

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

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Received: 17 July 2015 / Revised: 14 August 2015 / Accepted: 5 September 2015 / Published online: 8 October 2015
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Abstract Soluble *N*-ethylmaleimide-sensitive factor attachment receptor proteins (SNAREs) 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 (*Te*-Cel7A, a cellobiohydrolase) and the *Saccharomycopsis fibuligera* Cel3A (*Sf*-Cel3A, a β -glucosidase) expressed in *Saccharomyces cerevisiae* through single and co-overexpression of some of the endoplasmic reticulum (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. Improvements were largely independent of gene dosage as all strains only integrated single additional SNARE gene copies, with episomal variance between the most improved strains shown to be insignificant. 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 *S. cerevisiae* as protein production host.

Keywords SNAREs · Cellulases · Secretion · Yeast

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;

Electronic supplementary material The online version of this article (doi:10.1007/s00253-015-7022-2) contains supplementary material, which is available to authorized users.

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Den Haan et al. 2015). Two of the major price components contributing to its financial unfeasibility 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 (Stephen et al. 2012; Isola 2013; Pu et al. 2008; Aden and Foust 2009).

Several strategies have been employed in order 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 and 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 and 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 *N*-ethylmaleimide-sensitive factor (NSF) 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 distinguishes different SNAREs from each other (Hong and 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; Grote et al. 2000; Götte and Fisher von Mollard 1998; Malsam et al. 2008; Kloepper et al. 2008; Hou et al. 2012) (Fig. 1). SNAREs can be divided into two classes 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 and Söllner 2011).

Our previous findings (Van Zyl et al. 2014) illustrated that the overexpression of exocytic SNAREs involved in the terminal step of the secretion pathway, 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 overproduction 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 and 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 (Parlati et al. 2002; Stone et al. 1997; Sjøgaard et al. 1994). The t-SNARE Sed5p combines cooperatively with the two light chains, Bos1p and Sec22p, in order 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).

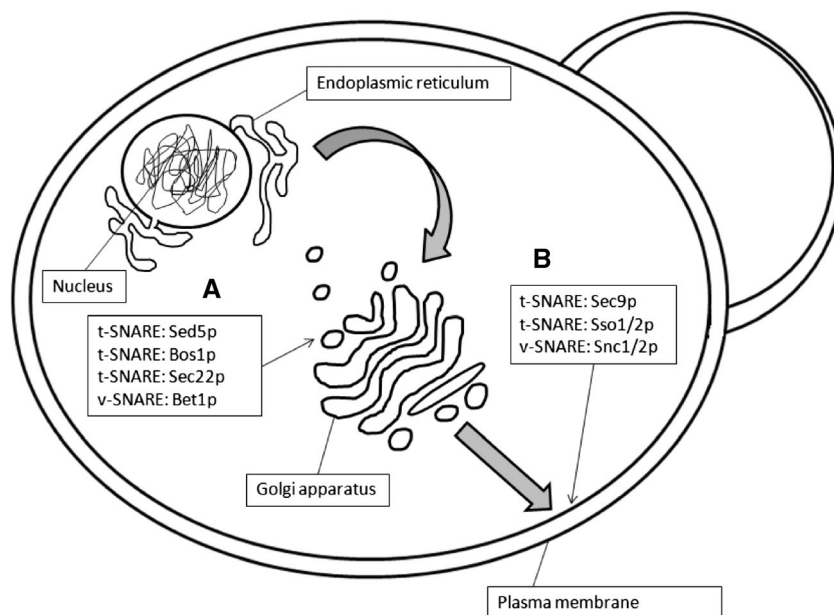
In this study, we investigated for the first time whether overexpression of the ER-to-Golgi SNARE components, facilitating vesicle fusion with the *cis*-Golgi, could increase heterologous protein secretion, with specific cellulolytic reporter proteins (*Sf*-Cel3A and *Te*-Cel7A) selected for expression in *S. cerevisiae*. The ER-to-Golgi t-SNARE components Bos1p, Sec22p and Sed5p, in addition to the v-SNARE Bet1p were constitutively overproduced, 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.

Materials and methods

Media and culturing conditions

S. cerevisiae strain Y294 (MAT α *his3* Δ *leu2* Δ *lys2* Δ *ura3* Δ) (ATCC 201160) was utilized as a background strain. Yeast cells were routinely cultivated at 30 °C in Yeast Extract-Peptone-Dextrose (YPD) medium (1 % yeast extract

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*-face of the Golgi. (B) The exocytic SNAREs facilitating fusion of the Golgi-derived secretory vesicles with the plasma membrane



(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 $\mu\text{g mL}^{-1}$ of the appropriate antibiotic (G418 disulphate (Melford Laboratories—Ipswich, UK), hygromycin B (Calbiochem—San Diego, USA), clonNAT (Werner BioAgents—Jena, Germany) and zeocin (Melford—Ipswich, UK)), whilst liquid cultures were cultivated on a rotary shaker (200 rpm) at 30 °C. For protein studies, yeast strains were grown on $2 \times \text{SC}^{-\text{ura}}$ (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 sulphate (Merck—Darmstadt, Germany) and 2 % glucose (Merck—Darmstadt, Germany), pH adjusted to pH 6.0 using 10 N sodium hydroxide). *Escherichia coli* DH5 α (Life Technologies—CA, USA) 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 $\mu\text{g mL}^{-1}$ ampicillin (Roche—Johannesburg, South Africa) at 37 °C.

Plasmid and strain construction

Standard DNA manipulation protocols were followed (Sambrook and 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—CA, USA) as instructed by the manufacturer, using forward and reverse primers that included *PacI* and *AscI* 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 *TEF1p-KanMX-TEF1t* selectable marker gene of pBKD1 with the *PFK2p-NAT-HXT3t* gene cassette. These vectors harboured different antibiotic selection markers (*KanMX* for G418 disulphate resistance, *hph* for hygromycin B, *NAT* for clonNAT and *Shble* 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 *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 (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 Online Table S1.

Plasmid isolations were carried out using the cetyltrimethylammonium bromide (CTAB) method (Sambrook and Russel 2001). All plasmids constructed and utilized in this study are summarized in Table 1. 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 gene (Kroukamp et al. 2013; GenBank

Table 1 Plasmids utilized in this study

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

AEV40916.1) on the ySFI episomal plasmid (Van Rooyen et al. 2005) and the other expressing the *T. emersonii* *CEL7A-cCBM* gene (Ilmén et al. 2011; GenBank AAL89553) on the pMI529 episomal vector (Ilmén et al. 2011) (Table 2). 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 and Da Silva 1997). Genomic DNA extractions were carried out using the method described by Hoffman and Winston (1987) and transformants were cultivated on selective YPD agar containing 100–200 $\mu\text{g mL}^{-1}$ of the appropriate antibiotic (G418 disulphate, hygromycin B or zeocin). All transformants were confirmed using PCR amplifications carried out using a *PGK1* promoter-specific forward primer and a gene-specific reverse primer (Online Table S1). All strains constructed for this study are described in Table 2.

Quantification of SNARE gene copy number and relative episomal quantities

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 genes of interest. Two reference genes, *ALG9* and *TFCl*, were selected to normalize the copy numbers of our genes of interest, as they are only represented at single copy 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 (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 quantities were carried out using the relative standard curve method (Applied Biosystems 2008). All primers utilized for real-time quantitative PCR are detailed in Online Table S1.

Enzyme assays

Yeast strains were inoculated in triplicate at an OD_{600} of 1.0 into 20 mL YPD in 125-mL Erlenmeyer flasks and cultivated for 72 h 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-h 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*-nitrophenyl- α -D-glucopyranoside (*p*NPG, Sigma—St. Louis, USA) as substrate. The extracellular cellobiohydrolase activity of the recombinant Y294 (*CEL7A*) strains was evaluated at 24-h intervals according to an adapted method described by La Grange et al. (2001), using *p*-nitrophenyl- β -D-cellobioside (*p*NPC, 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 a Bio-Rad xMark™ Microplate Spectrophotometer (Bio-Rad Laboratories—CA, USA) at $A_{400\text{nm}}$ and a reference strain (containing a vector with no cellulase-encoding gene) and media blanks were included to normalize activity readings. Some variability was observed between identical strains measured in separate assays, though the use of separate batches of substrate over a prolonged period of time accounted for this.

Table 2 Yeast strains utilized in this study

Yeast strain	Relevant genotype	Reference
<i>S. cerevisiae</i> Y294	MAT α his3 Δ leu2 Δ lys2 Δ ura3 Δ	ATCC 201160
<i>S. cerevisiae</i> Y294 (CEL3A parental)	ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-fur1::LEU2	Den Haan et al. (2007) ^a
Y294_CEL3A_BET1 (overexpressed)	ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-his3/HIS3-PGK1p-BET1-PGK1t-kamMX-fur1::LEU2	This work
Y294_CEL3A_BOS1	ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-his3/HIS3-PGK1p-BOS1-PGK1t-kamMX-fur1::LEU2	This work
Y294_CEL3A_SEC22	ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-his3/HIS3-PGK1p-SEC22-PGK1t-kamMX-fur1::LEU2	This work
Y294_CEL3A_SED5	ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-his3/HIS3-PGK1p-SED5-PGK1t-kamMX-fur1::LEU2	This work
Y294_CEL3A_BOS1_BET1	ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-his3/HIS3-PGK1p-BOS1-PGK1t-kamMX-PGK1p-BET1-PGK1t-hph-fur1::LEU2	This work
Y294_CEL3A_BOS1_BET1_SEC22	ura3/URA3-PGK1p-XYNSEC-CEL3A-PGK1t-his3/HIS3-PGK1p-BOS1-PGK1t-kamMX-PGK1p-BET1-PGK1t-hph-PGK1p-BET1-PGK1t-hph-PGK1p-SEC22-PGK1t-Shble-fur1::LEU2	This work
Y294_CEL3A_SSO1_SED5	ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3-PGK1p-SSO1-PGK1t-kamMX-PGK1p-SED5-PGK1t-NAT-fur1::LEU2	This work
<i>S. cerevisiae</i> Y294 (CEL7A parental)	ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3-	Ilmén et al. (2011) ^b
Y294_CEL7A_BOS1 (overexpressed)	ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3-PGK1p-BOS1-PGK1t-kamMX-fur1::LEU2	This work
Y294_CEL7A_BET1	ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3-PGK1p-BET1-PGK1t-kamMX-fur1::LEU2	This work
Y294_CEL7A_SEC22	ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3-PGK1p-SEC22-PGK1t-kamMX-fur1::LEU2	This work
Y294_CEL7A_SED5	ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3-PGK1p-SED5-PGK1t-kamMX-fur1::LEU2	This work
Y294_CEL7A_BOS1_BET1	ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3-PGK1p-BOS1-PGK1t-kamMX-PGK1p-BET1-PGK1t-hph-fur1::LEU2	This work
Y294_CEL7A_BOS1_BET1_SEC22	ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3-PGK1p-BOS1-PGK1t-kamMX-PGK1p-BET1-PGK1t-hph-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-kamMX-PGK1p-BET1-PGK1t-hph-PGK1p-BET1-PGK1t-hph-PGK1p-SEC22-PGK1t-Shble-fur1::LEU2	This work
Y294_CEL7A_BET1_SEC22	ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3-PGK1p-BET1-PGK1t-kamMX-PGK1p-SEC22-PGK1t-Shble-fur1::LEU2	This work
Y294_CEL7A_SED5_SEC22	ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3-PGK1p-SEC22-PGK1t-kamMX-PGK1p-SEC22-PGK1t-Shble-fur1::LEU2	This work
Y294_CEL7A_SED5_SEC22_BET1	ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3-PGK1p-SEC22-PGK1t-kamMX-PGK1p-BET1-PGK1t-hph-fur1::LEU2	This work
Y294_CEL7A_SNC1_SED5	ura3/URA3-ENO1p-CEL7A-ENO1t-his3/HIS3-PGK1p-SNC1-PGK1t-kamMX-PGK1p-SED5-PGK1t-NAT-fur1::LEU2	This work

^a GenBank accession nr of Sf-Cel3A: AEV40916.1

^b GenBank accession nr of Tz-Cel7A: AAL89553

Sodium dodecyl sulphate polyacrylamide gel electrophoresis, *N*-deglycosylation and densitometry

Strains were inoculated at an initial OD₆₀₀ of 1.0 into 20 mL double-strength buffered SC^{-ura} medium in 125-mL Erlenmeyer flasks and cultivated for 72 h on a rotary shaker (200 rpm) at 30 °C. Extracellular protein fractions (20 µl) were analysed using 10 % sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), 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 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 OD₆₀₀ of 0.05 into 20 mL Yeast Extract-Peptone ((YP)—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 OD₆₀₀ readings were taken using the Bio-Rad xMark™ Microplate Spectrophotometer and the data normalized with the use of media blanks. 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 tubes, inoculated from plate-grown cultures, on rotation (200 rpm) for 72 h. These cultures were each diluted to an OD₆₀₀ of 0.5 and subsequently serially diluted to 1:10 and 1:100. The initial dilution, the 1:10 and 1:100 serially 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 ethanol (4 and 6 %) and the secretion stress indicator tunicamycin (Sigma—St. Louis, USA) (0.2 and 0.5 µg mL⁻¹) with cultures incubated for 72 h at 30 °C prior to analysis.

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 strain constructed, with the strain yielding the highest extracellular enzyme activity being selected for further study. Further details regarding the selection of transformants are given in Online Fig. S1. Colonies were inoculated and cultivated for 72 h, after which they were normalized with regard to their OD₆₀₀ 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 following overexpression, superior with regard to extracellular enzyme activity for *Sf*-Cel3A and *Te*-Cel7A. The total collection of strains constructed is summarized in Table 2.

The maximum increase in extracellular *Sf*-Cel3A activity we were able to facilitate through the single overexpression of the respective ER-to-Golgi SNAREs was 22 % after 72 h compared to the parental strain, through the overexpression of the *SED5* t-SNARE component (Fig. 2a). All subsequently mentioned percentage increases refer to activity readings at 72 h, relative to the parental strain expressing either *Sf*-Cel3A or *Te*-Cel7A. The rest of the SNAREs investigated yielded no improved 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 lead 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 (22 % for *SED5* and 49 % for *SSO1*) (Fig. 3a). In an attempt to provide a 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 %) more activity relative to the parental strain (Fig. 3b).

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* leads to increases of 40 and 22 %, respectively.

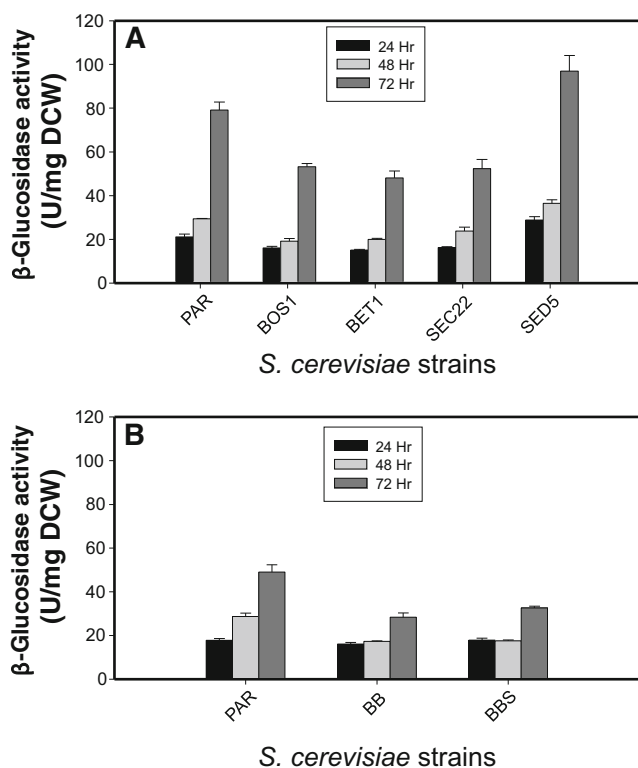


Fig. 2 The supernatant enzyme activities of the recombinant *S. cerevisiae* Y294 strains harbouring the ySFI episomal plasmid expressing the *Sf-Cel3A*, at 24-h intervals. **a** The respective SNARE genes individually overexpressed in each of the strains are indicated below (*BOS1*, *BET1*, *SEC22* and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

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 and 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 (Van Zyl et al. 2014) for *Te-Cel7A* improvements, with the top ER-to-Golgi candidate (*SED5*). The results, however,

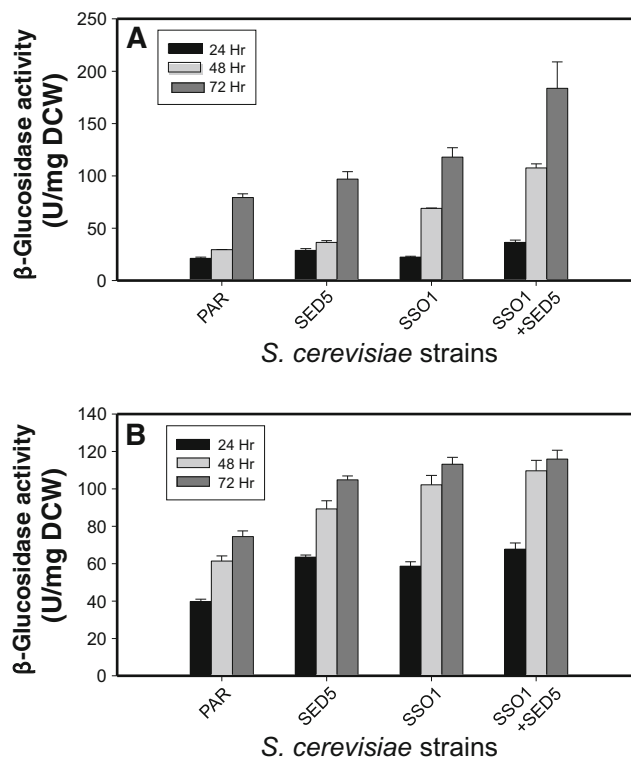


Fig. 3 The supernatant enzyme activities of recombinant *S. cerevisiae* Y294 strains harbouring the ySFI episomal plasmid, expressing the *Sf-Cel3A*, at 24-h intervals. **a** The respective SNARE genes overexpressed in each of the strains are indicated below (*SSO1*, *SED5* and a combinatorially expressing strain), whilst the parental (*PAR*) strain (not overproducing any of the SNARE genes) has also been included. **b** The total enzyme activities (cell and supernatant fractions) of the abovementioned *S. cerevisiae* Y294 strains, at 24-h intervals. All values represent mean values of assays done in triplicate with *error bars* indicating standard deviation

differ from the distinctive phenotypic increases achieved by overexpressing the individual SNARE genes for *Sf-Cel3A* with no significant improvement detected (Fig. 5).

The relative copy numbers of the overexpressed SNARE ORFs (in addition to the native copy) were determined relative to the *ALG9* and *TFC1* reference genes and results are depicted in Online Table S2. 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 (supplementary data), confirming that improvements in extracellular activity could be principally attributed to SNARE overexpression as opposed to differences in episomal expression levels.

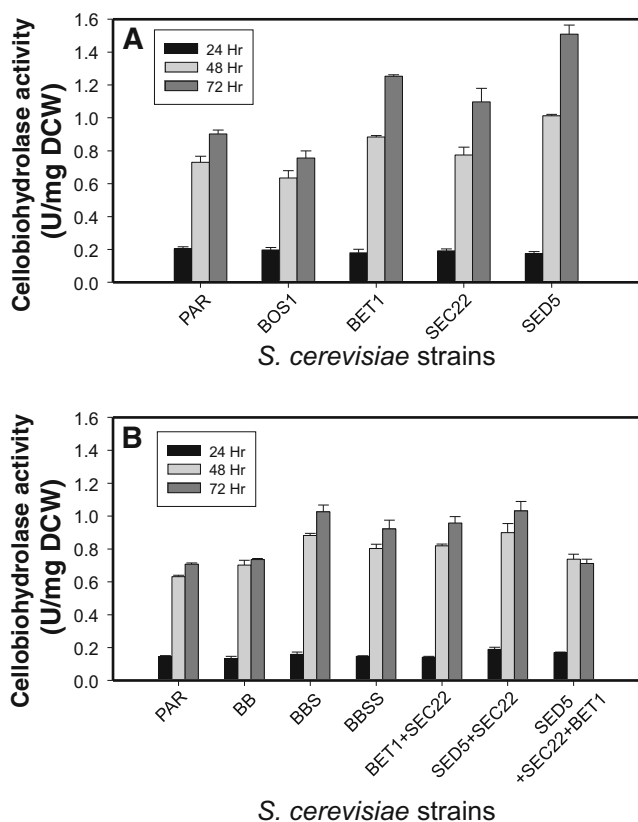


Fig. 4 The supernatant enzyme activities of recombinant *S. cerevisiae* Y294 strains harbouring the pMI529 episomal plasmid and expressing the *Te*-Cel7A at 24-h intervals. **a** The respective SNARE genes individually overexpressed in each of the strains are indicated below (*BOS1*, *BET1*, *SEC22* and *SED5*), whilst the parental (*PAR*) strain (not overproducing any of the SNARE genes) is also included. **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 standard deviation

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 recombinant, SNARE-overexpressing strains illustrating some of the most improved phenotypes (*Sf*-Cel3A-*SED5*, *Sf*-Cel3A-*SSO1*, *Sf*-Cel3A-*SIS5*, *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 appears at approximately 65 kDa. Deglycosylated extracellular protein fractions of the selected strains expressing the *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

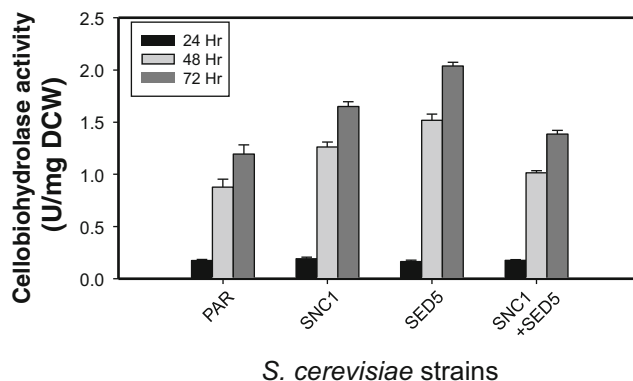


Fig. 5 The supernatant enzyme activities of recombinant *S. cerevisiae* Y294 strains harbouring the pMI529 episomal plasmid and expressing the *Te*-Cel7A at 24-h intervals. The SNARE genes being overexpressed (*SNC1*, *SED5* and a combinatorially overexpressing strain) along with the parental (*PAR*)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

target protein on a 10 % acrylamide gel (Fig. 6a). Kroukamp et al. (2013) illustrated that the *Sf*-Cel3A separates 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-*SIS5* strain (Fig. 6b). 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 in order 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.

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. 7a and b, respectively. The maximum specific growth rates (μ_{\max} (h^{-1})) and the final culture densities of these strains are summarized in Online Table S3. 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-*SIS5*) for an extended lag phase (Fig. 7a) and a decreased maximum specific growth rate (Online Table S3), 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-*SIS5*) was significantly affected following the diauxic shift at approximately 24 h, exhibiting a decrease in growth vigour until ultimately reaching an inferior terminal optical density to the rest of the investigated repertoire (Online Table S3). In

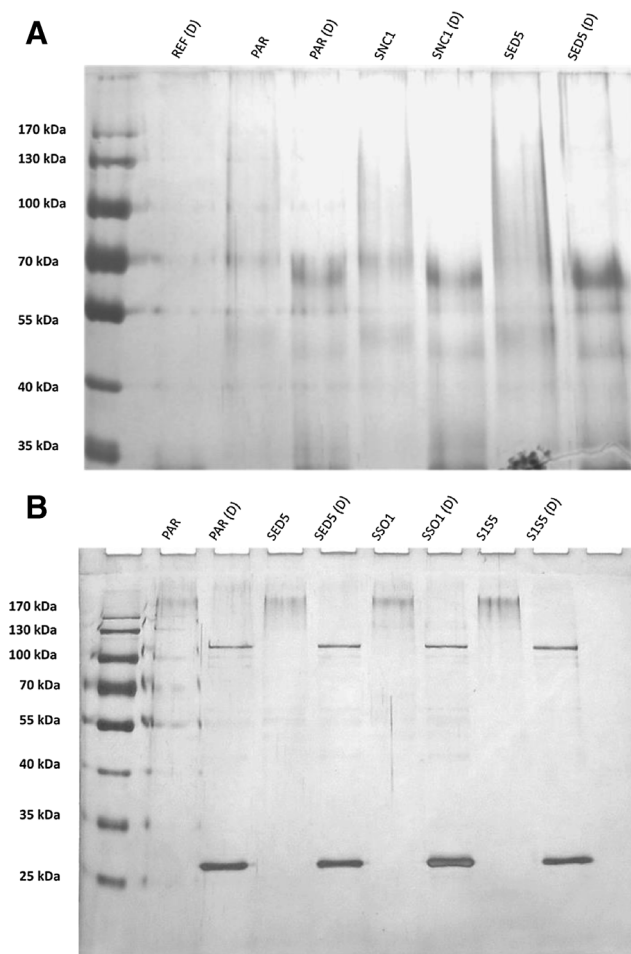


Fig. 6 **a** A silver-stained 10 % SDS-PAGE gel illustrating the quantitative differences in extracellular *Te-Cel7A* secretory titers between some of the most improved *S. cerevisiae* strains expressing this reporter protein (overexpressing *SNC1* and *SED5*) along with the parental (*PAR*) strain and 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 (*SIS5*) along with the parental (*PAR*) strain

contrast to the strains expressing the *Sf-Cel3A*, most of the *Te-Cel7A*-producing strains illustrated no signs of deleterious growth effects brought about by the respective SNARE overexpressions (Fig. 7b). 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 h, but reached the same endpoint optical density as the rest of the strains.

The overexpression of *SED5*, the most promising secretory enhancing SNARE component we have identified, led to detectable decreases in osmotic and ethanol tolerance for strains secreting both the *Sf-Cel3A* and the *Te-Cel7A* (Fig. 8a, b). Simultaneous overexpression (*BOS1*, *BET1* and *SEC22*) also

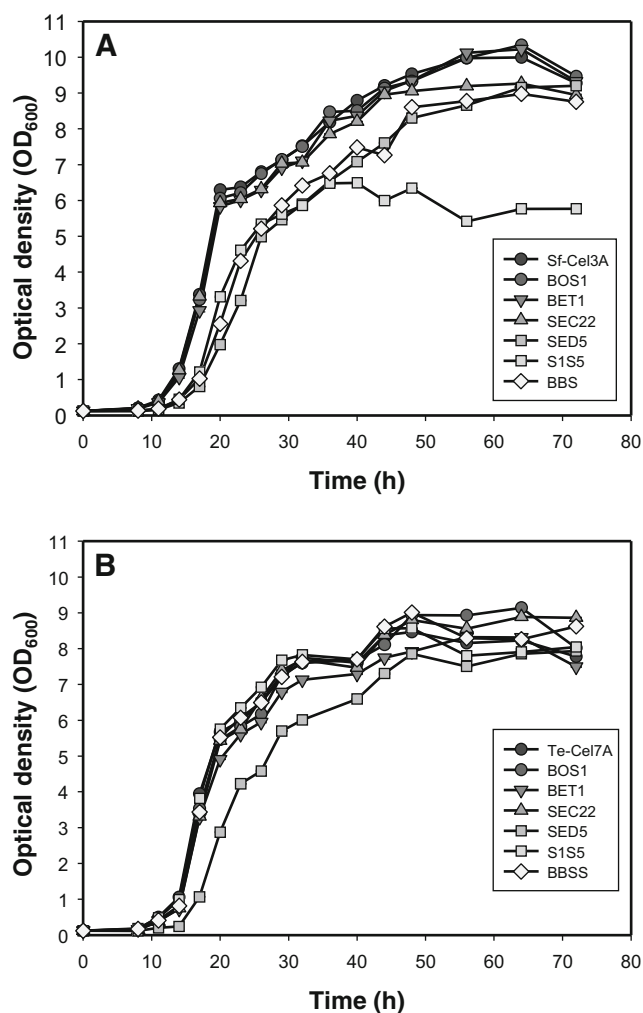
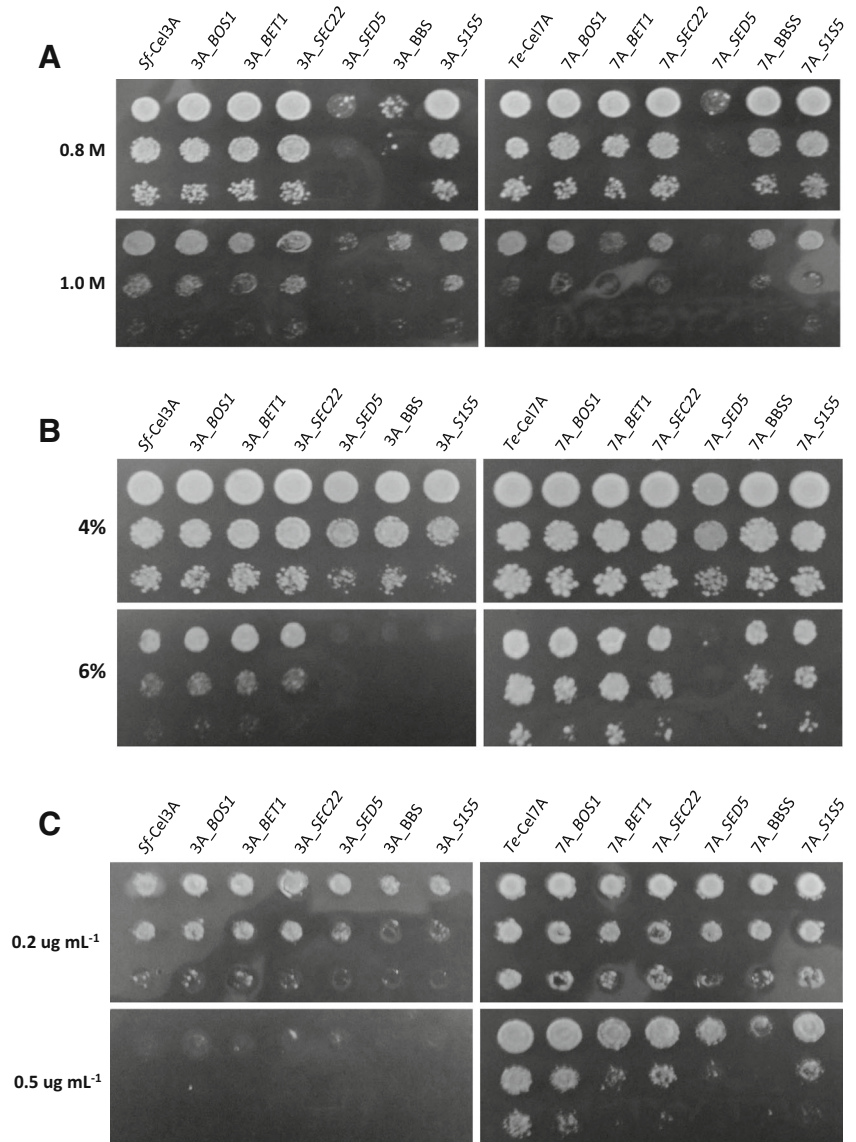


Fig. 7 **a** Growth trends for the parental (*Sf-Cel3A*) *S. cerevisiae* strain and all the singly overexpressing recombinant strains, along with the simultaneously overproducing (BBS—*BOS1*, *BET1* and *SEC22*) and combinatorially overexpressing (*S1S5*—*SSO1* and *SED5*) strains. Strains were evaluated over a period of 72 h, 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 overproducing (BBSS—*BOS1*, *BET1*, *SEC22* and *SED5*) and combinatorially overexpressing (*S1S5*—*SNC1* and *SED5*) strains. Strains were evaluated over a period of 72 h, with values representing the mean of triplicate cultures tested. Standard deviations were <5 %

decreased osmotic and ethanol tolerance in the *Sf-Cel3A*-expressing strain (Fig. 8a), whilst the most improved strain expressing this reporter protein (*Sf-Cel3A-SIS5*) also illustrated a decreased tolerance to increased ethanol concentrations (Fig. 8b). Enhancements in extracellular *Sf-Cel3A* activity were associated with greater ER-stress as higher tunicamycin concentrations ($0.2 \mu\text{g mL}^{-1}$) lead 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

Fig. 8 **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, with ethanol concentrations within the growth media (4 and 6 %) also illustrated. **c** A representation of the tolerance of the recombinant, SNARE-overexpressing strains to increased ER stress, induced by increasing concentrations of tunicamycin (0.2 and 0.5 $\mu\text{g mL}^{-1}$) in the growth media



strains, which also illustrated sensitivity in this regard (Fig. 8c). Neither of the two sets of strains grew when tunicamycin concentrations were raised to 0.75 $\mu\text{g mL}^{-1}$ (data not shown).

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 and Chen 2008; Singhania et al.

2013) and the bottlenecks they pose to lignocellulosic fuels (Sørensen et al. 2013), contributed to 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 and 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-Golgi 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 and Pelham 1992; Wooding and 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 leads to inferior phenotypes with regard to extracellular *Sf*-Cel3A activity (Fig. 2a), whilst the latter two components yielded less prominent increases (40 and 22 %, respectively) for the *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 overproduction 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), whilst attempts to simultaneously overexpress all four ER-to-Golgi SNAREs in a strain producing the *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 have investigated and that SNAREs may be an essential contributory element to these improvements.

The gene dosage for SNARE overexpression did not necessarily lead 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 (Online Table S2). 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 differential transcriptional regulation and expression (Rockman and Kruglyak 2006), 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 episomal expression variance (Online Fig. S2).

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 Sso1p, identified as the most effective exocytic SNARE candidate for the improvement of this particular cellulolytic reporter protein (Van Zyl et al. 2014), and Sed5p (Fig. 3a). However, given the marked decrease in growth capability (Fig. 7a) of this recombinant strain, coupled with a plateau in 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 during basal growth 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). 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. 6b). 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-overproducing Sso1p and Sed5p (*Sf*-Cel3A-*SIS5*), as well as the strain overproducing multiple SNARE components simultaneously (*Sf*-Cel3A-*BBS*) all illustrated a decreased maximum specific growth rate (Online Table S3), an extended lag phase and a lower terminal culture density (*Sf*-Cel3A-*SIS5*) (Fig. 7a). The strain simultaneously overproducing Sed5p and Sso1p (*Sf*-Cel3A-*SIS5*) became severely affected following the diauxic shift at 24 h, 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 have illustrated for this particular strain, in addition to osmotic and ER stress sensitivities (Fig. 8a–c). The most promising *Te*-Cel7A-secreting strain, overproducing Sed5p, illustrated an extended lag phase (Fig. 7b), a lower maximum specific growth rate (Online Table S3) and slightly diminished culture densities following the diauxic shift at 24 h, 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. 7b) was confirmed as increased concentrations of both ethanol and sodium chloride significantly inhibited the growth of the *Te-Cel7A-SED5* strain (Fig. 8a, b).

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). *S. cerevisiae* is 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 and El-Moghaz 2010). It is therefore conceivable that incorrect intracellular production and distribution of this solute could lead to the perceived osmotic vulnerabilities we have been able to illustrate in the most improved strains, 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. 2010). 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 and Thiede 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 $0.5 \mu\text{g mL}^{-1}$ 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—displaying 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 $\approx 130 \text{ U mg}^{-1}$) 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*.

A minimum of five different types of transport vesicles emerge from the late Golgi compartment (Harsay and 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 have attained for different reporter proteins. In addition, general polypeptide discrepancies such as protein size, levels of 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 catabolizing 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 et al. 2013). Our current research adds credence to the idea 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.

Acknowledgments Funding for this project was provided by the National Research Foundation (South Africa).

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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