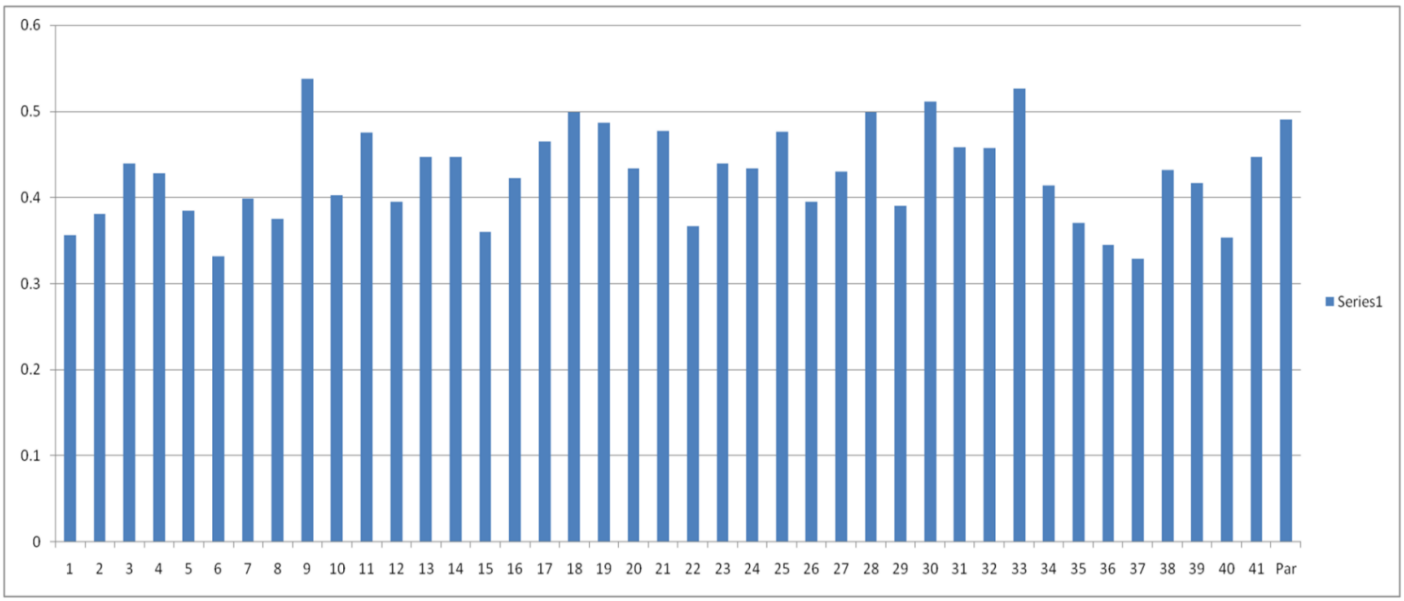

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Fig. S1.10 ***Te*-Cel7A_*SED5***

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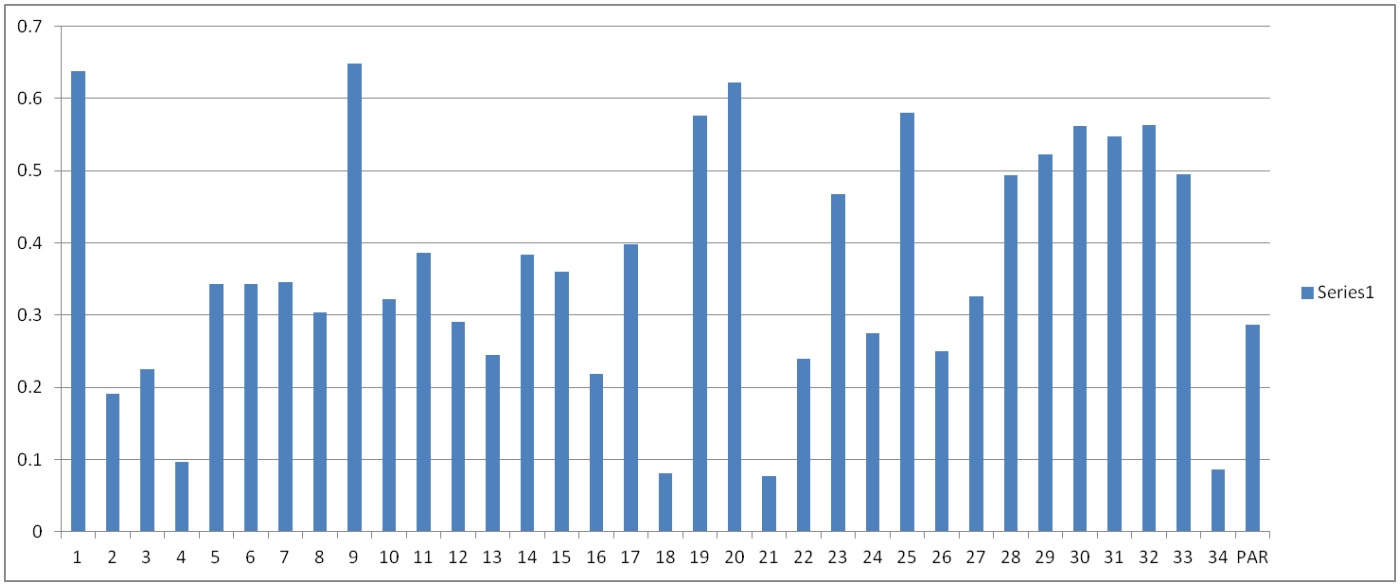

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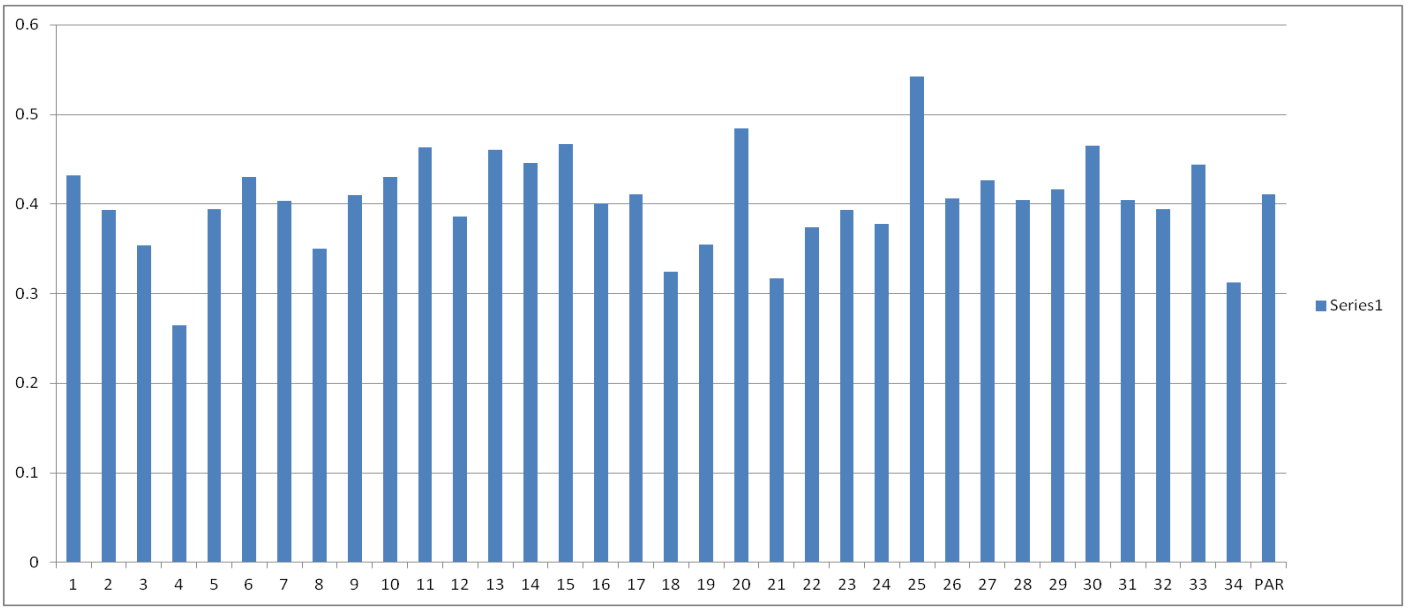

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Fig. S1.11 ***Te*-Cel7A_*BOS1_BET1_SEC22_SED5***

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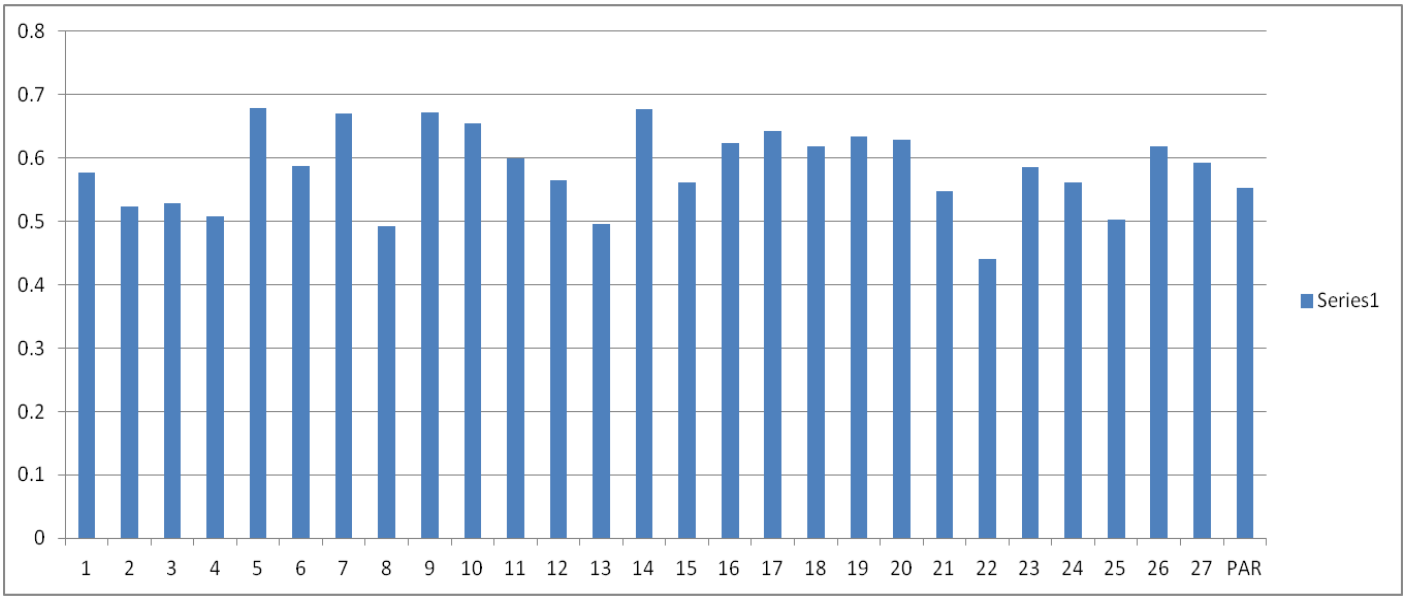

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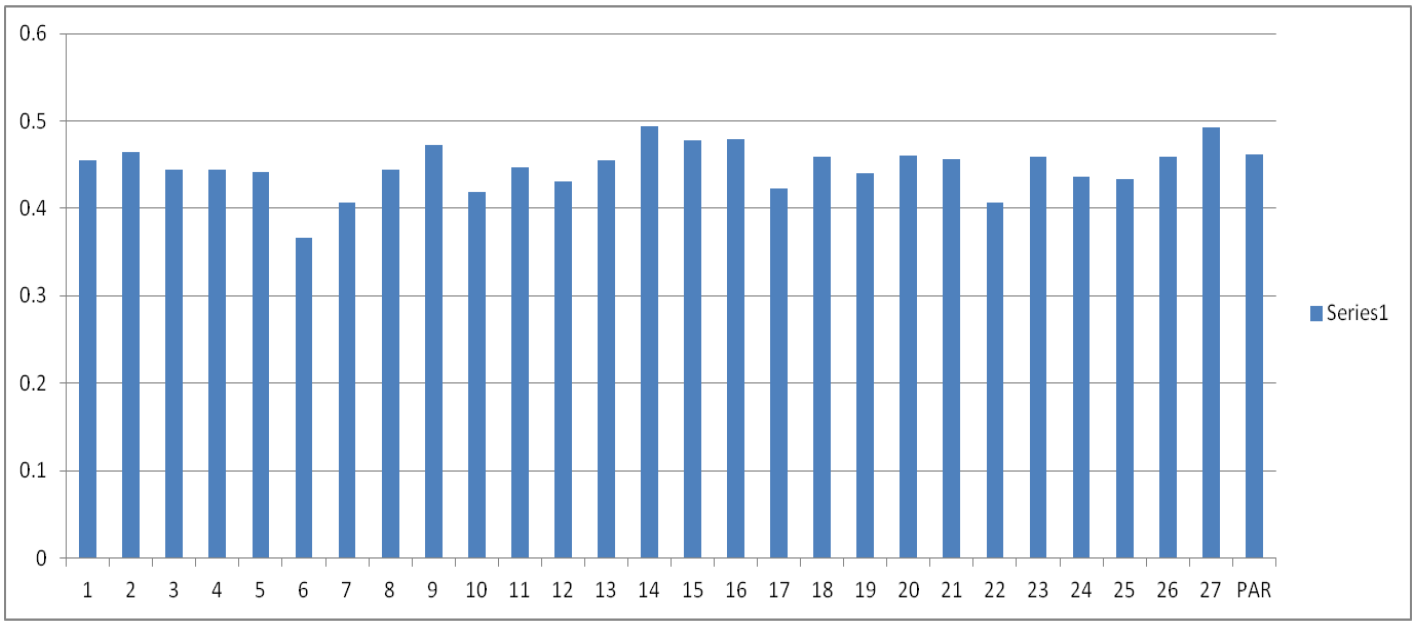

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Fig. S1.12 ***Te*-Cel7A_*SNC1_SED5***

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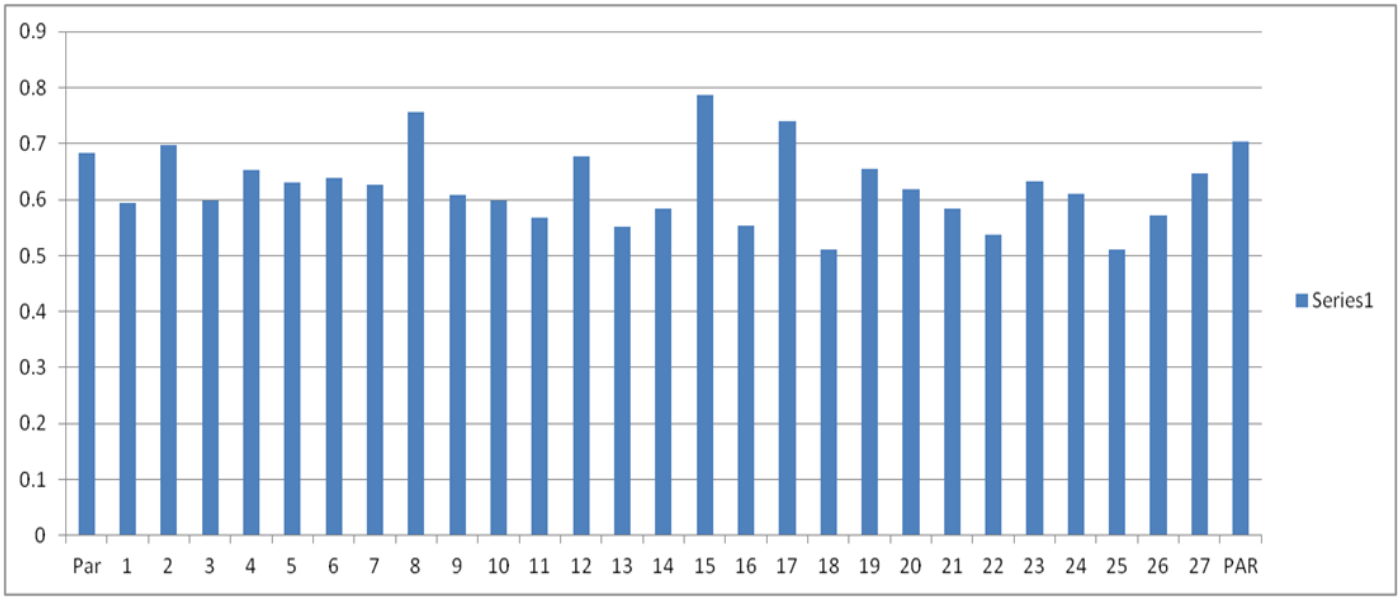

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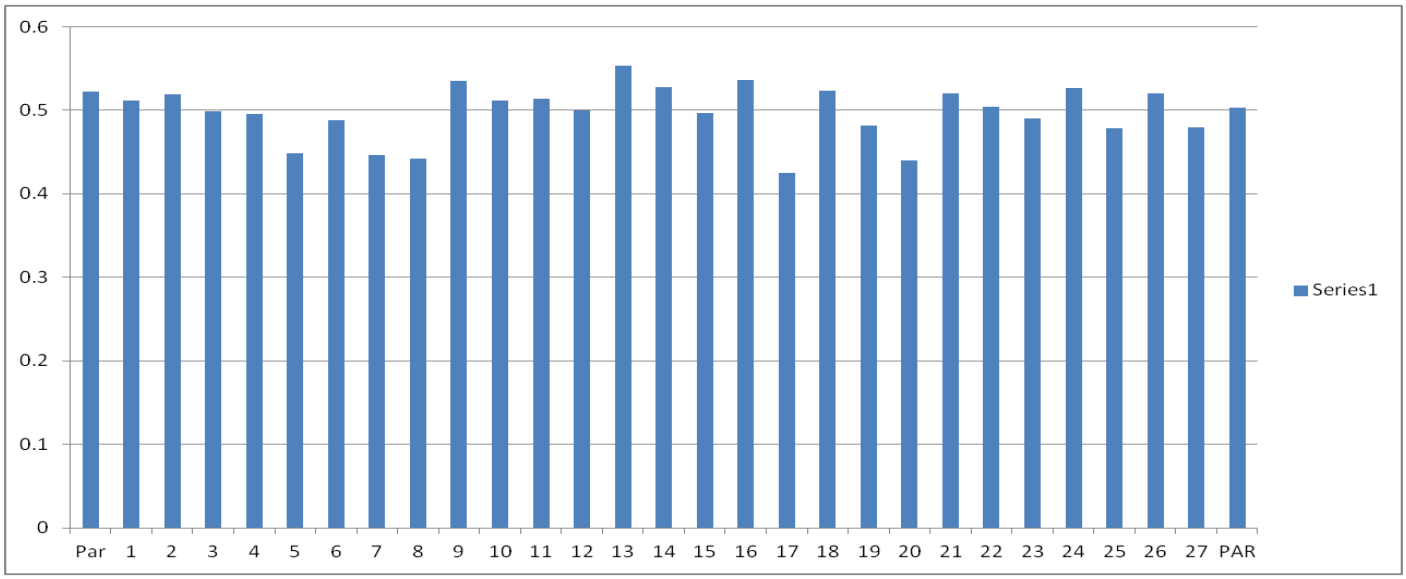

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Addendum B

Review Article:

Cellobiohydrolase secretion by yeast: Current state and prospects for improvement

Riaan den Haan[1†], Heinrich Kroukamp[1], John Henry D. van Zyl[1], Willem H. van Zyl[1]

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Abstract

Lignocellulose is an abundant and renewable feedstock for the production of such commodities as fuels and chemicals, provided that a low-cost technology can be developed to overcome its recalcitrance. Organisms that hydrolyze the sugar polymers in lignocellulose to produce a valuable product at a high rate would significantly reduce the costs of current conversion technologies. To develop yeasts, such as *Saccharomyces cerevisiae*, for such consolidated bioprocessing (CBP), a secreted heterologous cellulolytic enzyme system must be engineered into it. While considerable progress has been made in this regard, the secretion of cellobiohydrolases (CBHs) at levels required for crystalline cellulose hydrolysis has remained elusive until recently. Recent results suggest the existence of a compatibility factor for the expression of foreign genes in a host and that expression of some genes or their products exerted varying degrees of stress on the cell. The secretion machinery of yeasts is a multi-step process and each step is directed and regulated by several proteins, providing a vast array of targets that can be manipulated to enhance heterologous protein secretion. This review assesses the current state of the field with respect to CBH secretion in yeast and the options for enhancing yeast secretion capacity through strain engineering.

Keywords: Consolidated bioprocessing, cellulase, secretion, cellobiohydrolase

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Introduction

Lignocellulosic biomass is an abundant and potentially sustainable resource for the production of fuels and chemicals if technologies are established to overcome its recalcitrance [1]. Biofuels produced from lignocellulosic biomass may displace large proportions of liquid fuels that are currently used if the feedstock supply and process integration steps are optimally managed and if the technical barriers of the conversion technologies are overcome. In the biological conversion route, the sugar component of lignocellulose is converted to ethanol, butanol or energy-rich sugar derivatives through fermentation, which can subsequently be blended into existing fuels or used without blending in compatible engines. Alternatively, thermo-chemical procedures can be used to process lignocellulose, including fast pyrolysis and gasification; however, these procedures yield products that require further processing before they can be used as fuels [2].

Lignocellulose consists mainly of matrices of lignin (10-40%), cellulose (40-55%) and hemicelluloses (25-50%), with the exact composition varying according to the plant origin [3]. Liberation of the sugars in lignocellulose can be achieved by acid hydrolysis, but this reaction often yields large amounts of chemicals that inhibit downstream fermentation. Lignocellulosic sugar polymers are continuously and efficiently hydrolyzed by microbial enzymes in nature [4]. However, in an industrial setting, thermal and/or chemical pretreatment steps are essential to open the fibrous structure of lignocellulose to allow for the enzymatic hydrolysis of cellulose and hemicellulose in a timely manner [5]. The origin of the feedstock determines the optimal type of pretreatment required, which, in turn, defines the optimal enzyme mixture required in subsequent hydrolysis steps and the composition of the hydrolysis products [2]. The rapid and complete hydrolysis of crystalline cellulose is crucial to unlocking the potential of lignocellulosic biomass as feedstock for the production of fuels and chemicals. The hydrolysis of cellulose is achieved by the synergistic actions of (i) endoglucanases (EGs), which act in the amorphous regions of cellulose to release cellodextrins and providing free chain ends; (ii) exoglucanases, including cellodextrinases and cellobiohydrolases (CBHs), which act on crystalline cellulose in a processive manner starting at the free chain ends and releasing mainly cellobiose; and (iii) β-glucosidases (BGLs), which hydrolyze cellobiose and small cello-oligosaccharides to glucose [4,6]. The heterogeneous and chemically diverse polysaccharides that are commonly referred to as hemicellulose comprise a number of structures, such as (arabino)xylan, galacto(gluco)mannan, and xyloglucan [3]. These polymers are linked through covalent and

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hydrogen bonds and can be bound to lignin. Different pretreatment protocols remove variable amounts of hemicelluloses, but it remains imperative, from an economic perspective, that the sugars contained in the hemicellulose fraction of lignocellulose, including the pentoses xylose and arabinose, are also converted to ethanol [7]. The composition of hemicelluloses and the large variety of enzymes required to hydrolyze them have been reviewed elsewhere [8,9].

Following pretreatment, four biologically mediated events are required for the conversion of lignocellulose to ethanol: (i) the production of hydrolyzing enzymes, (ii) the enzymatic hydrolysis of the polysaccharide components of pretreated biomass, (iii) the fermentation of the hexose (C6) sugar fraction, and (iv) the fermentation of the pentose (C5) sugar fraction [4]. Improvements in biomass conversion technologies commonly involve combining two or more of these steps. The hydrolysis and fermentation steps can be combined in the simultaneous saccharification and fermentation (SSF) of hexoses or the simultaneous saccharification and co-fermentation (SSCF) of both hexoses and pentoses, assuming that an optimal fermentative organism is available. The ultimate objective is one-step consolidated bioprocessing (CBP) of lignocellulose to bioethanol, in which a single microorganism or microbial consortium converts pretreated biomass to a commodity product, such as ethanol, without the need for added enzymes. CBP represents a breakthrough for low-cost biomass processing due to the economic benefits of process integration [2,10,11] and by avoiding the high costs of enzymes that make the biological conversion route unattractive [12,13].

While several microorganisms possess the ability to produce the complete range of enzymes required to hydrolyze all of the polysaccharides found in lignocellulose, none display the ability to directly convert these to a desired product, such as ethanol, at economically feasible rates and titers [14,15]. Conversely, microorganisms with favorable product-producing qualities often cannot hydrolyze polysaccharides, utilize all of the sugars available in biomass, or exhibit sensitivity to the inhibitors present in pretreated lignocellulosic biomass. Three different approaches have been utilized to develop CBP organisms: (i) engineering product-forming ability into organisms that are efficient biomass degraders, (ii) engineering cellulolytic ability into organisms that have attractive product-producing attributes and (iii) engineering both product-forming and cellulolytic abilities into organisms with other particular positive attributes [2,15].

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The yeast *Saccharomyces cerevisiae* has long been used as an industrial ethanologen [16,17]. Attributes that render this organism suitable for industrial ethanol production include a high rate of ethanol production from glucose (3.3 g/L/h), high ethanol tolerance and its favorable GRAS (generally regarded as safe) status. *S. cerevisiae* has adapted to stress conditions, such as high ethanol and sugar concentrations (hence, osmotolerance), in fermenting simple hexose or disaccharide (sucrose and maltose) streams. This yeast also has a natural hardiness against the inhibitors produced in biomass pretreatment and has the ability to grow at low oxygen levels. These features confer a general robustness to *S. cerevisiae* in industrial process conditions and make it a good candidate for CBP [9]. However, the drawbacks that must be overcome before this organism can be used in CBP are also significant. *S. cerevisiae* does not utilize the pentose sugars (xylose, arabinose) that are available in lignocellulosic biomass [7] and does not produce the necessary enzymes to hydrolyze cellulose or hemicellulose. Several other yeast species have innate properties that make them attractive as possible CBP organisms, such as thermotolerance, a wider substrate range, process robustness and the ability to secrete large amounts of heterologous enzymes [14]. In the following sections, we will discuss the importance of CBH in cellulase mixtures, the secretion of this enzyme in yeast and the options for increasing CBH secretion levels in yeast in the light of developing superior strains for CBP.

The role of cellobiohydrolase in cellulase mixtures

Cellobiohydrolase enzymes are key components in fungal cellulase systems as they are required for the hydrolysis of crystalline cellulose [18]. The *Trichoderma reesei* cellulase mixture consists of many catalytically active proteins that have been identified by 2D-electrophoresis, including two cellobiohydrolases (CBH1-2), five endoglucanases (EG1–5), and a β-glucosidase [19,20]. CBH1 (Glycoside Hydrolase family 7, GH7), CBH2 (GH6), and EG2 (GH12) are the three main components of the *T. reesei* cellulase system, representing ~60%, ~20% and ~12% of the total cellulase protein secreted by this organism, respectively [6,20]. The structure of both cellobiohydrolases is modular, featuring a catalytic domain and a carbohydrate-binding module (CBM) that are connected by a glycosylated peptide linker [6,21]. CBHs are naturally *N*- and *O*-glycosylated. The catalytic domain structures of CBH1 and CBH2 are entirely different, but both feature tunnel-shaped structures formed by disulfide bridges, supporting a structural interpretation of the processive action of exoglucanase [22,23]. The catalytic sites of both cellobiohydrolases are within the tunnel near the outlet so that the β-glucosidic bonds are

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cleaved by retaining (CBH1) or inverting (CBH2) mechanisms from the reducing or non-reducing ends, respectively. This property is typical of GH7 and GH6 enzymes (http://www.cazy.org/Glycoside-Hydrolases.html). CBHs can cleave several bonds following a single adsorption event before the dissociation of the enzyme substrate complex, resulting in a gradual decrease in the degree of polymerization of the substrate [6,24].

The structures of the catalytic domains of different GH7 enzymes have been resolved, including those of CBH1 from *T. reesei* [23] and *Talaromyces emersonii* [25]. The protein sequences of these two CBHs are 66% homologous, and they have remarkably similar architectures with regard to their catalytic tunnels. In both cases, the tunnel spans approximately 50 Å with ten well-defined sites for glycosyl units (**Fig. 1A, B, D and E**). Both CBHs consist of two β-sheets that are packed face-to-face to form a β-sandwich with long loops that enclose the cellulose binding tunnels [25]. The tunnel structure of *T. emersonii* CBH1 is slightly more open and straight, presumably to allow shorter chain oligosaccharides better access to the active site, which is supported by the observation of a comparatively higher catalytic rate and efficiency with the oligosaccharide 4- nitrophenyl-lactopyranoside [26]. The structure for *T. reesei* CBH2 (GH6) also revealed a catalytic tunnel; however, this shorter tunnel only accommodates four glycosyl units at the non-reducing end of cellulose chains (**Fig. 1C, F**). The CBH2 tunnel is formed via an α/β fold that forms a barrel with seven parallel β-sheets, and the roof of the tunnel is enclosed by two loops [27].

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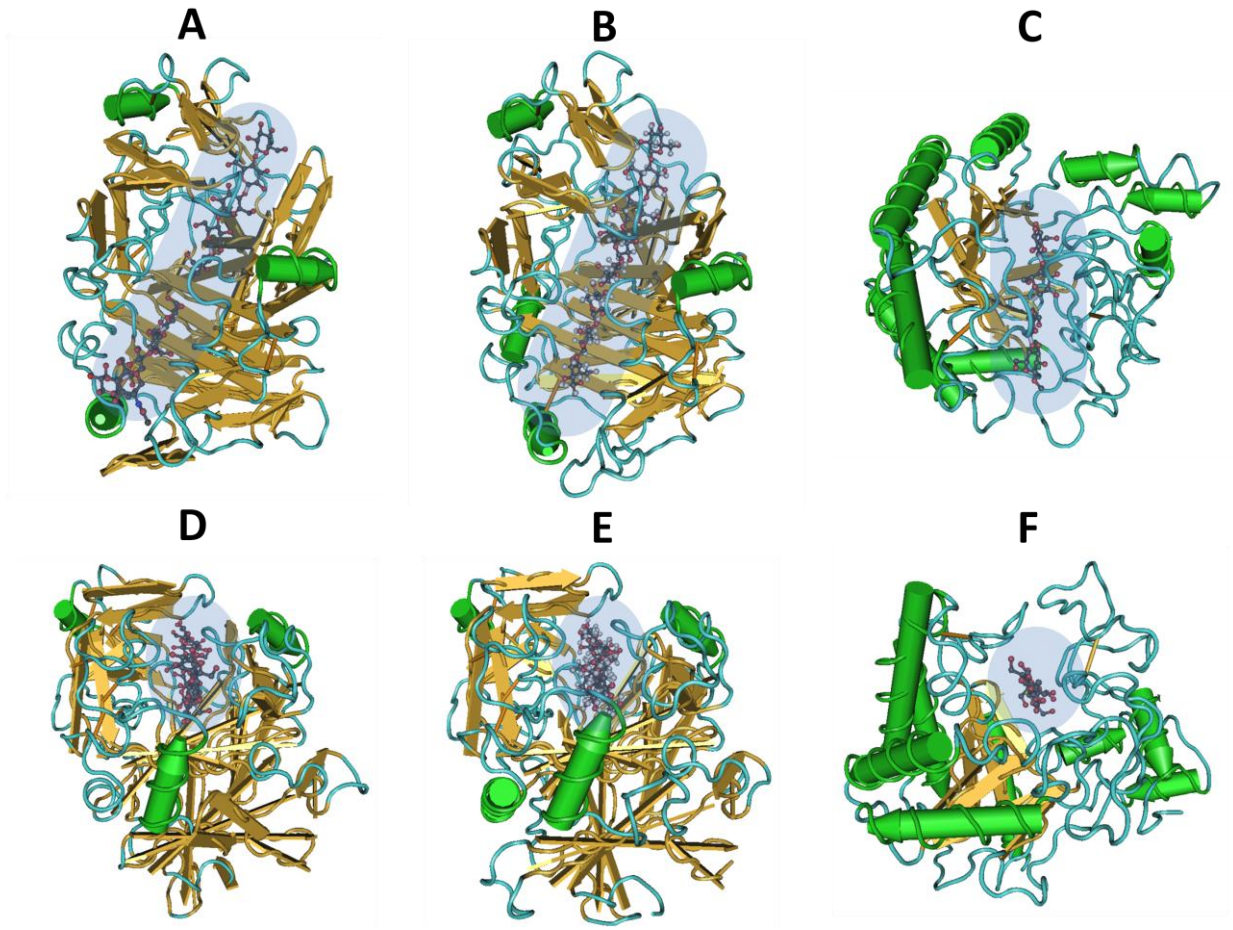

Fig. 1: Ribbon diagrams depicting the structures of the catalytic domains of (A,D) *T. reesei* CBH1, (B,E) *T. emersonii* CBH1 and (C,F) *T. reesei* CBH2 bound to cello-oligosaccharides. Diagrams A–C depict top views, demonstrating the positioning of the glycosyl groups in the tunnels (shaded areas), whereas diagrams D–F show side views into the respective tunnels that accommodate the glycosyl groups. The diagrams were created using Cn3D (version 4.3) software from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and the high-resolution crystal structures for *T. reesei* CBH1 (PDB ID: 6CEL; [23]), *T. emersonii* CBH1 (PDB ID: 3PL3; [25]) and *T. reesei* CBH2 (PDB ID: 1QK2; [27]).

The CBM modules of *T. reesei* CBH1 and CBH2 are located at the C-terminus and N-terminus, respectively. Removal of the CBMs of cellulases results in a several-fold reduction in the rate of hydrolysis of insoluble substrates but had little effect on the hydrolysis of soluble substrates [6,28]. *T. reesei* CBMs belong to CBM family 1 (CBM1), characterized by a small wedge-shaped fold that features a cellulose binding surface with three exposed aromatic amino acid residues that are thought to be critical for binding crystalline cellulose. The spacing of the three aromatic residues coincides with the spacing of every second glucose ring on a β-1,4-glucan

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chain. It was postulated that the aromatic amino acids of the CBMs form van der Waals interactions and aromatic ring polarization interactions with the pyranose rings on the surface of cellulose, causing a disturbance in the substrate surface to allow entry of the glucan chain into the tunnel of the catalytic domain [29]. Several naturally occurring CBHs, such as the *T. emersonii* CBH1b, lack a CBM and have comparatively lower specific activities on insoluble cellulose substrates [25,30].

The stability of the folded conformation of a protein depends on its primary structure and is determined by many local and long-term interactions [31]. One of these interactions is the formation of disulfide bonds. Most GH7 cellobiohydrolases contain ten disulfide bonds in the catalytic domain. *T. reesei* CBH1 contains ten disulfide bonds in its catalytic domain and two more in its CBM [23,32]. The single module CBH1 from *T. emersonii*, which consists only of the catalytic module, contains 9 disulfide bonds [33].

CBH expression in yeast: protein effects

Over the last 25 years, several authors have reported the expression of many cellulase-encoding genes in yeast strains [2,9] (**Table 1**). Several researchers produced cellulases in organisms that would not yield interfering activities to gain insight into the mechanism of the original cellulolytic enzyme, whereas others enabled the yeast to hydrolyze cellulosic substrates, as has been reviewed elsewhere [2,9,34]. Thus, strains of *S. cerevisiae* were created that could convert cellobiose [35,36], amorphous cellulose [37-40] and even crystalline cellulose, at least partially, [30,41,42] to ethanol. CBHs have been successfully produced and secreted by *S. cerevisiae* and other yeasts and were tested for their activity on a variety of substrates ranging from small synthetic molecules to amorphous and crystalline forms of cellulose (**Table 1**). A general feature among most reports of heterologous CBH production in yeast is that a relatively low titer of secreted CBH was found, although the range of reported values is quite large—0.002 to >1 % of total cell protein [9,30]. This feature, coupled with the low specific activity of CBHs on polymeric substrates, has led to the identification of CBH expression as a limiting factor for CBP using yeast [14]. Den Haan et al. [43] attempted to calculate the amount of CBH1 required to enable growth on crystalline cellulose and determined that this amount was within the capacity of heterologous protein production in *S. cerevisiae* in terms of total cellular protein, *i.e.*, 1 - 10% of the total cellular protein (tcp) [43-45]. Recently, the expression of relatively high levels of CBHs in *S. cerevisiae* was reported for

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the first time [30,41]. The CBH expression levels achieved in these studies of up to 4% tcp met the calculated levels sufficient for growth on cellulose at the rate required for an industrial process [34].

High and variable levels of glycosylation were consistently observed with CBHs produced in *S. cerevisiae* [30,43,52]. CBH1s originating from *Thermoascus aurantiacus*, *T. emersonii*, *Neosartorya fischeri*, and *T. reesei*, among others, and CBH2s originating from *Chrysosporium lucknowense*, *Acremonium cellulolyticus* and *T. reesei*, among others, all showed significant levels of hyperglycosylation. Band shifts could be observed for these protein species on SDS-PAGE gels following deglycosylation reactions with Endo H or PNGase F [30]. The clear bands generated with these *N*-deglycosylating enzymes also indicated that most heterogeneity was caused by *N*-attached hyperglycosylation. Although variable levels of these CBHs were produced, it was found that the fundamental attributes of the enzymes were generally retained, such as their activity on crystalline cellulose substrates and, for example, the thermostability of the *T. aurantiacus* enzyme. Hyperglycosylation was also shown to occur on CBHs that were heterologously produced in *P. pastoris*, although to a lesser extent than in *S. cerevisiae* [57]. Several reports have shown a decreased specific activity for certain heterologous CBHs on polymeric substrates, presumably as a result of hyperglycosylation, although this has not always been reported to be the case [33,43,46,47]. The *N*-glycans added by yeast to recombinant *T. emersonii* CBH1 seemed to improve the stability of the enzyme and the activity on crystalline cellulose (at 70°C) to some extent; however, its ability to bind Avicel seemed to decrease.

Ilmén et al. [30] expressed synthetic genes encoding 14 CBH1s and 10 CBH2s of fungal origin, which were codon-optimized for expression in *S. cerevisiae*. Remarkable variation was found in the secreted protein levels and activities between the different recombinant strains. For example, activities of the CBH1-expressing strains on 4-methylumbelliferyl-β-D-lactoside (MULac), a substrate for the fluorometric assay of galactosidase and CBH1, ranged over two to three orders of magnitude. Heinzelman et al. [60] expressed the *cbh1* genes of *T. reesei*, *T. emersonii*, *A. thermophilum*, *Chaetomium thermophilum* and *T. aurantiacus* and found a similar activity-based ranking. The enzyme secretion levels were lower in this report, which was probably due to differences in codon optimization, the strain used, construct-specific effects or a difference in the amino acid sequences initially chosen. The level of variation in the activities between strains was somewhat surprising, given that the overall sequence homology of the

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GH7 proteins and the GH6 proteins produced were both over 60% and that the genes were expressed under the control of identical promoters on identical episomal plasmids.

A strain producing a recombinant *T. emersonii* CBH1 produced the highest secreted MULac activity and the highest amount of secreted CBH protein of the CBH1s tested by Ilmén et al. [30]. Despite this result, the activity of the protein on Avicel was relatively low, likely because the native enzyme lacks a CBM. To add a CBM, constructs were designed in which the linkers and CBMs originating from the CBH1s of *Humicola grisea*, *T. reesei* or *C. thermophilum* were fused to the C-terminus of the *T. emersonii* CBH1. Alternatively, the linker and CBM of *T. reesei* CBH2 were fused to its N-terminus. The fusion proteins were produced in an enzymatically active form, even though the production level was reduced relative to that of the enzyme with no CBM, suggesting that the presence of the CBM increased the complexity of CBH production in yeast. Avicel conversion by yeast culture supernatants containing the fusion of *T. emersonii* CBH1 and the *T. reesei* CBH1 CBM exceeded that of the non-fused protein, indicating that the addition of the CBM leads to enhanced crystalline cellulose hydrolysis. It was also repeatedly observed that the different fusion proteins were secreted at different levels, suggesting that the choice of the fusion partner or the design of the fusion had a large effect on the level of secreted protein. Estimations of the protein concentrations of recombinant *T. emersonii* and *A. thermophilum* CBH1 that were based on total protein and the estimation of the protein concentration of active CBH1 that was based on its specific activity on MULac were fairly consistent. In comparison, the enzymatic activities of the *T. reesei* and *C. thermophilum* CBH1s were not proportional to the amount of protein measured, suggesting that only a small fraction of the secreted enzyme pool was enzymatically active, similar to the *T. reesei* CBH1 expressed in *P. pastoris* [57,61]. For this CBH1 produced in *P. pastoris*, circular dichroism assays indicated that the lack of active enzymes was due to the improper formation of disulfide bonds. These findings highlight how different genes may vary in their compatibility to heterologous expression in different hosts, as the *C. thermophilum* CBH1 was found to be well-expressed and displayed the highest specific activity of the CBH1 candidates tested for heterologous expression in *T. reesei* in a recent study [62] and *C. thermophilum* CBH3 (GH7) was also produced at high levels in *P. pastoris* [58]. Co-expression of the most successfully produced CBH1s and CBH2s lead to Avicel conversion efficiencies that exceeded that of the corresponding strains expressing only one enzyme in most cases – likely due to a synergistic effect [30]. Lower MULac activities in co-expressing strains indicated that CBH1 production in these strains was

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lower than when it was produced alone; this effect also varied depending on the co-expression partner.

CBH expression in yeast: cellular effects

To investigate why CBHs with high degrees of homology are secreted at such vastly different levels and why co-expression alters the production levels of CBHs in comparison to single expression, relative differences in *cbh* mRNA levels, the copy number of the expression vector, and secretion stress-induced responses were investigated in strains with both high and low cellulase production [30]. A correlation was found between mRNA levels, vector copy number and MULac activity for strains expressing CBH1s. The strain producing the *T. emersonii* CBH1, for example, had much higher mRNA levels, a higher vector copy number and a higher level of MULac activity than the strain producing the poorly expressed *T. reesei* CBH1. The same criteria were also greater for the strain producing the *T. emersonii* CBH1 than those for any strain co-producing this enzyme, or for its CBM-attached derivative with any CBH2. The mRNA levels of both *cbh1* and *cbh2* were decreased when compared with the corresponding strains expressing only one *cbh*, and this was consistent with the plasmid copy numbers. The strain co-expressing the combination of the most highly expressed *cbh1* and *cbh2* genes had a copy number intermediate to strains expressing these genes alone. Thus, irrespective of its larger size, more copies of the plasmid with two *cbh* expression cassettes were found than the plasmids with only one expression cassette. Pakula et al. [63] noted that when the folding and secretion of CBH1 in *T. reesei* was disrupted by the reducing agent dithiothreitol, the unfolded protein response (UPR) was activated and *cbh1* transcript levels were drastically decreased. This finding suggested a novel type of feedback mechanism that is activated in response to an impairment of protein folding and secretion, which is called repression under secretion stress (RESS). A similar response was observed for glucoamylase production by *Aspergillus niger* [64]. However, RESS is a transcriptional response that should be avoided by using constitutive glycolytic yeast promoters during heterologous expression.

To test the induction of cellular stress by CBH production, the transcript levels of the UPR regulator *HAC1* was studied [30]. The unspliced *HAC1* transcript, which does not code for a functional protein, was detected in all cells. Spliced *HAC1* mRNA coding for the UPR-inducing transcription factor was not detected in the strain containing the empty vector but appeared in all CBH-producing strains, indicating that the UPR was induced. As additional evidence,

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expression of the genes *KAR2* and *PDI1*, which are both known to be induced by the UPR, was also upregulated in CBH-producing strains in comparison to control strains. Levels of the spliced *HAC1* transcript varied between the CBH-producing strains; the strain producing *C. lucknowense* CBH2 (the best produced CBH2) had the lowest level, suggesting that the expression of this protein was the least stressful for the cell's secretion machinery. Similarly, other strains producing efficiently secreted enzymes also had relatively low levels of the spliced *HAC1* transcript. The strain producing *T. emersonii* CBH1 also produced less of the spliced *HAC1* transcript than its CBM-attached derivative, suggesting that the production of the fusion protein resulted in higher ER stress. While the strain producing the *T. emersonii* CBH1 with a CBM fusion had levels of *HAC1* mRNA that were comparable to that of the strain producing *T. reesei* CBH1, the latter protein caused a stronger UPR induction and far less active enzyme was secreted. This finding suggests that the post-translational processing in the secretory pathway, such as hyperglycosylation or incorrect folding, resulted in the secretion of notably little active enzyme, as had been shown previously [46,57,65]. Pakula et al. [66] made the interesting observation that CBH secretion in *T. reesei* was slower than that in *S. cerevisiae*. The *O*-glycosylation of CBH1 was found to be essential for its secretion in *T. reesei* [66]. The first steps of *O*-glycosylation occur in the ER in *S. cerevisiae* [67], and aberrant *O*-glycosylation of the CBH1 of *T. reesei* early during secretion in yeast could adversely affect this process.

Taken together, these results indicate that there are certain gene candidates that are more compatible with expression in yeast than others. However, the features that lead to incompatibility, which is marked by low levels of plasmid, mRNA, and secreted protein and a strong induction of the UPR, are difficult to define. High level CBH secretion generally corresponded with high plasmid copy number and the *cbh* gene inserts significantly influence plasmid copy number, but the mechanism by which this occurred remains obscure [30]. It is possible that the *cbh* inserts affect plasmid replication or transcription, or indirect cellular effects caused by the translation of specific CBHs may be involved. Transcriptional down-regulation of histone genes under replication-stress conditions has been observed for *S. cerevisiae* [68]. Individual gene- and/or protein-specific features and compatibility with the host are important, not only for the efficient production of the individual protein but also for the efficient production of the accompanying protein when two or more genes are simultaneously expressed from one plasmid. CBH expression induced the UPR, with the

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strength of its activation depending on the expressed gene. A negative correlation was shown between the spliced *HAC1* mRNA levels and the amount of secreted active enzyme.

Manipulation of yeast secretion: cultivation conditions

The model of the yeast protein secretory pathway postulates that only budding cells actively secrete extracellular proteins [69], which translate to approximately 30% of an exponential yeast culture actively secreting the protein of interest [70]. The intricate relationship between yeast growth and protein secretion has been intensively exploited in the industrial scale production of commercially significant proteins by optimizing media composition, temperature, pH and osmolarity.

Extrapolating from the findings of Schekman [69], the correlation between growth and secretion implies that media composition, preferential for high growth rates of the cells, should correspond to high secreted protein yields. Indeed, higher protein yields are generally obtained when yeasts are grown in complex media [71,72,73]. Crous et al. [73] obtained up to 70-fold higher levels of an α-L-arabinofuranosidase in complex YPD media compared to yeast nitrogen base media, possibly due to differences in regards to available amino acids, salts and nitrogen sources. The influence of un-complemented auxotrophies could also have a profound impact on secreted protein yields as shown in a study by Görgens et al. [74]. A significantly higher biomass and secreted xylanase yield was observed in this study in a auxotrophic strain with increased additions of histidine, leucine and tryptophan to the growth media. Yields were similar to those of a prototrophic variant, at a 10.8 mM total amino acid concentration. However, at a concentration of 4.3 mM amino acid mix, the low biomass yield of the auxotrophic strain was mostly alleviated, but it still yielded low xylanase activity [74]. This suggests that, although growth and secretion are fundamentally linked processes, the availability of certain media components could independently effect the production and secretion of proteins products. The phenomenon of metabolic burden, caused by protein overproduction, as seen in many heterologous protein production systems, could also be partly relieved by incorporation of specific amino acids in the growth medium, even though they were not necessary to complement yeast auxotrophies [75].

Although carbon sources which allows for the highest biomass yields are routinely used on laboratory scale fermentations, the availability of certain metabolizable carbon sources, such as lactate and trehalose, in complex growth media has also been shown to improve recombinant

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protein production in yeast up to 3-fold [76]. Alterations in the cell wall composition [77] and the prevention of protease induction [78], due to media composition, also significantly enhance secreted protein yields. Although these processes do not directly enhance the efficiency of secretion, their influence on cell morphology and prevention of protein degradation results in higher secreted protein yields.

In classical taxonomy, the optimal growth temperature of an organism has often been utilized as a distinguishing factor and is routinely applied for cultivation. Although the optimal temperature allows for the fastest growth rates, this specific temperature does not always translate to maximal protein yield [79]. It is not surprising that temperature could have a significant influence on yeast protein yields because its impact on cell metabolism is profound, as it affects the regulation and abundance of ER foldases and chaperones among many other proteins involved in the stress response and protein processing [80].

Hackel et al. [79] demonstrated the effect of temperature on the secretion of single-chain antibody fragments (scFv) produced in *S. cerevisiae*; a 4.5- and 3.3-fold increase was observed in scFv secretion, respectively, at 20°C and 30°C compared to that at 37°C. Although the increase in protein secretion at lower growth temperatures is often attributed to lower specific growth rates, which allows for more time for the folding of recombinant proteins, differential gene regulation at these temperatures could also play a major role. Changes in the transcriptional levels of many genes involved in protein folding and trafficking have been reported, such as the up-regulation of genes involved in vesicular trafficking (*BMH1/2*), the down-regulation of ER-associated protein degradation (ERAD), the reduced transcription of folding-related genes (*SSA1/2, ERO1, HSP82, KAR2* and *CPR5*) and the up-regulation of the membrane SNARE-complex genes (*SSO1/2* and *SNC1*) [80]. In contrast, the expression of β-glucosidase from the hyperthermophilic *Pyrococcus furiosus* in *S. cerevisiae* showed an increased secreted enzyme titer with an increase in temperature [81]. It is tempting to hypothesize that the efficient folding of certain proteins is linked with the optimal temperature of its native host, although more in-depth studies are required to elucidate this subject. Many other putative secretion-enhancing events induced by temperatures shifts have been suggested, including changes in the cell cycle and the composition of the cell wall. One example of such a change is the decrease in the percentage of cell wall chitin levels and cell wall-linking β-glucans when *S. cerevisiae* is grown at 22°C compared to 30°C and 37°C [77], which could have a significant effect on the secreted protein titer [82].

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When cells are exposed to osmotic changes, the cell wall architecture is also modified [80]. Changes in cell wall integrity could, in turn, affect protein secretion, since many yeast cell wall mutants display osmo-sensitivity and changes in protein secretion levels [82-85]. The improvement of single-chain antibody titers when *P. pastoris* cells are grown with salt stress [86] contradicts other studies reporting lowered titers of antibodies that were also produced in *P. pastoris* with salt stress [87], making the relevance of osmotic stress in fermentations a topic of debate and a poorly understood area of research.

In industrial scale fermentations, the main advantage of low pH values is the reduced activity of host proteases and a reduced chance of contamination [88-90]. The inhibition of proteases is probably the main contributor to higher product yield by reducing protein degradation, but lowered extracellular pH increases the energy requirements of the cell to maintain a constant intracellular pH, delaying cell growth and promoting cell wall rigidity, both of which are negative effectors for protein secretion [77,80,91]. Wang et al. [88] demonstrated this balance between efficient protein production and protease activity. These authors expressed the *Plasmodium falciparum* merozoite surface protein 3 (MSP3) in *P. pastoris* and determined MSP3 yield and protease activity at different media acidities. Although the protease activity increased with an increase in pH (pH 3.5 to pH 6.8), the highest MSP3 yield was obtained in cells grown at pH 6.8 and almost no MSP3 was produced at pH 5 and lower.

Kapteyn et al. [91] demonstrated an increase in the resistance of *S. cerevisiae* cells to cell wall degrading β-1,-3-glucanase when cultivated in media with a decreasing pH. This result was due to physical changes in the structure of the cell wall with a novel type of modification, an alkali-labile linkage of glycosylphosphatidylinositol (GPI)-dependent cell wall proteins to cell wall β-1,3-glucans. Additionally, microarray studies revealed changes in expression of many high-osmolarity glycerol (HOG) pathway-dependent genes and four cell wall-related genes, *CWP1, HOR7, SPI1* and *YGP1*, both of which have been previously linked to secretion [91,92].

Given this evidence, it can be assumed that several changes in cultivation conditions can be made to optimize heterologous cellulase production in recombinant yeast strains. However, this idea remains to be studied in detail and the optimal conditions are likely to vary depending on the specific cellulase of interest.

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Manipulation of yeast secretion: strain engineering

The secretion pathway of *S. cerevisiae* is generally described as the path that a newly synthesized peptide follows to reach the cell membrane, and the continuing elucidation of this complex pathway has introduced directed engineering approaches to improve the rate of protein export [93]. The yeast secretion pathway and frequent engineering targets are illustrated in **Figure**

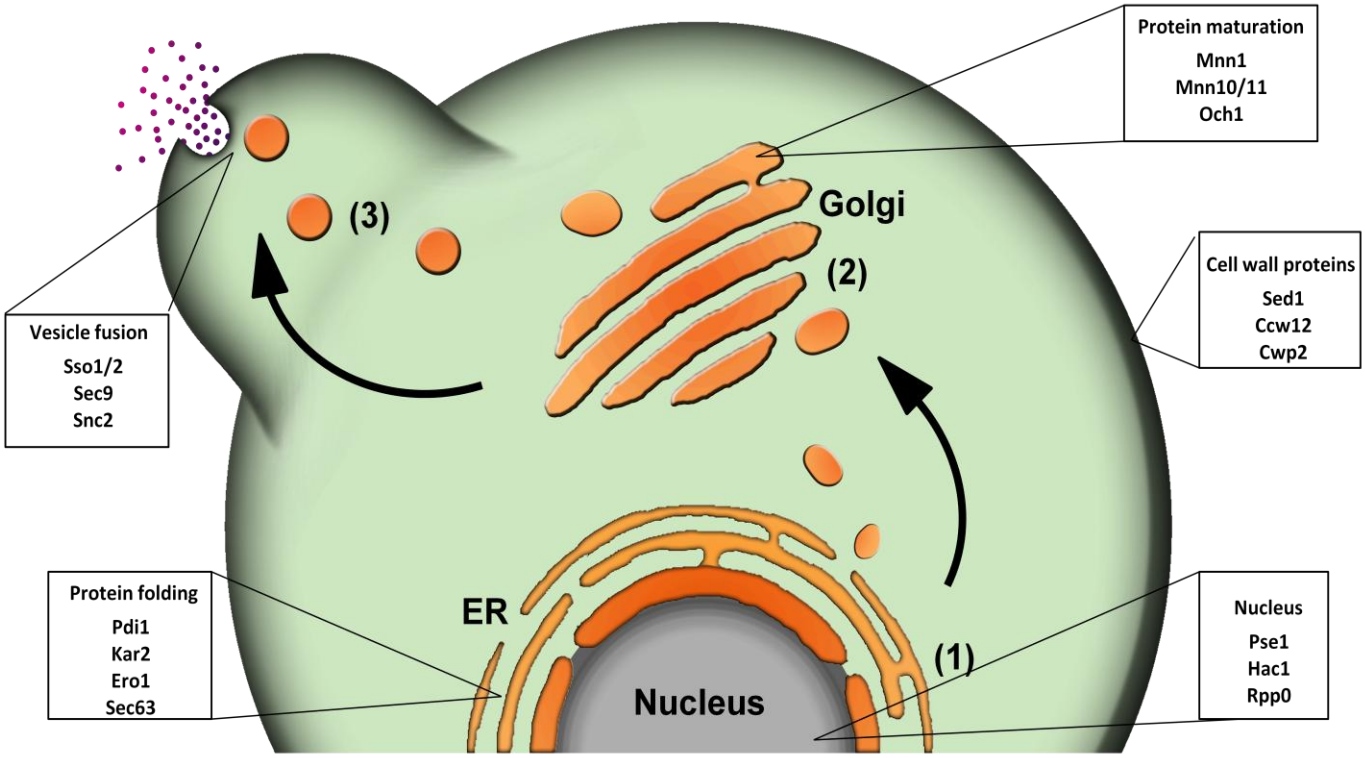

Fig. 2: A simplified diagram depicting the secretion pathway of yeast. (1) The ER: newly synthesized unfolded polypeptides, which are destined for secretion, enter the ER via co-translational translocation in a SRP (signal recognition particle)-dependent fashion. The polypeptides are correctly folded by numerous ER foldases and chaperones [94] and subsequently transported in COPII-coated vesicles to cis-Golgi compartments. (2) The Golgi apparatus: the Golgi is responsible for additional protein maturation modifications, such as signal peptide cleavage and the extension of *N*-linked glycan chains, a process that is initiated in the ER. (3) Vesicle fusion events: e.g., secretion vesicles with mature proteins are directed toward the tip and collar of the growing bud. In this process, the action of a number of SEC proteins [95] facilitates membrane fusion events to allow the release of the vesicle contents into the extracellular environment [96].

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The cell-specific productivity of the host organism can be improved by increasing the cellular abundance of a certain protein product, ensuring that more of this product is available for secretion [97]. This increased abundance is commonly achieved by ensuring efficient transcription along with subsequent efficient translation. Significant increases in heterologous protein titers have been obtained by codon optimization [98], increasing the gene copy number [99] and using modified or strong constitutive promoters [100,101]; however, protein specific effects have also been reported for these approaches. The abundance of the intracellular product, however, is never the complete solution to solve low secreted protein titers [102-104]. Efficient protein secretion is often stalled during the quality control steps of protein folding and membrane crossing events, contributing to the estimate that yeast secretory expression is 100- to 1000-fold lower than the theoretical protein yield [105].

Quality control machinery and chaperones are in place to ensure that misfolded or immature proteins do not escape the ER and enter the next steps of the secretory pathway [106]. Since polypeptides that initially enter the ER are unfolded, their hydrophobic amino acid residues are exposed; this, in combination with the elevated protein concentrations in the ER lumen, makes many nascent polypeptides prone to aggregation – a scenario that is common for many heterologously over-expressed proteins [107]. To avoid the accumulation of unfolded proteins, it is necessary for the cell to have tightly regulated quality control mechanisms, such as the UPR and ERAD, which are able to increase the folding capacity of the ER and remove misfolded or aberrant proteins from the secretion pathway, preventing the secretion of dysfunctional proteins under conditions of ER stress. The induction of these processes has been reported for many yeast production models that over-express native or heterologous proteins of interest [30,108,109].

The immunoglobulin heavy-chain binding protein (BiP) is a well-known example of an ER chaperone that is up-regulated under ER stress conditions, and if secretory proteins are incorrectly folded, they are often retained in the ER lumen in BiP-containing clusters [110-114]. This retention is one of the main bottlenecks known to limit the secretion of heterologous proteins in yeast [115]. Conversely, the over-expression of BiP in *S. cerevisiae* has also lead to increases in heterologous protein secretion of up to 8–fold [116]. Engineering strategies that focus on the post-translational folding of nascent polypeptides have also yielded improvements in heterologous protein secretion [117]. The over-expression of the protein disulfide isomerase

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PDI1, which is involved in protein folding and isomerization reactions, the UPR transcription factor *HAC1* [118] and other chaperones, such as *SEC63*, have previously led to improvements in heterologous protein secretion. Additionally, over-production of the PDI1 oxidant protein ERO1 was shown to increase the secretion of single-chain T-cell receptor (scTCR) by up to 5.1-fold. Wentz & Shusta [92] also illustrated that the over-expression of the ribosomal subunit encoded by *RPP0* or the cell-wall-associated proteins *CCW12, CWP2* or *SED1* all lead to significant secretion improvements for scFv (single-chain antibody fragment) and scTCR.

Possible secretory improvements relating to glycosylation have also been investigated [119,120]. The introduction of additional *N*-glycosylation sites within the N-terminal domain of a polypeptide increased the secretion of a selection of heterologous proteins, including cutinase, by up to 5-fold. These improvements, however, were largely protein-specific and were applicable mainly to proteins that are prone to aggregation [107]. The roles of *N*-glycosylation and cell wall integrity in secretion are becoming more evident following the observation of increased secreted protein yields in *N*-glycosylation-deficient mutants [120, Kroukamp et al. unpublished].

Another important bottleneck that has been demonstrated to significantly limit heterologous protein secretion in yeast expression systems is the exit of the nascent polypeptide from the ER and its subsequent targeting and transport to the Golgi [121-123]. The influence of the leader sequence used to target the secretion of heterologous proteins has been tested. Native *S. cerevisiae* leader sequences, foreign leader sequences and synthetically designed leaders have all been implemented with varying degrees of success. Native leader sequences provided particularly beneficial results, having been successfully utilized for the secretion of HAS (human serum albumin), IFN (human interferon) and *A. niger* GOD (glucose oxidase) [124-126]. However, heterologous leader sequences have been used for the highly successful secretion of xylanases and cellulases [30,72]. The presence of secretion signal peptides necessitates the action of specialized signal peptidases and cofactors [127]. Often, when secreted proteins are over-expressed, these cofactors and additional processing proteins become limiting, occasionally resulting in the accumulation of the precursor protein and a resulting decrease in absolute secretion titers [128]. Consequently, the over-expression of heterologous proteins in *S. cerevisiae* often leads to the intracellular accumulation of folded and misfolded proteins as different components of the secretory pathway are saturated [116,129].

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Native yeast proteases often limit the secretion titers of heterologous proteins by inducing proteolytic degradation to varying degrees [130]. The latter can be attributed to the proteolytic cleavage of vulnerable heterologous proteins that can occur through the action of native host-specific proteases, which are often present in relatively high levels in yeast and are often induced by environmental stresses [44,93]. The proteolytic activity of *S. cerevisiae* has been described in detail [131]. Lower heterologous protein yields can also occur because secreted proteins are inevitably exposed to vacuolar proteases (e.g., Proteinase A (*PEP4*), carboxypeptidase Y (*PRC1*), and proteinase B (*PRB1*)) following cell lysis after high density fermentation [132]. For this reason, protease-deficient strains have been engineered that alleviate this problem to a certain extent and increase the overall secreted protein yield [133,134].

Hou et al. [135] reported the intracellular accumulation of heterologous secretory proteins in *S. cerevisiae* due to limitations of the folding machinery. Many heterologous proteins that are intended for secretion are mis-sorted to the vacuole. Deletion of the vacuole protein sorting receptor VPS10 leads to recombinant protein secretion improvements [93,109,136]. Thus, it is clear that there are limitations at specific points in the secretion pathway that collectively limit heterologous protein secretion.

SNARE (Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins are required in the majority of vesicle fusion events during intracellular transport and play crucial roles in facilitating protein trafficking between the various membrane-enclosed organelles and the plasma membrane [135,137]. SNARE proteins have been identified on the ER, Golgi membrane, vacuole/lysosome, plasma membrane and the vesicles that are derived from these respective membranes [138]. Vesicle- and Target-SNARES have the fundamental characteristic of being able to recognize one another and form SNARE complexes to facilitate SNARE-mediated exocytosis [139] by bringing the respective lipid bilayers into close proximity, which is an energetically favorable state for fusion [140,141].

The over-expression of genes associated with vesicle fusion events, especially those promoting the Golgi to exocytosis phase, was applied to increase heterologous protein secretion with varying degrees of success [93,135,142]. The over-expression of a number of late-secretory pathway-acting genes, including *SSO2* (t-SNARE), had positive effects on native and heterologous protein secretion in *P. pastoris* [80], while the over-expression of *SSO2, SNC2* (v-

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SNARE) and *SEC9* (t-SNARE) in *S. cerevisiae* yielded improvements in the secretion of native and heterologous reporter proteins [van Zyl et al. unpublished]. Both yeast syntaxin homologs, *SSO1* and *SSO2*, have been reported to increase protein secretion 4- to 6-fold when over-expressed in *S. cerevisiae* using multicopy plasmids [142]. In our laboratory, we demonstrated that the secreted titers of a *T. reesei* EG1 were increased by the over-expressing of *SEC1*, but we obtained no significant increase in yields with *Saccharomycopsis fibuligera* BGL1 or *T. emersonii* CBH1 as reporter proteins [Kroukamp et al. unpublished]. Hou et al. [135] illustrated that the overproduction of either of the two SM proteins SEC1 and SLY1, which play different roles in SNARE assembly and fusion, resulted in notable increases in the secretion levels of native invertase and α-amylase. While SEC1 is involved in vesicle transport from the Golgi to the plasma membrane, SLY1 is responsible for the regulation of vesicle fusion from the ER to the Golgi.

Conclusions and perspectives

To develop yeasts, such as *S. cerevisiae*, for CBP conversion of lignocellulosic biomass to ethanol, a secreted heterologous cellulolytic enzyme system must be engineered into these yeasts. Heterologous EG and BGL secretion by a variety of yeast strains has been relatively successful, and *S. cerevisiae* strains have been developed that could grow on and convert a variety of cellulosic substrates to ethanol. However, the secretion of high levels of CBHs has proved to be particularly challenging. While the first report of the successful expression of a CBH in yeast was published over 24 years ago, the high level production that is required for successful crystalline cellulose hydrolysis has remained elusive until recently [30,41,42]. The study by Ilmén et al. [30] revealed several interesting phenomena that occur during CBH expression. (i) This group suggests the existence of a compatibility factor for the expression of certain genes or the production of certain proteins in a host, even when they are highly homologous. This idea echoes observations that heterologous protein titers often vary greatly between different expression hosts and that engineering to enhance protein secretion is highly reporter protein-specific. The evaluation of contributing factors, such as protein folding, glycosylation and the elucidation of other underlying factors responsible for this "compatibility", should allow us to predict which expression platform will be most suited for a given protein product or which protein may be more suitable for expression in a particular host. (ii) Ilmén et al. [30] also demonstrated that the expression of various CBHs exerted varying

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degrees of stress on the cell, observed as the induction of the UPR to varying degrees. This finding suggests a method by which the expression compatibility of a specific gene may be assessed. (iii) This group also showed that the cell has a means of down-regulating the plasmid copy number when a "stressful" gene is present on an episomal plasmid, but the method by which this occurs is unknown. This phenomenon suggests another reason why multi-copy gene integration is a superior method of strain construction for heterologous protein production. While CBHs that are compatible with expression in yeast were identified in several studies and strains that are able to grow on cellulosic substrates were generated, full CBP of real world substrates remains elusive. The optimal co-expression of enzymes for several other activities, including enzymes that are active on hemicelluloses, is still required. It is likely that significant strain engineering will be required to enable the optimal secretion of this plethora of enzymes.

Improvements in the levels of secreted heterologous proteins, including cellulase titers in several yeast strains, have been demonstrated. The secretion machinery of yeasts is a multi-step process and each step is directed and regulated by several proteins, providing a vast array of targets that can be manipulated in isolation or in concert to enhance heterologous protein secretion. Furthermore, several chaperone- and foldase-encoding genes from higher fungi with vastly better secretion qualities compared to yeast remain to be tested for their effect on yeast cellulase secretion. An increasing number of these genes are being constantly unveiled due to the mounting number of available genome sequences. There is an increased shift towards rational strain engineering approaches due to the availability of extensive transcriptomic, proteomic and metabolomic data. The potential of "-omics" data and other post-genomic technologies to identify possible gene candidates or pathways to be used in strain engineering strategies is clearly illustrated by the transcriptomics-based work of Gasser et al. [80]. Another recent approach to identify genes responsible for specific host strain characteristics is the pooled segregant whole genomic sequencing (PSWGS) of strains with a specific superior phenotype. PSWGS allows for the identification of the genes responsible for complex traits, such as high ethanol [143] or acetic acid tolerance or even high secretory capacity [144]. The principle of PSWGS is based on the recombination of chromosomes from genetically diverse parental strains, which differ from the phenotype of interest, during meiosis. The allelic variants that are present in the progeny that still display the superior phenotype are subsequently identified. Putative genomic variations that confer a phenotype of high CBH secretion were identified through this method [Kroukamp et al. unpublished]. The advantages

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of identifying secretion-enhancing genes through a polygenic analysis approach were highlighted by Kroukamp et al. [145]. In this study, the authors demonstrated a synergetic 4.5-fold increase in *S. fibuligera* BGL1 production with the over-expression of native *S. cerevisiae PSE1* and *SOD1*, encoding a karyopherin that interacts with the nuclear pore complex and a cytosolic superoxide dismutase, respectively. They also demonstrated how some genes might enhance secretion in combination with other effectors but not in isolation.

Therefore, there is a great scope for further improvement in heterologous protein secretion. One drawback is the observation that most of the positive effects observed thus far have been highly protein-specific, signifying that the effects should be asserted on a case-by-case basis for different proteins of interest. However, it is predicted that the identification of compatible gene candidates for all required activities, and the combination of these genes in a strain engineered for their optimal secretion, will enable the construction of ideal CBP yeast strains.

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Table 1. A selection of CBH genes secreted by yeast

Organism & Gene/ Enzyme	Titer (mg/L)	% Of Total Cell Protein	Activity/Substrate(s)	Specific Activity (U/mg)	Reference
S. cerevisiae:					
CBH1 (GH7)					
T. reesei CBH1	2	1.5	MUC, AC	NR	[46]
	5	*0.123*	MUL, BMCC	0.26 (BMCC)	[47]
	0.22	*0.006*	0.06 U/L (PASC), 0.06 U/L (BMCC)	0.22 (PASC)	[48]
	0.1	NR	0.6 U/L (MUL), <3% (Avicel)		[30]
A. niger CBHB	NR	NR	0.035 U/L (AC), 0.03 U/L (BMCC)	NR	[43]
P. chrysosporium CBH1-4	NR	NR	12 U/L, ~3.3 U/gDCW (BBG), 10 U/gDCW (PNPC)	NR	[48]
	NR	NR	22 U/gDCW (AC)	NR	[49]
	NR	NR	18 U/gDCW (PNPC)	NR	[50]
	NR	NR	0.035 U/L (AC), 0.03 U/L (BMCC)	NR	[50]
P. janthinellum CBH1	NR	NR	MUL	NR	[51]
T. aurantiacus CBH1	0.1	*0.002*	Avicel, AC, PNPC, PNPL	0.03, 0.04, 0.11, 0.29 (same order as activity)	[52]
A. aculeatus CBH1	7	*0.173*	Avicel, MUL	0.007 (Avicel)	[53]

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C. fimi cex	2.5	*0.03*	8 U/L, ~1.0 U/gDCW (PNPC)	3 (PNPC)	[54]
H. grisea CBH1	57		3.3 U/L (MUL), 9 % (Avicel)	NR	[30]
C. thermophilum CBH1	10		32 U/L (MUL), 7% (Avicel)	NR	[30]
T. emersonii CBH1	74		145 U/L (MUL), 7% (Avicel)	NR	[30]
T. emersonii CBH1-CCBM	300	1.2	84 U/L (MUL), 11% (Avicel)	NR	[30]
CBH2 (GH6)					
T. reesei CBH2	100	2.6	BBG, AC	NR	[46]
	10	*0.33*	24 U/L, 3 U/gDCW (AC)	0.7 (AC)	[55]
	NR	NR	0.15U/ gDCW (AC)	NR	[38]
	NR	NR	0.14 U/L (AC), 0.09 U/L (BMCC)	NR	[43]
	41	NR	6% (Avicel)	NR	[30]
A. bisporus CEL3	NR	NR	0.06 U/g DCW (AC), 0.033 U/gDCW (CC), 0.008 U/g DCW (BBG)	NR	[56]
C. heterostrophus CBH2	NR	NR	6% (Avicel)	NR	[30]
C. lucknowense CBH2b	1000	4	9% (Avicel)	NR	[30]
P. pastoris:					
CBH1 (GH7)					
T. reesei CBH1	0.5 (AOX1p)	NR	MULac, CNPLac, BMCC	NR	[57]

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T. reesei CBH1	0.1 (GAPp)	NR	MULac, CNPLac, BMCC	NR	[57]
C. thermophilum CBH3	1700	NR	2.5 U/ml (pNPC)	1.4 (BMCC); 1.1 (FP)	[58]
CBH2 (GH6)					
T. reesei CBH2	27	NR	0.27 U/mL (PASC)	1.72 (PASC)	[59]
Y.lipolytica:					
CBH2 (GH6)					
T. reesei CBH2	50	NR	0.36 U/mL (PASC)	2.4 (PASC)	[59]

U = micromole substrate released/min, NR = not reported. Italics indicate calculation based on the following assumptions: 0.45 g DCW/g glucose, 0.45 g protein/g DCW, 1.3×10^7 cells/mg DCW, and 1 OD(600) = 0.57 g DCW/L. CBH = cellobiohydrolase, EG = endoglucanase, BGL = β-glucosidase, AC = amorphous cellulose, BMCC = bacterial microcrystalline cellulose, BBG = barley beta-glucan, CC = crystalline cellulose, PNPC = p-nitrophenol cellobioside, PNPL = p-nitrophenol lactoside, MUC = methylumbelliferyl cellobioside, MUL = methylumbelliferyl lactoside, PASC = phosphoric acid swollen cellulose.