Expression of stress-tolerance related genes in *Saccharomyces cerevisiae* producing heterologous cellobiohydrolase.

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I declare that **"Expression of stress-tolerance related genes in *Saccharomyces cerevisiae* producing heterologous cellobiohydrolase"** is my own work, that it has not been submitted for any degree or examination in any other university, and that all sources I have used I have used have been indicated and acknowledge by complete references.
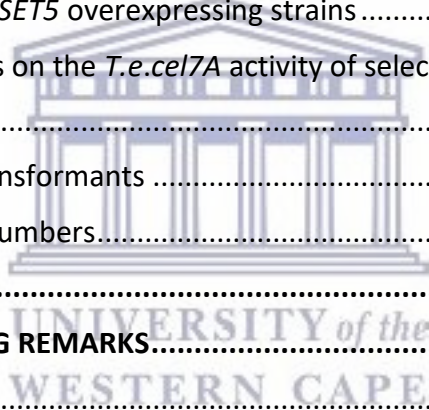
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B. <u>LIST OF ABBREVIATIONS</u>

1G first generation

2G second generation

ATP adenosine triphosphate

BGL β-glucosidase

C$_t$ cycle threshold

CBH cellobiohydrolase

CBP consolidated bioprocessing

CO$_2$ carbon dioxide

DCW dry cell weight

DMSO dimethyl sulfoxide

DNA deoxyribonucleic acid

DTT dithiothreitol

ESR environmental stress response

EG endoglucanase

ER endoplasmic reticulum

ERAD endoplasmic reticulum associated degradation

ESR environmental stress response

HMF hydroxymethyl furfural

HSP heat shock protein

HSE heat shock element

HSR heat shock response

H$_2$O water

H$_2$O$_2$ hydrogen peroxide

mRNA messenger ribonucleic acid

mtDNA mitochondrial deoxyribonucleic acid

OD optical density

PASC phosphoric acid swollen cellulose

PCR polymerase chain reaction

pNP p-nitrophenol

pNPC p-nitrophenyl-β-D-cellobioside

qPCR quantitative polymerase chain reaction

ROS reactive oxygen species

SC synthetic complete

SDS-PAGE sodium dodecyl sulphate - polyacrylamide gel electrophoresis

SNARE soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor

UPR unfolded protein response

USA United States of America

VGH Very high gravity

YPD yeast extract, peptone, glucose

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E. <u>ABSTRACT</u>

Cellulose is the most abundant naturally occurring renewable biopolymer on earth and a major structural component in plant cell walls, making it an ideal source of renewable energy. Consolidated bioprocessing (CBP) is a cost effective method of converting cellulose to liquid fuels such as ethanol. For CBP to be achieved an organism needs to be able hydrolyze cellulose and produce high yields of ethanol. The yeast *Saccharomyces cerevisiae* is an ideal CBP candidate, however wild type strains do not produce cellulases and these activities need to be engineered into yeast. In addition, the generally low secretion titers achieved by this yeast will have to be overcome. It has been demonstrated that heterologous cellulase secretion causes stress responses in yeast. Expression of stress tolerance genes were shown to enhance heterologous cellulase secretion. In this study two native *S. cerevisiae* genes, *YHB1* and *SET5*, were individually overexpressed by placing each gene under the transcriptional control of the constitutive *PGK1* promoter. The effect of these genes on heterologous protein secretion of cellobiohydrolase encoded by *cel7A Talaromyces emersonii* was investigated by integrating the *PGK1*$_{P/T}$-*YHB1* and *PGK1*$_{P/T}$-*SET5* cassettes into *S. cerevisiae* strains. Transformants were obtained that showed significantly higher secreted protein yield, with a resulting heterologous protein activity that ranged from 22% to 55% higher compared to the parental strains when grown in complex media. These increases in activity did not lead to any significant deleterious growth effects. The Y294-[*cel7A*]-*YHB1* strain also demonstrated multi-tolerant characteristics desirable in bioethanol production, i.e. high tolerance to osmotic stress, increased tolerance to secretion stress (tunicamycin) and high temperature stress. This study shows that cellulase secretion in *S. cerevisiae* could be greatly improved with strain engineering. These strains are a significant step toward creating an efficient cellulase secreting yeast for 2nd bioethanol production.

CHAPTER 1

LITERATURE REVIEW

1.1   Bioenergy

1.1.1   Introduction to biofuels

Bioenergy can be defined as renewable energy that is derived from biological sources and has gained a lot of attention in recent years due to concerns over energy security and the negative impact of fossil fuels that cause global warming (van Zyl *et al*., 2011b). Products derived from fossil feed stocks include plastic, fertilizers, pesticides, waxes, detergents and, importantly, fuel. Therefore a lot of urgency is put into research to find "cleaner" alternatives that can meet the demand of the growing population. This has lead towards making renewable energy that can be derived from biomass (Naik *et al*., 2010). Biomass can be defined as organic matter of plant or animal origin. It is also more predictable than solar and wind energy as the energy is stored in the chemical bonds of carbon and hydrogen (Bioenergy, 2009). Biomass can be found in forestry by-products, and agricultural and municipal waste. Biofuels are any liquid, gas or solid fuels derived from biomass. Solar energy is collected by plants via photosynthesis and stored as lignocellulose. Decomposition of the cellulosic material into simple 5- and 6-carbon sugars is achieved by physical and chemical pretreatment, followed by exposure to enzymes from biomass-degrading organisms. The simple sugars can be subsequently converted into fuels by microorganisms (**Figure 1.1**).

These fuels include, but are not limited to, ethanol, biodiesel, methanol, methane and hydrogen (Demirbas, 2008). Biofuels can broadly be classified into primary and secondary fuels (Nigam and Singh, 2011). Primary biofuels are those used in an unprocessed form and include the use of fuelwood, wood chips and pellets. Primary biofuels are generally used for heat, electricity generation and cooking. Secondary biofuels are produced by using processed biomass and converting it into ethanol, diesel, biogas, etc., that is mainly used in vehicles and industrial processes. Secondary biofuels can further be classified into first, second and third generation biofuels based on the source of the biomass and technology used to produce it.
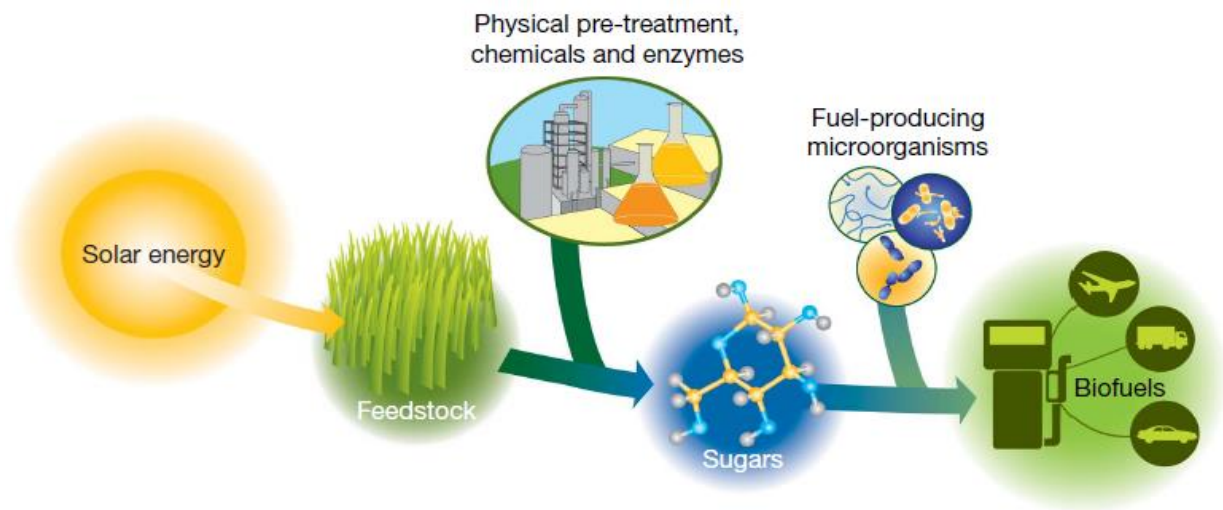
Figure 1.1: The biological conversion of solar energy to biofuels (Rubin, 2008).

The gap between the energy requirements of the industrial world and the incapability to renew this energy is growing as fossil fuels are being depleted, which in turn increases the threat of global warming (Balat and Balat, 2009). Biofuels are a strategically important sustainable fuel source that can help alleviate greenhouse gas if produced with carbon neutral technologies (Jaecker-Voirol *et al.*, 2008). An ever growing global population will always have a need for fuel for living, heating and transportation, leading to an increased demand of fossil fuels. One worrying statistic is that oil and gas production is approaching its maximum and that for every four barrels of oil consumed only one new barrel is found (Aleklett and Campbell, 2003). According to information from BP, we have approximately 53 years before our current oils reserves run dry (Tully, 2014). Therefore biofuels can be seen as an alternative future supplier of energy that will reduce carbon dioxide (CO_2) emissions, increase the security of the supply of energy and provide a source of income.

Many countries have implemented ways to introduce biofuels into the economy, however this is often influenced by the location, feedstock availability, political agenda and environmental concerns (Nigam and Singh, 2011). In South Africa the government's main rationale to implement biofuels is to diversify the energy industry and alleviate the risk of an energy crisis. In Western Europe the main focus for developing bioenergy is to decrease CO_2

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emission, and in America to decrease the reliance on fossil fuel and ensuring energy security (Balat and Balat, 2009). Aside from Africa, other countries and regions such as Brazil, USA and Europe rely on feedstock's such as corn, sugarcane and wheat to produce 1st generation biofuels. In Africa however, food security is a major issue, therefore finding an alternative feedstock to produce biofuels would ensure a sustainable future. In the past decade lignocellulosic materials and algal biomass have shown promise for conversion to biofuels.

1.1.2 Bio-ethanol

Ethanol is one of the most important industrial solvents that is used to synthesize organic chemicals such as ethylene and as a biofuel many countries currently add it to gasoline (Balat and Balat, 2009). Bioethanol is categorized as a secondary fuel because it is produced by processing biomass (Nigam and Singh, 2011). About 95% of bio-ethanol produced globally relies on biotechnological applications using glucose as a carbon source and microorganisms such as yeast and bacteria as biocatalysts. Currently bioethanol production can only become economically feasible if the carbon source (feedstock) price is low, conversion technology improves, the price of oil increases, or if it's regulated by the government. In Brazil the carbon source of choice is saccharose (sucrose) from cane molasses, while in the USA glucose obtained from corn starch is used. Since corn can be used for animal and human nutrition, a conflict about the use of this source (food versus fuel) is countered by research which aims to produce ethanol from biomass (Balat and Balat, 2009).

Ethanol was used in the first car engines in the late nineteenth century and received a resurgence in appeal as a viable candidate to replace fossil fuels ever since the energy crisis in the 1970s (Iodice et al., 2016). Bioethanol is also a very attractive biofuel for the automotive industry due to its miscibility with petroleum gasoline and the fact that it can be used in low concentration blends (< 10%) in vehicles with no modifications (Hamelinck *et al*., 2005). The use of ethanol blends has benefits of reducing water contamination and poses no significant adverse impacts on public health or the environment, generating lower emissions of CO_2, non-combusted hydrocarbons, carbon monoxide, nitrogen oxides and volatile organic compounds (Al-Baghdadi, 2003). Another advantage of using ethanol as a transportation fuel is that it offers high octane and high heat of vaporization, resulting in a greater energy output and

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improved net performance. The yeast *Saccharomyces cerevisiae* is the most important organism that is used to produce ethanol. This organism produces ethanol through fermentation, where it forms two moles of ethanol per mole of glucose. Using *S. cerevisiae* and other closely related yeast strains as ethanologens, industrial ethanol titers from sucrose are up to 93% of the stoichiometric maximum have been achieved (Weber *et al*., 2010). However yeast can metabolize saccharose, but not starch. Therefore if starch is used as the carbon source for ethanol production, depolymerisation to glucose must precede fermentation.

1.1.3 Bio-ethanol production according to technologies

1.1.3.1 First generation bio-ethanol production

First generation (1G) biofuels refers to the fuels that are derived from food crops rich in sugar or oil like corn, wheat, animal fats, and vegetable oil (Clark, 2007). Some of the crops suggested for 1G biofuel technology in South Africa include canola, sunflower oil and soybeans (Ryan et al., 2011). Starch based crops are normally processed first by breaking macromolecular starch into simpler glucose polymers by being mixed with water and ground into a mash (Hahn-Hägerdal *et al*., 2006). The mash is then cooked at or above boiling point and three enzymes are added for hydrolysis. The first enzyme, amylase, breaks maltodextrin down to oligosaccharides by liquefaction. The dextrin and other oligosaccharides are then hydrolyzed by pullulanase and glucoamylase to produce glucose, maltose and isomaltose. Once the fermentation broth is cooled to 30°C, yeast is added to convert the glucose into ethanol. Corn ethanol production can also be classified into two groups, i.e. wet & dry mill processes (Christophe *et al*., 2012). Wet mill processes usually produce other high-value products such as nutraceuticals, pharmaceuticals and other solvents aside from ethanol. Conversely dry milling focuses mainly on ethanol production.

1.1.3.2 Second generation bio-ethanol production

Second generation (2G) biofuels are produced from biomass, mainly plant biomass which consists mostly of lignocellulosic material (van Zyl *et al*., 2007). The material mainly represents the cheap and abundant non-food materials available from plants (La Grange *et al*., 2010).

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This untapped resource is the most abundant and underutilized renewable resource on earth. Lignocellulosic material can be divided into three different categories: forest residues, agricultural residues (sugar cane bagasse, crop waste etc.) and woody energy crops. In South Africa potential biomass resources vary from maize stover, sugarcane bagasse, and wheat straw to paper sludge and invasive plant species (Ryan *et al*., 2011).
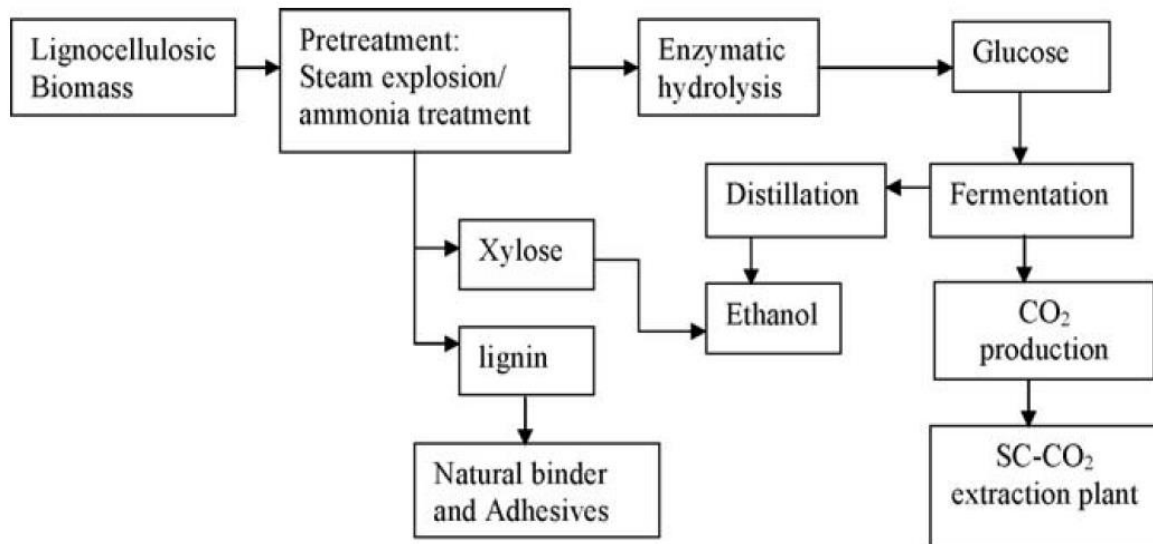
Figure 1.2: The biological conversion of lignocellulosic biomass to ethanol (Naik et al., 2010).

Currently 2G bioethanol produced on a commercial scale involves separate hydrolysis of lignocellulose polymers by the addition of cellulases followed by fermentation (Balan, 2014) (**Figure 1.2**). There is currently no ideal microorganism that exists that is able to produce all the hydrolysing enzymes required to saccharify lignocellulosic sugar polymers and that produces ethanol at the rates and titres required by industry. Therefore the ultimate goal of industry is to develop a single microorganism that is capable of producing these hydrolysing enzymes and is able to ferment the resulting sugars to ethanol, a process known as consolidated bioprocessing (CBP). One of the most successful strategies thus far has been the metabolic engineering of yeast species to secrete these enzymes as they already possess fermentation capabilities (Hasunuma *et al*., 2015).

In context of this study, we will look at the development of highly fermentative, robust yeast strains with the ability to produce recombinant cellulolytic enzymes to hydrolyse cellulosic substrates and ferment them to bioethanol.

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1.2   Biomass break down

1.2.1   Cellulose and Hemicellulose structure

The word 'cellulose' was given to the fibrous component of higher plants cells that had a unique chemical structure by Anselme Payen, as early as 1838 (Wertz *et al*., 2010). Cellulose is a homopolymer of glucose and the main molecule in the cell wall of higher plants. It can also be produced by certain algae, bacteria, fungi, animal tunicates and protozoa. There is more cellulose in the biosphere than any other biological substance. A cellulose molecule is a linear polymer of D-anhydroglucopyranose units linked together by β-1, 4-glucosidic bonds and differs from starch which contains α-1, 4-glucosidic bonds (**Figure 1.3**). Hemi-cellulose is a highly branched heteropolymer that consists of pentoses (xylose and arabinose) and hexoses (glucose, galactose, and mannose) as well as other sugar acids. Another component found in biomass is lignin which is a complex polymer of aromatic alcohols and does not contribute to the carbohydrate pool (Galbe and Zacchi, 2002).
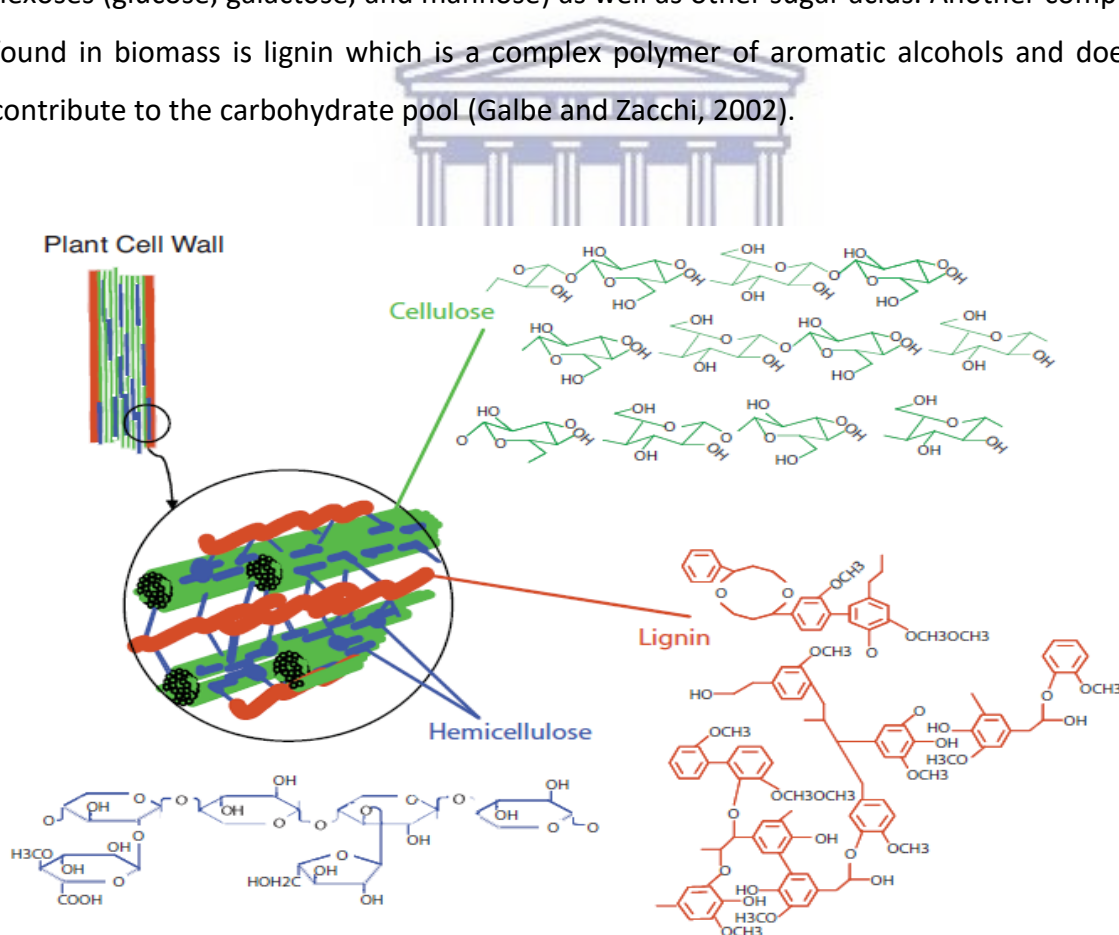
Figure 1.3: Schematic representation of the chemical structure of major compounds in lignocellulose. Retrieved from: https://microbewiki.Lignocellulose_biodegradation_in_Asian_long-horned_beetle.

The breakdown of cellulose in lignocellulose is inhibited by physiochemical, structural and compositional factors (Kumar *et al.*, 2009). Cellulose can exist in two different forms, the first is a tightly packed crystalline homo-polymer while the other has non-organized soluble amorphous regions depending on the source. The tightly packed crystalline structure is highly resistant to enzymatic hydrolysis and the presence of lignin and hemicellulose acts as a further barrier for cellulolytic enzymes to reach the cellulose (Margeot *et al.*, 2009).

1.2.2 Pre-treatment and enzymatic breakdown of cellulose

The breakdown of lignocellulosic feedstock requires an initial pre-treatment step due to its recalcitrant nature (Yang and Wyman, 2008). The pre-treatment is necessary to alter the structure of lignocellulose and make the cellulose accessible to cellulases during the hydrolysis step. The pre-treatment processes can be classified into two major groups: non-biological and biological (Kumar and Sharma, 2017). Non-biological methods can roughly be divided in physical, chemical and physico-chemical methods as illustrated in **Figure 1.4.** Biological methods include the use of bacteria and fungi as it is a more eco-friendly process and there is no inhibitor generation during the process.

The most commonly used pre-treatment methods used include ammonia fibre explosion, chemical treatment, biological treatment, and steam explosion (Kumar *et al.*, 2009). The type of pre-treatment defines the optimal enzyme mixture to be used and the composition of the hydrolysis products (Stephanopoulos, 2007). Biomass pre-treatment and hydrolysis are areas that need drastic improvement. A disadvantage of the pre-treatment of lignocellulosic biomass is the release and generation of a broad range of undesirable by-products discussed further in Section **1.5.5.** More information on the state of the art pre-treatment options is reviewed by Kumar and Sharma (2017).
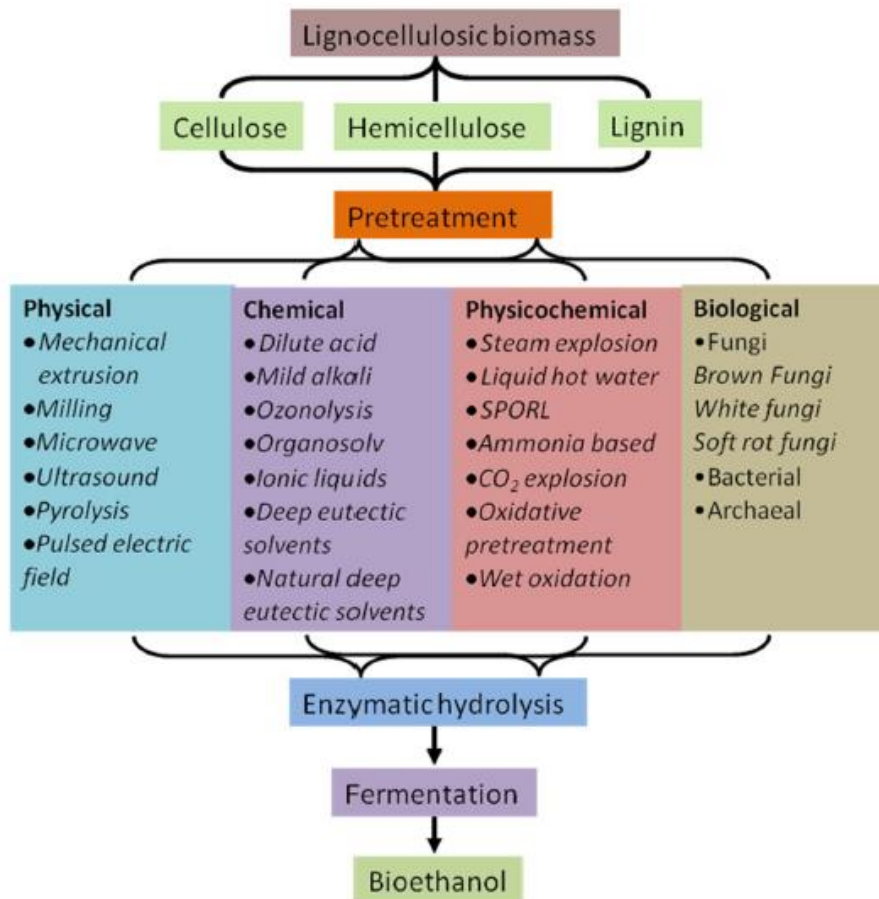
Figure 1.4: Pre-treatment methods used for the breakdown of lignocellulose and its conversion to bioethanol (Kumar and Sharma, 2017).

After pre-treatment, the biomass suspension is exposed to cellulolytic enzymes that can digest cellulose and hemi-cellulose to release primarily six- and five-carbon sugars (Stephanopoulos, 2007). Enzyme hydrolysis of cellulose requires the synergistic action of three major classes of cellulases, namely endoglucanases (EGs), exoglucanases or cellobiohydrolases (CBHs), and β-glucosidases (BGLs) (**Figure 1.5**) (van Zyl *et al*., 2011a). EGs begin by cleaving cellulose at random amorphous regions that have been exposed by pre-treatment and yields cellodextrins. This leads to a decrease in the degree of polymerization of the fibre and allows for new chain ends to be exposed. CBHs hydrolyze crystalline regions by acting on reducing or nonreducing chain ends to release the disaccharide cellobiose. BGLs are then able to hydrolyze the β -1,4 glycosidic bond of cellobiose and cellodextrins to release glucose.

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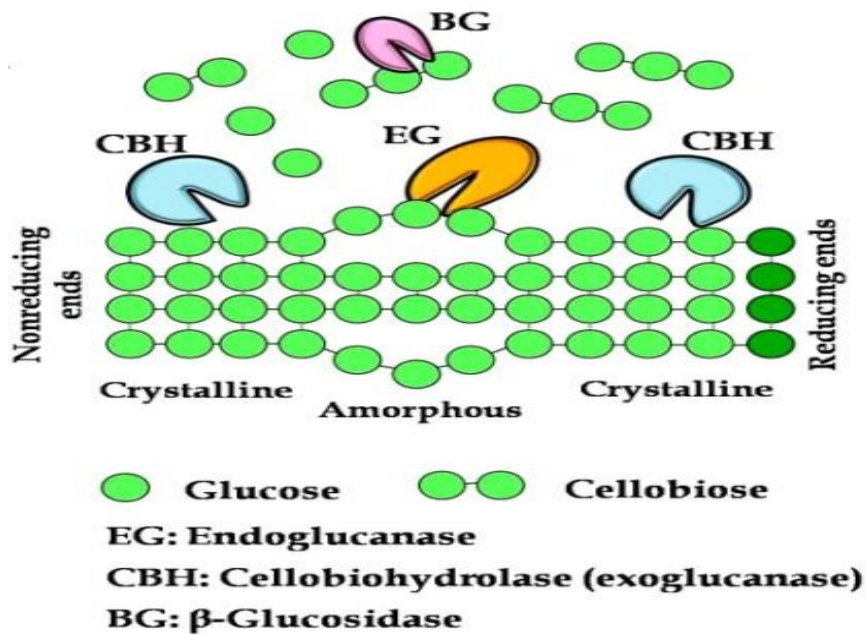
Figure 1.5: Schematic representation indicating the mode of action of the cellulase enzymes of non-complexed cellulase systems in the hydrolysis of amorphous and microcrystalline cellulose **(Waeonukul, 2013).**

In this study we are focusing on the cellobiohydrolase *cel7A* which is produced by the filamentous fungi *Talaromyces emersonii.* The enzymes cellulose binding domain is attached to the C-terminus of the catalytic domain through a linker which enhances hydrolysis of crystalline cellulose (Voutilainen *et al.*, 2010). Cellobiohydrolases are processive enzymes as they hydrolyse cellulose chains from reducing and nonreducing chain ends in a continuous manner (Teeri, 1997). The processive action of cellobiohydrolases is intrinsically slow and a major bottleneck in cellulose hydrolysis (Ilmén et al., 2011, Horn et al., 2012).

1.3 Consolidated bioprocessing (CPB) organismal development

1.3.1 Recombinant protein production in *S. cerevisiae*

Microorganisms are ideal hosts for the production of some heterologous proteins used both medically and industrially because of their rapid growth (Idiris *et al.*, 2010). Bacteria are one of the most efficient protein producers; however they do not perform some of

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co-/post translational modifications that eukaryotic organisms do such as removal of introns, glycosylation, phosphorylation and proper protein folding. Yeasts are able to perform some of these translational modifications to secrete heterologous proteins in their native form. Yeast expression systems also offer a number of other advantages including: simple handling, growth on inexpensive media, quickly reaching high cell densities, post-translational modifications and being free from pathogens or viral inclusions (Van Zyl *et al*., 2014). Yeast expression systems are also beneficial as they have many of the advantages of bacterial systems coupled to the advantages of eukaryotic systems. This is of particular importance in industrial scale production of proteins where secretion plays an important role in downstream purification. *S. cerevisiae* has thus been used for the industrial scale production of several proteins as is shown in **Table 1.1**.

Another advantage when cloning and expressing foreign proteins with yeast is the variety of vectors available (Clark and Pazdernik, 2011). These are generally classified into three main classes: (i) Episomal vectors which are designed to act as shuttle vectors between *E.coli* and yeast, (ii) integrating vectors that integrate into the yeast chromosome which is advantageous because episomal plasmids may often be lost in large-scale cultures and (iii) yeast artificial chromosomes (YACs) which can be used for cloning and analysing large regions from eukaryotic genomes. Most episomal vectors for *S. cerevisiae* are based on the high copy number 2μm plasmid found in most wild type strains and contains a prokaryotic origin of replication and a sequence for a specific antibiotic resistance for propagation in a bacterial host (Glick *et al*., 2010). The yeast sequences of the plasmid contains genes encoding markers such as β-isopropylmalate dehydrogenase (*LEU2*) and/or oritidine 5'-decarboxylase (*URA3*) as auxotrophic markers to select for yeast transformants (Gellissen and Hollenberg, 1997). It has also been observed that linear DNA fragments can undergo homologous recombination in *S. cerevisiae* which can be used to clone *in vivo* by using a fragment whose ends bear homology to plasmid sequences (Oldenburg *et al*., 1997). Shao *et al*., (2009) demonstrated how a fully functional biochemical pathway could be assembled through such *in vivo* homologous recombination.

S. cerevisiae also contains a number of promoters used for efficient transcription of heterologous genes in yeast vectors (Den Haan *et al*., 2007b, Jeon *et al*., 2009). Tightly

regulatable inducible promoters are mostly preferred for producing large amounts of protein within a specific time. A good examples of these are galactose-regulated promoters which increase transcription 1000-fold by the addition of galactose. Promoters from genes encoding glycolytic enzymes such as *PGK1* and *ENO1* are also commonly used for heterologous expression in yeast. Another common constitutive promoter is the glyceraldehyde-3-phosphate dehydrogenase (GAPD, GAPDH) gene promoter which is expressed in the presence of 2-5% glucose.

Table 1.1: Recombinant proteins produced by *S. cerevisiae* expression systems adapted from Glick et al., (2010)

Vaccines	Diagnostics	Human therapeutic agents
Hepatitis B virus surface antigen	Hepatitis C virus protein	Epidermal growth factor
Malaria circumsporozoite protein	HIV-1 antigens	Insulin
HIV-1 envelope proteins		Platelet-derived growth factor
		Fibroblast growth factor
		Blood coagulating factor XIIIa
		Hirudin
		Human growth factor
		Human serum albumin

S. cerevisiae does not contain any human pathogen sequences or produce fever-stimulating pyrogens and a number of human therapeutic proteins are produced by this organism (**Table 1.1**) (Glick *et a*l., 2010). This makes experimentation less extensive and cheaper than producing these proteins in unapproved host cells. It was found that more than 50% of the world's insulin supply is currently being produced by *S. cerevisiae* and a number of engineered strains are also major producers of the hepatitis B vaccine, which was the first commercialized recombinant protein of its kind (Gellissen and Hollenberg, 1997, Glick *et al.*, 2010).

All glycosylated proteins (*O*-linked or *N*-linked) can be secreted by *S. cerevisiae* provided they have a leader sequence to pass through the secretion system (Glick *et al.*, 2010).

Leader sequences derived from the yeast mating type α-factor gene usually allow for correct disulphide bond formation and endoprotease removal of this sequence so that the active recombinant protein can be secreted. Over the last decade the amount heterologous protein produced per liter of yeast culture has increased from ~0.02 to 2g/L mainly due to improvements in fermentation technology that allow growing cells to high densities. The amount of protein produced per cell remained unchanged due to issues such as incorrect folding, cellular mechanisms not coping with stress of protein overproduction, hyperglycosylation, codon usage, vector choice, leader sequences and cultivation conditions (Glick *et al*., 2010, Idiris *et al*., 2010). Due to *S. cerevisiae's* limited secretion capacity a lot of strain engineering is being done to increase secretion of heterologous proteins. However, the results vary based on the reporter protein characteristics which influences their progression through the secretion pathway (Den Haan *et al*., 2015, Kroukamp *et al*., 2013, Van Zyl *et al*., 2014).

1.3.2   Expression of cellulases in *S. cerevisiae*

One of the most successful strategies for production of bioethanol from biomass has been through using ethanologenic yeast species to ferment sugar released from lignocellulose (Kricka *et al*., 2014). *S. cerevisiae* in particular has many characteristics that make it appealing for industrial applications including high sugar consumption rate, tolerance of high osmolality, resistance to low pH (Temnykh *et al*., 2000, Den Haan *et al*., 2007, Gibson *et al*., 2007, Hasunuma *et al*., 2011, van Zyl *et al*., 2011a). Wild type *S. cerevisiae* strains do not produce cellulases or hemicellulases and are unable to convert either xylose or arabinose to ethanol. These activities need to be engineered into the yeast for optimal second generation ethanol production (Den Haan *et al*., 2013).

Yeasts can generally secrete reasonable titers of recombinant proteins and are more likely to secrete active forms of fungal cellulases compared to bacterial cellulases as their protein secretory machinery are similar to fungi such as *Trichoderma* species and *Aspergillus* species (Lambertz *et al*., 2014, Várnai *et al*., 2014, Young and Robinson, 2014). Fungal cellulases can generally be secreted by yeast using the native secretion signal peptide but can also be fused with cell-surface proteins such as α-agglutinin to form chimeric proteins (Hasunuma *et al*.,

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2015). One strategy to engineer cellulolytic *S. cerevisiae* strains is thus to produce multiple heterologous cellulases via the secretory pathway to allow their secretion as free enzyme, as yeast cells cannot take up cellulose. Another method involves displaying these cellulases on the yeast cell surface. However there is no significant quantitative data available to determine which option is most suitable (Van Rensburg *et al*., 2014).

Table 1.2: Recombinant cellulases produced by *S. cerevisiae* expression systems adapted from (Kricka et al., 2014)

Host strain	cellulase enzyme	Tethered or Secreted	PASC (g/L)	Ethanol (g/L)	Yield (g/g)	References
S. cerevisiae **Y294**	*T. reesei* (EGI)	Secreted	10	1.00	0.10	(Den Haan et al., 2007)
S. cerevisiae **Y294**	*Saccharomycopsis fibuligera* (BGLI)	Secreted				
S. cerevisiae **BY4742**	*C. thermocellum* CelA (EG)	Tethered to Cellulosome	10	1.25	0.12	(Goyal et al., 2011)
	T. aurantiacus (BGLI)	Tethered to Cellulosome				
S. cerevisiae **BY4742**	*C. thermocellum* CelA (EG)	Secreted	10	0.43	0.04	(Goyal et al., 2011)
S. cerevisiae **MT8-1/ cocdBEC3**	*T. reesei* (EGII)	Tethered	20	7.6	0.38	(Yamada et al., 2011)
	T. reesei (CBHII)	Tethered				
	A. aculeatus (BGLI)	Tethered				
S. cerevisiae **BY4741**	*T. reesei* (EG2)	Tethered	20	6.7	0.34	(Liu et al., 2016)
	T. emersonii (CBH1)	Tethered				
	C. lucknowense (CBH2)	Tethered				

There have been several studies that have successfully demonstrated heterologous production of EGs and CBHs both separately and in combination by *S. cerevisiae* (Ilmén *et al*., 2011, Olson *et al*., 2012)*. S. cerevisiae* strains producing both BGL and EG activities were able to ferment amorphous cellulose and the addition of CBH activity to these should enable the conversion of crystalline cellulose (Buijs *et al*., 2013). These recombinant strains can utilize a diverse range of synthetic substances such as carboxymethyl cellulose, phosphoric acid swollen cellulose (PASC) and Avicel microcrystalline cellulose through secreted cellulases (Lambertz *et al*., 2014, Yamada *et al*., 2013). It has been reported that the ratio of the three cellulases affects the efficiency of cellulose hydrolysis. This was examined by expressing different ratios of the three cellulases in a recombinant *S. cerevisiae* strain*,* where different

number of copies of the genes were integrated into multiloci delta sites in the *S. cerevisiae* genome (Yamada *et al.*, 2010b). The highest yield of glucose obtained from PASC was from a strain containing *EG1*, *BGL1* and *CBH2* genes in a ratio of 16:2:6. Recent work done by Liu *et al.*, (2016) demonstrated that assembling a cocktail of cellulases containing *EG2* and *CBH1* on the cell wall of a BGL-displaying S*. cerevisiae* resulted in 3.1 g/L of ethanol being produced from 20 g/L PASC. To further increase the cellulose degradation efficiency a new strain was constructed that also contained *CBH2* and this strain generated an ethanol titer of 6.7 g/L (**Table 1.2**). Liu *et al.*, (2017) were also able to construct a yeast strain displaying *EG1*, *BGL1*, *CBH1* and *CBH2* through ratio optimization that was able to produce 2.9 g/L ethanol from 10 g/L Avicel crystalline cellulose. While this represents the best results yet reported for crystalline cellulose hydrolysis without the addition of external enzymes, the conversion level is still only ~60% of the theoretical maximum. One way to improve conversion levels is to significantly improve the amount of heterologous secreted cellulases. In the next section we will explore various options of how this can be achieved.

1.3.3   Strategies for engineering *S. cerevisiae* for improved cellulase conversion

Researchers have tried different methods to increase cellulase production/secretion that include using different promoter and terminators, constructing artificial transcription factors, increasing gene copy number, codon optimization, designing secretion leader sequences and disrupting protease genes (Lambertz *et al.*, 2014, Yamada *et al.*, 2013). Other methods involved engineering the protein itself through structure based engineering, directed evolution and protein fusion (Fischer *et al.*, 2008). Both strategies have enabled promising advances, however, recombinant protein secretion is highly protein specific (Ilmen *et al.*, 2011). **Figure 1.6** summarizes some of the rational design strategies that have been attempted to improve CBP yeasts.

1.3.3.1 Promoter optimization

Many different promoters have been used to increase recombinant gene expression (Den Haan *et al.*, 2007c, La Grange *et al.*, 2010, Van Zyl *et al.*, 2014). Constitutive promoters such as the *PGK1* (Yamada et al., 2011), *TEF1* (Kricka *et al.*, 2014), *SED1* (Inokuma *et al.*, 2014), and

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ENO1 (Den Haan *et al.*, 2007a) gene promoters have been utilized for continuous expression of cellulase genes. Inducible promoters such as the *GAL1/10* promoter have also been used (Jeon *et al.*, 2009). These promoters drive significantly higher gene expression, however, they are repressed by glucose, the end product of cellulose hydrolysis and require the addition of an expensive starting substrate (galactose) to the medium.

1.3.3.2 Overexpression of native genes for enhanced secretion

Overexpression of native *PSE1* in *S. cerevisiae* lead to a 3.7- fold and 1.25 increased in secreted enzyme of recombinant *Saccharomycopsis fibuligera* Cel3A [BGL] and *Neocallimastix patriciarum* Cel6 [EG], respectively (Kroukamp *et al.*, 2013). Overexpression of exocytic soluble *N*-ethylmaleimide-sensitive factor attachment receptor protein (SNARE) genes such as *SNC1* and *SSO1* increased the secretion of *Talaromyces emersonii* c*el7A* [CBH] and *S. fibuligera* Cel3A (Van Zyl *et al.*, 2014). 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 (Van Zyl *et al.*, 2014).

Increased *N*-glycosylation of recombinant cellulases reduces their activity and might also play a role in less protein being secreted (Greene *et al.*, 2015). Knockout of the inherent glycosylation-related genes *MNN10* and *PMT5* increased the extracellular levels of *Phanerochaete crysosporium* excocellulase PCX up to 6.0-fold and 4.3-fold, respectively (Wang *et al.*, 2013). When different recombinant proteins were expressed in *S. cerevisiae* they were shown to exert varying degrees of stress, sometimes leading to the production of reactive oxygen species (ROS) (Jamieson, 1998). Simultaneous overexpression of *SOD1* (encoding a superoxide dismutase involved in ROS detoxification) and *PSE1* resulted in a 4.5-fold increase in secreted BGL compared to the parental strain (Kroukamp *et al.*, 2013). These genetic modifications in recombinant yeast can only be considered successful when an

acceptable level of enzyme production is achieved (Ilmen *et al*., 2011). The negative effects of recombinant protein expression on yeast metabolism is referred to as a metabolic burden and should be taken into account when developing CBP organisms (van Rensburg *et al*., 2012).
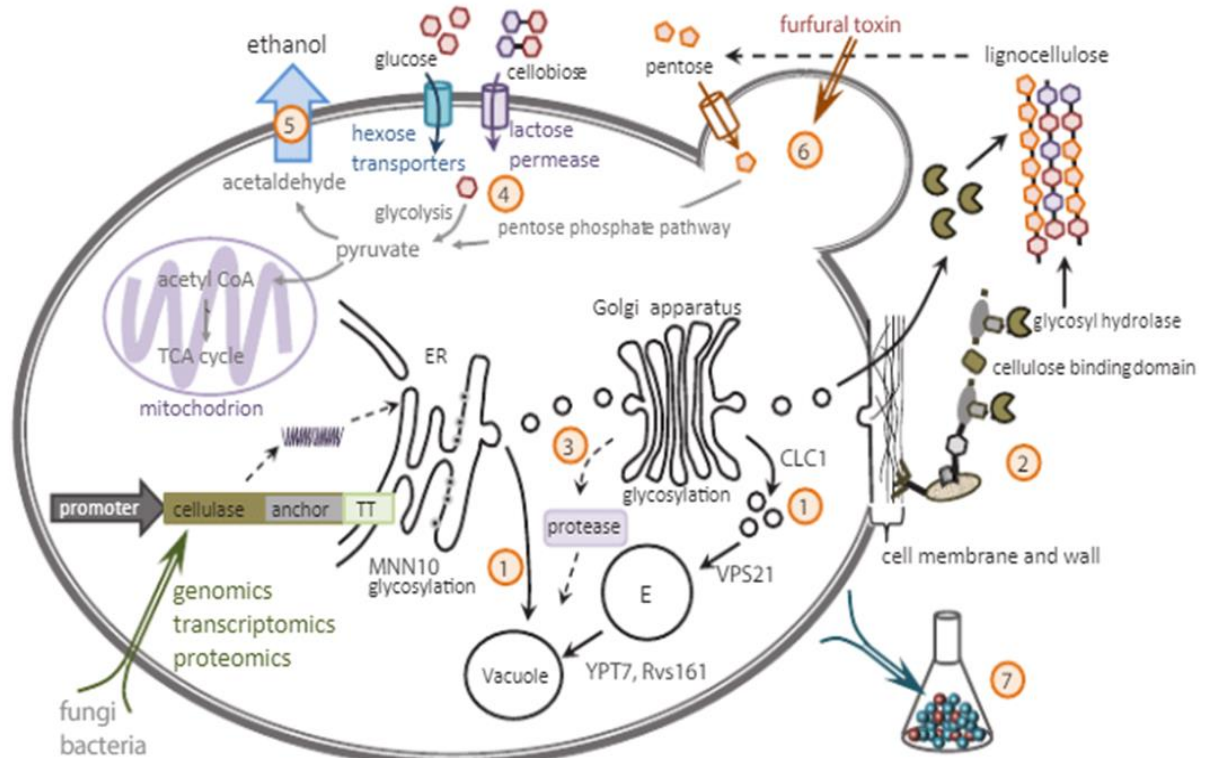
Figure 1.6: Strategies for engineering *S. cerevisiae* for enhanced protein secretion and CBP (1) deglycosylation and secretion improvement; (2) cell surface engineering; (3) protease deficient strain; (4) multiple carbon source utilization; (5) increased ethanol production; (6) tolerance adaptation; (7) immobilization and high cell density (Wang, 2015).

The engineering and expression of cellulases in yeast has progressed significantly in the last few years, however no ideal process-ready organism that can produce high levels of desired product without the addition of exogenous enzymes is available yet (Den Haan *et al*., 2015). Low hydrolysis rates remain the main obstacle, which can be improved through using more digestible feedstock's in combination with increasing cellulase secretion or activity. The cellulase secretory pathway involves many complex interactions, and overproduction and misfolding of recombinant proteins can trigger stress which results in increased metabolic burden and retarded growth (Hasunuma *et al*., 2015). To understand how rational engineering of the yeast secretion pathway and stress responses can improve heterologous cellulase secretion, we will now broadly explore these topics.

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1.4   Protein secretion and stress response

1.4.1   Introduction to the protein secretion pathway

Transport of newly synthesized proteins to specific cellular destinations is generally referred to as protein targeting or sorting and consists of two different processes (Lodish, 2004). The first process involves targeting of proteins to membranes of intracellular organelles which occurs during or after translation. The second process applies to proteins that are targeted to the endoplasmic reticulum (ER) and transported through the cellular membrane to specific organelles or cell membranes and this is referred to as the protein secretion pathway (**Figure 1.7**). The pioneering work done by Palade (1975) showed that in order for proteins to be secreted they must cross the ER before transportation to the plasma membrane. Further work done by Novick *et al.*, (1981) showed that proteins intended to be secreted first entered the ER lumen and were then transported to the Golgi apparatus via vesicles, glycosylated and finally transported in vesicles to the plasma membrane.

Proteins enter the secretion pathway through co- or posttranslational translocation into the ER lumen and may be bound by the chaperone protein BiP to facilitate folding into native structures (Idiris *et al.*, 2010). A number of other modifications take place in the ER including the processing of the signal sequence, disulfide bond formation, glycosyl-phosphatidyl-inositol addition, degradation and sorting. Misfolded or aggregated proteins bind to the BiP complex which acts as a quality control system and redirects these proteins to the cytosol for degradation, a process called ER-associated protein degradation (ERAD).

After proper folding and correct modifications, proteins are transported from the ER to the Golgi apparatus via special vesicles that bud from the surface of specialized ER domains (Farquhar and Palade, 1981). Proteins that contain retention signals are then recycled via retrograde transport of coat protein complex I coated vesicles, whereas proteins moving to the cell membrane are sorted into clathrin coated vesicles (Mellman and Warren, 2000). The membranes of the Golgi are thought to have an important role in maturation and transportation of proteins to secretion vesicles.
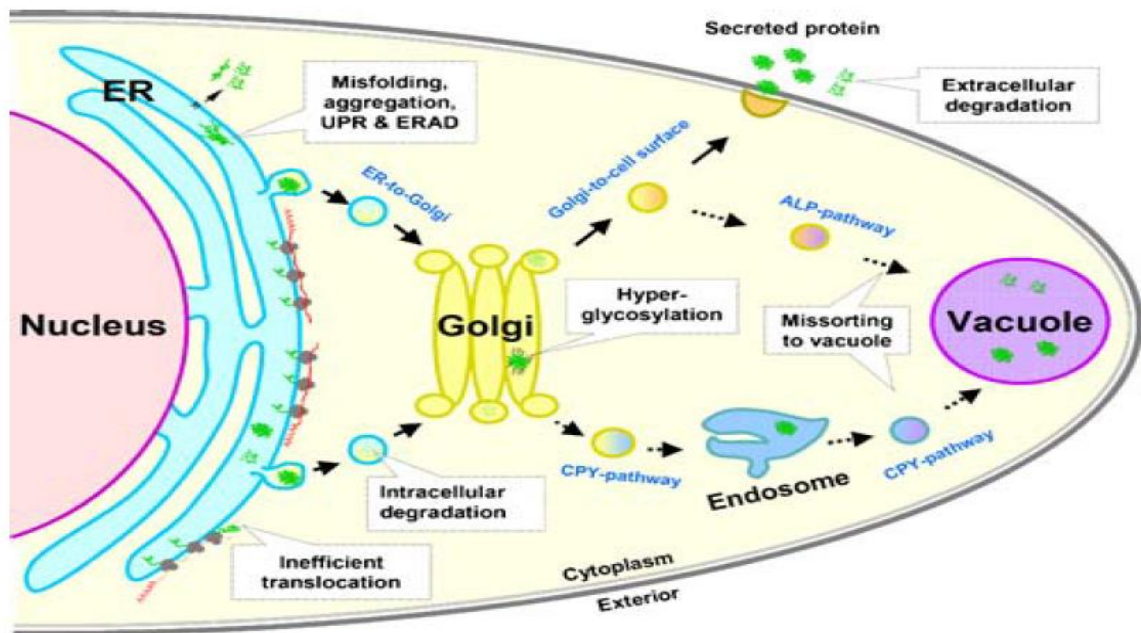
Figure 1.7: A schematic diagram representing the secretion pathway. Some of the major bottlenecks of heterologous protein secretion in yeast are indicated at various stages of the pathway (Idiris et al., 2010).

To create an efficient CBP organism that can effectively produce 2G bioethanol, the host organism should be able to secrete a vast amount of different recombinant enzymes to completely hydrolyse cellulose into fermentable sugars (Lynd *et al.*, 2005). One of the major bottlenecks is the low efficiency of expression and secretion of cellulolytic enzymes (Kroukamp *et al.*, 2013). Producing recombinant proteins in fungal species is less efficient than producing native proteins as several steps in the secretory pathway are potential bottlenecks during recombinant protein production (**Figure 1.7**) (Wang *et al.*, 2013). Theoretically the yeast secretory system should be able to secrete 100 to 1000 fold higher yields, however it is theorised that protein secretion is hampered during the quality control steps of protein folding and membrane crossing events (Cudna and Dickson, 2003, Idiris *et al.*, 2010, Wang *et al.*, 2013).

1.4.2   Protein secretion stress
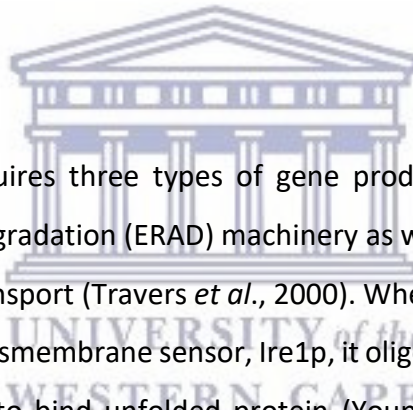
Yeast cells have stress-adaptation mechanisms, such as the induction of stress-related proteins (Gasch, 2003), changes in membrane composition (lipid composition and membrane fluidity)

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(Swan and Watson, 1999), repression of translation mechanisms (kinases that inhibit translation initiation and protein synthesis) (Harding *et al.*, 2000), accumulation of stress protectants (trehalose, proline, glycogen, sterols and intracellular glycerol) (Majara *et al.*, 1996), and by regulation of gene expression through signal transduction pathways (Gasch and Werner-Washburne, 2002, Kauffman *et al.*, 2002, Nicolaou *et al.*, 2010). These mechanisms help cells adapt to survive and even thrive in conditions that would otherwise be harmful to the cell. During heat-shock and ethanol stress, cells are known to alter plasma membrane properties, by reducing plasma membrane fluidity and increasing the degree of saturation of membrane lipids (Verduyn *et al.*, 1990). Stress protectants such as proline and trehalose are accumulated in the cell during stressed conditions and have stress-protective activities. They serve multiple functions *in vitro* such as stabilizing proteins and membranes, lowering the T_m of DNA, and scavenging reactive oxygen species (Takagi, 2008).

The response and adaptation mechanisms that occur in cells under stress are highly complex and therefore research on stress responses plays an important role in recombinant protein production (Mager and Ferreira, 1993). A lot of the fundamental principles of cellular and molecular biology have been discovered while studying how cells respond to stressful conditions. *S. cerevisiae* is one of the most established heterologous host systems in terms of genetic and physiological background and it is assumed that stress situations of the host cells can largely influence the productivity of an expression system (Ghaemmaghami *et al.*, 2003). Some of the stress encountered in a recombinant yeast strain arises from increasing gene copy number, codon usage of the expressed gene, transcription using strong promoters, translation signals, processing and folding in the ER and Golgi, and finally secretion out of the cell (Mattanovich *et al.*, 2004). These stresses caused by the exploitation of the cellular system to produce a recombinant protein often hampers the secretion of the final protein product due to the metabolic burden (van Rensburg *et al.*, 2012).

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1.4.3 ER stress – The unfolded protein response (UPR)

Approximately a third of the *S. cerevisiae* proteome transverses the secretory pathway before going to various destinations (Ghaemmaghami *et al.*, 2003). Within the secretory pathway lies an elaborate control system called the unfolded protein response (UPR) that regulates ER homeostasis to ensure proper protein synthesis and maturation. The UPR gets activated when a variety of exogenous and endogenous elements overwhelm the ER's processing capacity. These elements include chemical treatment, nutrient depletion and changes in redox status or calcium concentration. The UPR restores homeostasis by increasing the protein folding capacity, degrading unfolded proteins (through ER-associated degradation) and reducing translation and entry of new proteins into the ER (Gasch and Werner-Washburne, 2002). The cellular stress responses to unfolded proteins are known to play a significant role in the stress response to secretion of heterologous proteins (Cudna and Dickson, 2003, Kauffman *et al.*, 2002).

The UPR in *S. cerevisiae* requires three types of gene products which include molecular chaperones, ER-associated degradation (ERAD) machinery as well as key enzymes involved in lipid synthesis and protein transport (Travers *et al.*, 2000). When unfolded proteins stimulate the luminal domain of the transmembrane sensor, Ire1p, it oligomerizes when BiP (*KAR2* gene product) is removed from it to bind unfolded protein (Young and Robinson, 2014). Ire1p oligomerization facilitates the splicing of *HAC1* mRNA to enable synthesis of the Hac1p transcription factor that binds to unfolded protein response elements (UPREs) to induce the expression of several hundred genes (**Figure 1.8**) (Kohno *et al.*, 1993, Mori *et al.*, 1992). The Ire1-dependent *HAC1* mRNA is the only mechanism identified to date that signals from the ER lumen and triggers transcriptional changes in yeast.

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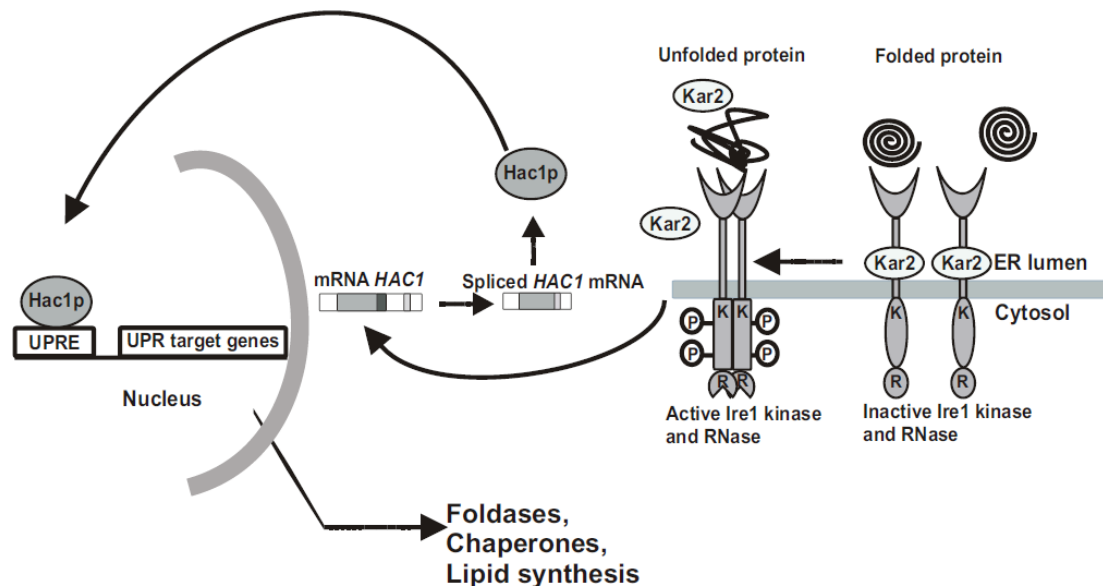
Figure 1.8: A schematic diagram representing UPR induction in *S. cerevisiae*. Ire1p oligomerization facilitates the splicing of *HAC1* mRNA to enable synthesis of the Hac1p transcription factor that binds to unfolded protein response elements (UPREs) (Guerfal et al., 2010).

Kauffman *et al.*, (2002) have described the induction of BiP in *S. cerevisiae* upon the overexpression of a secreted single chain antibody, which indicates that heterologous protein expression induces the UPR. Ilmén *et al.*, (2011) also demonstrated that expression and co-expression of two cellobiohydrolases in *S. cerevisiae* induced the UPR. The researchers found that spliced *HAC1* mRNA was not detected in the yeast strain containing an empty vector while it appeared in each of the strains expressing the cellobiohydrolase. Transcript levels of *KAR2* and *PDI1* (also known to be induced by UPR) were also elevated relative to the control strain.

In this study we induced the UPR in the laboratory yeast strain Y294 producing cellobiohydrolase by the additional of ER stress through chemical secretion 'blockers' such as the glycosylation inhibitor tunicamycin and the secretion stressor sodium orthovanadate which is known to prevent the release of secretion vesicles (Ballou *et al.*, 1991, Bull and Thiede, 2012, Arvas *et al.*, 2006, Berry *et al.*, 2011). We investigated how the responses of recombinant strains we constructed differed in the presence of different stresses in order to elucidate the mechanisms of the stress-tolerance genes.

1.5   Environmental stress and inhibitors

1.5.1   Environmental stresses

In addition to stress induced by heterologous protein secretion, yeasts may face numerous environmental stress factors. Yeast cells have evolved to be remarkably proficient at surviving sudden harsh changes in their external environment (Gasch, 2003). In the wild, yeast cells must adapt to sudden changes in temperature, osmolarity, acidity, the presence of radiation and nutrient starvation. When there is a sudden change in environment, cells must rapidly adjust their internal machinery to that required for growth in the new environment. For bioethanol production, the fermentation environment has very specific environmental challenges compared to the wild which include varying ethanol concentrations, high temperatures and the presence of lignocellulosic-derived inhibitors (Mukherjee *et al*., 2014). Yeast cells are often exposed to these stresses in a sequential manner (Nicolaou *et al*., 2010). When cells are under severe stress, yeast fermentation is often inhibited or limited, lowering the efficiency of product formation. Stress associated with fermentation also interferes with cell growth, internal secretory pathway mechanisms and the level of protein secreted in the medium (Kaufman, 1999, Bauer and Pretorius, 2000). It is therefore also important to focus on the effect of these environmental factors and not just on engineering the host strain or protein of interest when developing CBP yeast strains.

1.5.2   Ethanol toxicity

The main objective of 2nd generation bioethanol production is to produce ethanol from fermentable sugars (Den Haan *et al*., 2015). Under normal fermentation conditions the final ethanol concentrations range between 3-6%, and under high gravity fermentation the concentration may be >10% (Gibson *et al*., 2007). Ethanol is inhibitory to yeast at high concentration by disrupting the integrity of the cell membrane. The effects of ethanol on yeast may vary but the main site of ethanol damage seem to be the cellular membrane. More specific effects include growth inhibition, reduced cell size (Canetta *et al*., 2006), reduced viability, reduced respiration and glucose uptake (Pascual *et al*., 1988), reduced fermentation (Fernandes *et al.*, 1997), enzyme inactivation, lipid modification, loss of proton motive force across the plasma membrane and increased membrane permeability (Marza *et al*., 2002),

lowering of cytoplasmic pH and the induction of respiratory-deficient mutants (Jiménez *et al*., 1988). When yeast cells are exposed to ethanol stress, cells need to adapt either through transcriptional, translational or other types of regulations (Gasch *et al*., 2000). Different genes which are involved in metabolism, protein trafficking, ionic homeostasis and lipid metabolism to restore vital cellular functions get differentially up- or down-regulated (James *et al*., 2003).

Exposure of yeast cells to ethanol stress induces the production of trehalose (Ding *et al*., 2009). Trehalose is involved in reducing membrane permeability and increasing ethanol tolerance by inhibiting endocytosis in yeast cells exposed to toxic concentrations of ethanol (Lucero *et al.*, 2000). It has been demonstrated by Alexandre et al., (2001) that genes involved in trehalose synthesis in yeast are up-regulated within 30 min of ethanol induced stress and down-regulated as the fermentation continues. Other genes that play a role in ethanol stress can be seen in **Table 1.3.** The genes named in the table were deletions that conferred sensitivity to yeast grown in complex glucose-based medium containing 6% ethanol. It was shown recently that manipulation of ion transport systems could improve ethanol tolerance in yeast. Similarly Lam *et al*., (2014) demonstrated that changing potassium ion and proton electrical forces could improve yeast tolerance to ethanol. Transcription reprogramming using a transcription machinery engineering approach also lead to higher ethanol resistance (Alper *et al*., 2006). Mutagenesis of the transcription factor *SPT15* allowed for selection of the strain SPT15-300 that contained a mutation in a phenylalanine (Phe[177] Ser) as the dominant mutation which lead to increased tolerance to high concentrations of glucose and ethanol, as well as improved ethanol production (Davies, 1995).

Table 1.3: Gene deletions that conferred sensitivity to 6% ethanol on complex glucose-based medium (van Voorst et al., 2006)

Gene	Growth condition	Function
MSN2 and *MSN4*	No growth	Activator; binds to STREs (CCCCT) and mediates protein kinase A dependent gene expression
IMG1	No growth	Involved in mitochondrial function
SMI1 and *BEM2*	No growth	Encoding proteins involved in the down-regulation of signaling through the *PKC1* pathway
VPS15, 15, 34, 36, 39 and *VPH1*	No growth	Vacuolar function and vesicular transport to the vacuole
SLG1 and *ROM2*	Reduced growth	Encoding proteins involved in up-regulation of signaling through *PKC1* pathway
yGIM4 and *GIM5*	Reduced growth	Encoding subunits of the hetero-oligomeric co-chaperone GimC complex, involved in the function of actin/tubulin folding
FEN1, PLC1, ERG6, TPS1 and *SUR4*	Reduced growth	Involved in lipid biosynthesis, which is in addition to those involved in phosphatidyl inositol 3,5-bisphosphate synthesis (*VPS34*, *VAC14*, and *FAB1*), and they are up-regulated during ethanol stress by the general stress response pathway
ATP1, HMI1, MSK1, AND MTF2	Reduced growth	Involved in the mitochondrial function, which have positive function during ethanol stress

1.5.3   Oxidative stress

Oxidative stress occurs when there are toxic levels of oxygen-derived ROS (Jamieson, 1998)**.** ROS are represented by different oxidation states of dioxygen (O_2) and includes singlet oxygen, superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical (OH). Specific effects caused by oxidative stress include lipid peroxidation, protein inactivation and nucleic acid damage, including damage to mitochondrial DNA (mtDNA) which leads to the generation of respiratory deficient 'petites'(Gibson *et al*., 2007). Oxygen plays a contradictory role within cells, i.e being essential for aerobic respiration and other metabolic processes, while also being inherently toxic (Davies, 1995).

ROS are usually generated from environmental insults and side reactions of normal aerobic metabolism reactions (Davies, 1995). The main source of ROS in eukaryotic cells is through mitochondrial respiration via oxidative phosphorylation (Murphy, 2009). When ATP is generated, electrons are transported along protein complexes that constitute the electron transport chain to the acceptor oxygen to form water. When leakage of these electrons from the respiratory chain occurs it results in the reduction of oxygen, generating ROS in yeast cells. Expression of recombinant proteins causes ER stress and the use of oxygen as a terminal

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electron acceptor during oxidative protein folding means that the ER is also a significant source of ROS (Tu and Weissman, 2004).

S. cerevisiae responds to oxidative stress using a number of cellular responses that ensure the survival of the cell following exposure to oxidants (Morano *et al.*, 2012). These include defence systems that detoxify ROS, reduce their rate of production, and repair the damage caused by them. Many responses are ROS specific, but there are also general stress responses that are typically invoked in response to diverse stress conditions. *S. cerevisiae* can synthesize a vast array of antioxidant defence molecules which include nonenzymatic molecules D-erythroascorbic acid, flavohaemoglobin, glutathione, metallothioneins, polyamines, ubiquinol, trehalose and ergosterol, and enzymatic defences which include catalase, cytochrome c peroxidase, superoxide dismutase, glutaredoxin, glutathione peroxidase, glutathione reductase, thioredoxin, thioredoxin peroxidase and thioredoxin reductase (Gibson *et al.*, 2007).
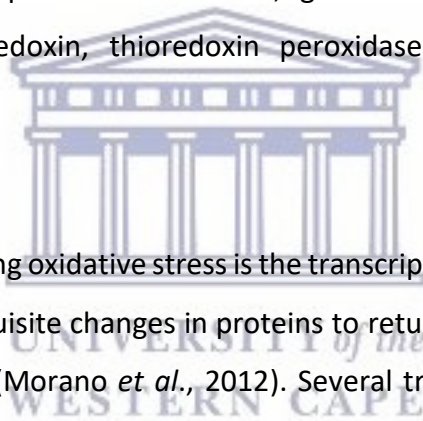
A key feature in cells undergoing oxidative stress is the transcriptional reprogramming of gene expression to provide the requisite changes in proteins to return the redox status of the cell back to an acceptable range (Morano *et al.*, 2012). Several transcriptional regulators have been identified that lead to the induction of antioxidant proteins. *YAP1* is a primary determinant in the antioxidant response (Harshman *et al.*, 1988). Several research groups have found that *YAP1* was critical for tolerance to oxidants such as H_2O_2 and diamide (Kuge and Jones, 1994). Another transcription factor *SKN7* was identified by screening in methylviologene, hyperbaric oxygen and hydrogen peroxide to identify mutations that cause sensitivity to peroxide (Krems *et al.*, 1996). The transcription factors encoded by *MSN2* and *MSN4* which are important participants in heat shock tolerance also play an important role in oxidative stress as well. Mutants lacking both *MSN2* and *MSN4* are highly sensitive to oxidative stress (Martínez-Pastor *et al.*, 1996).

1.5.4   Temperature

S. cerevisiae has an optimal growth temperature between 25°C and 30°C, however, at > 36-37°C, yeast cells activate the protective transcriptional program termed the heat shock response (HSR) which changes the membrane composition and carbohydrate flux (Morano *et al*., 2012). The HSR also plays a role in protein secretion stress by responding to disruptions of proteostasis (Akerfelt *et al*., 2010) and impacts cell physiology via oxidant defense, cell-wall remodeling, metabolism and transport (Hahn *et al*., 2004).

Microarray studies in *S. cerevisiae* demonstrated that the magnitude of HSR is proportional to the intensity of the stress (*e.g.* temperature shift) (Gasch *et al*., 2000), which implies that the organism is able to detect variations in temperature and control the transcriptional activation accordingly.In *S. cerevisiae*, heat shock transcription factor (Hsf1p) is encoded by a single, essential gene, *HSF1 (*Boy-Marcotte *et al*., 1998). The primary modulator involved in the HSR is Hsf1p, which identifies heat shock element (HSE) in the promoter regions of target genes.
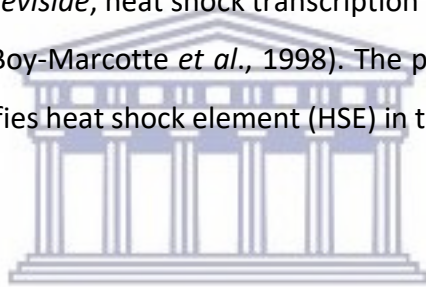
In addition to gene expression mediated by Hsf1p, a parallel pathway was discovered that responds not only to heat shock, but to a variety of cellular and environmental stress conditions (Brion *et al*., 2016). Two highly related and partially redundant zinc-finger transcription factors called Msn2p and Msn4p (*MSN*2/4) govern the majority of genes in heat and other environmental stress (**Figure 1.9**) (Boy-Marcotte *et al*., 1998). Both Msn2p and Msn4p bind to a nearly invariant five base pair sequence element (CCCCT) called the "stress response element".
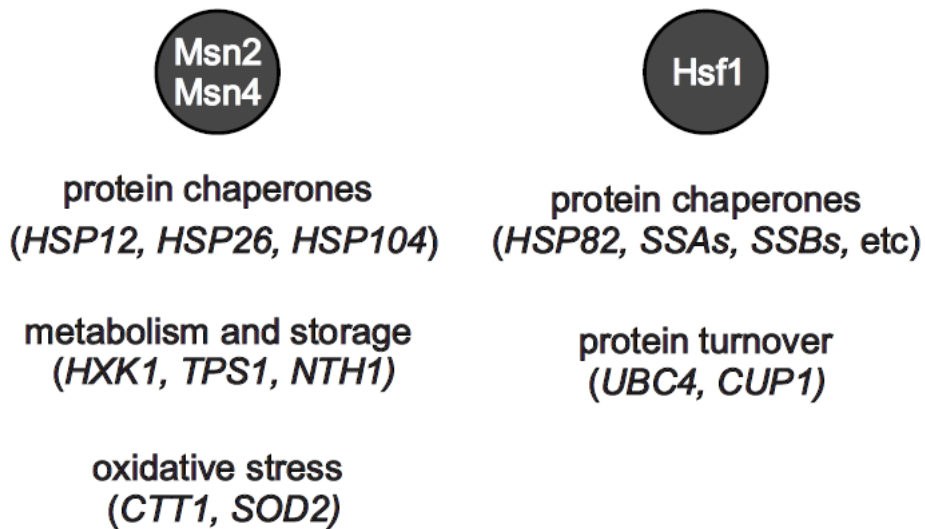
Figure 1.9: A schematic diagram representing the division of labour between Hsf1p and Msn2/4p. The general classes of stress genes that are induced by Hsf1p and Msn2/4p (Trott and Morano, 2003).

To distinguish the HSR from other stress response pathways, microarray experiments evaluated transcriptional changes in response to heat stress, osmotic stress and nutrient limitations (Morano *et al*., 2012). The findings indicated that ~10% of the genome is remodeled during one or more stresses and approximately 300 genes were up-regulated, mostly a result of transcription factors Msn2p and Msn4p, while approximately 600 genes were suppressed. This comprehensive cellular response has now been named the Environmental Stress Response (ESR) and the HSR is considered one subset thereof.

1.5.5   Inhibitors

Biomass contains microbial inhibitors that are released during pretreatment and enzymatic hydrolysis, affecting fermentation performance (Jönsson *et al*., 2013). There are at least four main classes of fermentation inhibitors encountered in biomass conversion: furfural and hydroxymethyl furfural (HMF), weak acids, and phenolic compounds (**Figure 1.10**). A major disadvantage to all pre-treatment methods is the production and release of various undesirable by-products such as acetic, formic and levulinic acids resulting from the hydrolysis of sugar molecules. These weak acids can affect cellular growth and ethanol yield through diffusion across the plasma membrane and altering cytosolic pH (**Table 1.4**) (Palmqvist and Hahn-Hägerdal, 2000).
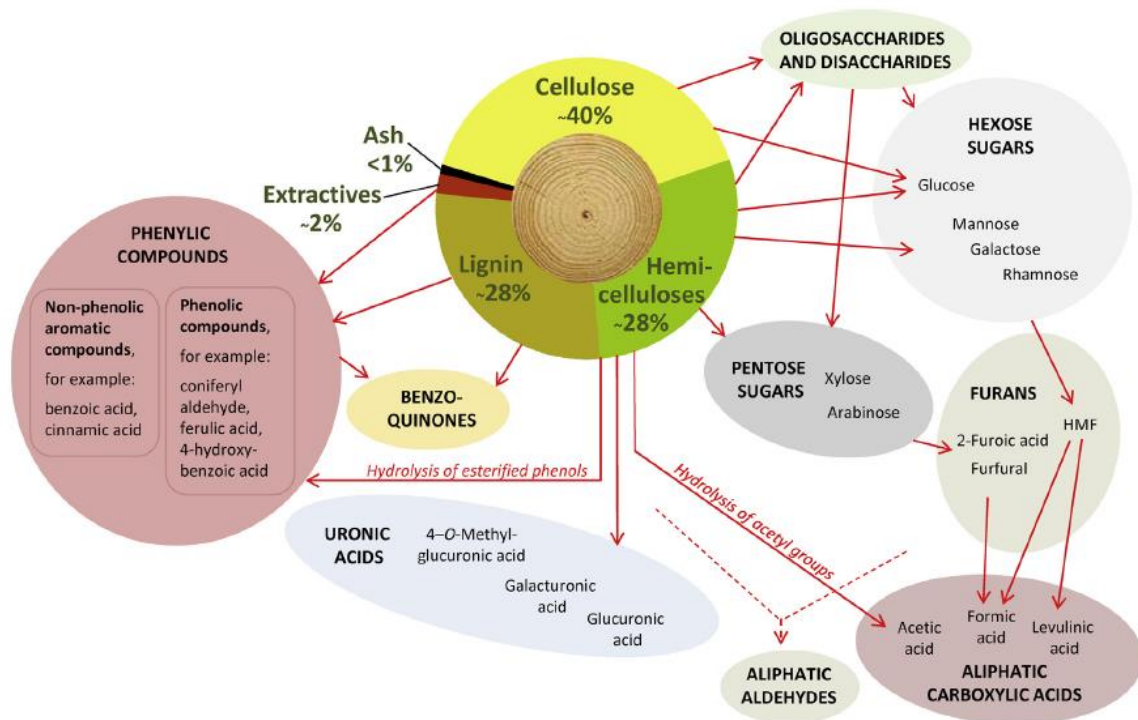
Figure 1.10: Schematic representation of inhibitors derived from lignocellulose resulting from pre-treatment under acidic conditions. Red arrows indicate tentative formation pathways (Jönsson and Martín, 2016).
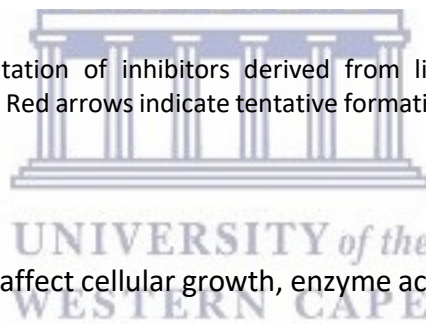
Furfurals have been shown to affect cellular growth, enzyme activity (Modig et al., 2002), and cellular redox balance (Ask *et al*., 2013), although, interestingly, glycolytic activity was maintained (Horváth et al., 2001, Sarvari Horvath et al., 2003). Transcriptome analysis of cells grown in the presence of inhibitors revealed reduced levels of transcripts coding for proteins required not only for carbohydrate metabolism but also for transcriptional and translational control, indicating the pleotrophic effect of inhibitors on cell metabolism (Li and Yuan, 2010).

Phenolic compounds, like vanillin, syringaldehyde and ferulate are a major constituent of lignin and are also linked to hemicellulose in some biomass substrates, for example grasses (Kumar and Sharma, 2017). These compounds are able to embed themselves into the cell membrane of organisms, causing a loss of integrity. Lower-molecular-weight phenolic acids behave in the same way as weak acids with respect to disruption of intracellular pH. To avoid these inhibitors, a pre-treatment process should be selected that either removes much of the lignin or leaves the lignin intact (Shi *et al*., 2015).

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Table 1.4: Effects of inhibitors found in lignocellulosic hydrolysates (Field *et al.*, 2015)

Inhibitor	Effect	Reference
Furfural	• Lag phase increase in lab strains.	(Kricka et al., 2015)
HMF	• Specific growth rate μ (h$_{-1}$) decreased in lab strain	(Yang and Wyman, 2008)
	• Specific ethanol production rate (g/g.h^{-1}) decreased in lab strain.	
Acetic acid	• Biomass formation decreased in lab strain.	(Field et al., 2015)
	• Specific xylose consumption rates decreased in xylose-consuming strains.	
Aromatic (Phenolics)	• Volumetric ethanol productivity (g/L.h) was reduced in commercial strain.	(Shi et al., 2015)
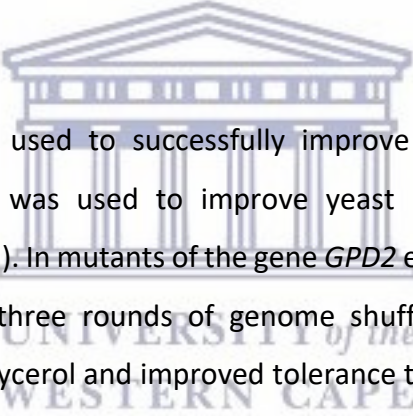
Several successful strategies have been used to improve tolerance to inhibitors. Adaptive laboratory evolution has been successfully used for selection of yeast strains tolerant to lignocellulose hydrolyzates containing furfural, HMF, and acetate (Keating *et al.*, 2006, Liu, 2011). Evolution of yeast populations in synthetic medium containing 3 mM furfural resulted in the selection of tolerant strains after 300 generations (Heer and Sauer, 2008). Research done by Greetham et al., (2016) demonstrated that by adding low concentrations of acetic acid increased *S. cerevisiae* tolerance to HMF. It has been also demonstrated that tolerance to furfural can be increased by the overexpression of *ADH7*, the ORF *YKL071W*, and *ARI1* genes, which encode reductases involved in furfural reduction (Heer *et al.*, 2009, Sehnem et al., 2013). Although there have been several successful strategies to improve tolerance to inhibitors, more research needs to be done to implement these or new strategies in the development of CBP yeast strains.

1.5.6   Osmotic stress

Osmotic stress is caused by changes in the concentration of dissolved molecules in the medium, such as high gravity fermentations where initial sugar concentration in the media is over 250g/L (Liu *et al.*, 2012a). High gravity fermentations are required for economic considerations in 2nd generation bioethanol production. Glucose concentrations in the range of 300g/L are needed to reach ethanol titers higher than 150g/L (Olsson and Hahn-Hägerdal, 1993). Osmotic shock disrupts the actin cytoskeleton and this disturbs MAP kinase cascades, which regulate the cell cycle (Chowdhury *et al.*, 1992). After being exposed to high osmolarity, yeast cells accumulate high amounts of glycerol which serves as an osmolyte to protect the

cell (Hohmann, 2002). Under osmotic pressure, the excretion of ethanol and glycerol is impaired, leading to the accumulation of intracellular ethanol and a decrease in cell viability (Panchal and Stewart, 1980, D'Amore *et al.*, 1988).

Glycerol is produced in yeast from the glycolytic intermediate dihydroxyacetonephosphate in two steps that are catalyzed by glycerol-3-phosphate dehydrogenase (Gpd) and glycerol-3-phosphatase (Gpp), respectively (D'Amore *et al.*, 1988). Both enzymes exist in two isoforms, Gpd1p and Gpd2p, as well as Gpp1p and Gpp2p. Deletion of *GPD1* and *GPD2* or *GPP1* and *GPP2* abolishes glycerol production and causes strong osmosensitivity (Karlgren *et al.*, 2005). The same transcription factors involved in HSR, namely Msn2p and Msn4p, are induced in osmotic stress, demonstrating ESR is interconnected with osmotic stress (Boy-Marcotte *et al.*, 1998).

Several strategies have been used to successfully improve osmotic tolerance in yeast. Genome-shuffling technology was used to improve yeast performance in high gravity fermentations (Liu *et al.*, 2012a). In mutants of the gene *GPD2* encoding glycerol 3-phosphate dehydrogenase subjected to three rounds of genome shuffling, a population of strains producing lower amounts of glycerol and improved tolerance to ethanol and high osmolality were selected (Tao *et al.*, 2012). These strains showed changes in fatty acid composition and higher accumulation of trehalose.

1.6   <u>Objectives of this study</u>

Osmotic stress and high gravity fermentation is of particular importance in this study. The stress modulation genes used in this study were identified by monitoring a hybrid yeast strain under high gravity fermentation (Liu *et al.,* 2012a). Using microarray analysis Liu et al., (2012a) saw that these genes were upregulated in cells that were growing well under VHG conditions. In this study we assess whether these genes would help alleviate some of the protein secretion stresses involved in cellulase production and other stresses encountered in the fermentation environment of second generation bioethanol production. Some of these objectives include:

- Transforming stress-tolerance related genes individually to the recipient yeast strains on integrative plasmids with the G418 resistance marker (pHO plasmids).
- Test the secreted protein and activity levels of yeast strains and monitor physiological changes including changed ethanol and osmotic tolerance, tunicamycin resistance and growth physiology.
- Combining various genes with positive effects to see if further enhancements can be achieved.
- Monitoring the UPR using qPCR.

The following chapter will cover the materials and methods used during this study. This will be followed by the results and discussion (Chapter three), and a final summative discussion in Chapter four.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Recombinant yeast strain construction

Standard protocols were used for DNA manipulations (Sambrook and Russell, 2001). All enzymes and kits were used as recommended by the manufacturer. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs. Digested DNA was eluted from 1% agarose gels with the Zymoclean Gel DNA Recovery Kit (Zymo Research). For polymerase chain reactions (PCR), Phusion DNA polymerase was purchased from ThermoScientific and reactions were performed using an Applied Biosystems 2720 Thermal cycler. Genes associated with improved growth in very high gravity fermentations were previously identified by a Chinese collaborator (Liu et al., 2012a). Several of these genes were cloned into integrating expression vectors under the control of the yeast *PGK1* promoter by these researchers. These plasmids, pHO-SET5, pHO-PPR1, pHO-YCR049C, pHO-YDJ1, pHO-ATX1, pHO-PRB1 and pHO-YHB1 were a kind gift received from Prof. Xinqing Zhao (Shanhai Jiao Tong University) and used in the initial part of this study (**Table 2.1**). The pHO-based plasmids were linearized with *NotI* and transformed to the *S. cerevisiae* Y294-*cel7A* strain with selection on Geneticin containing YPD plates.

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Table 2.1: Plasmids carrying stress-tolerance related genes and their known functions. These plasmids were kindly provided by Prof. Xinqing Zhao (Shanhai Jiao Tong University).

Plasmid name	Known function of gene expressed*
pHO-PPR1	Involved in De Novo pyrimidine biosynthesis
pHO-YCR049C	Unlikely to encode a functional protein, based on available experimental and comparative sequence data
pHO-YHB1	Flavohemoglobin involved in nitric oxide detoxification
pHO-YDJ1	Type I HSP40 co-chaperone; involved in regulation of HSP90 and HSP70
pHO-ATX1	Transports copper to the secretory vesicle copper transporter
pHO-PRB1	Involved in protein degradation in the vacuole
pHO-SET5	Methyltransferase involved in methylation of histone

***Data retrieved from*: www.yeastgenome.org**

Strains utilized and constructed is summarized in **Table 2.2**. Details of the primers used in this study is given in **Table 2.3**. For the construction of *YHB1* and *SET5* overexpressing strains, the open reading frames of the *YHB1* and *SET5* genes of *S. cerevisiae* Y294 were amplified using the primer sets YHB1-L/R and SET5-L/R, respectively. A 1200-bp PCR fragment for *YHB1* and a 1581-bp PCR fragment for *SET5* were digested with *Asc*I and *Pac*I, and ligated into the yeast expression vector pBKD1 – to yield pBKD1-YHB1 and pBKD1-SET5. These integration plasmids were linearized with *Bst*1107I after which transformation of Y294 [*cel7A*] was conducted according to a LiOAc/DMSO protocol (Hill et al., 1991). Transformants were plated out on geneticin-containing plates after an expression step of one hour in liquid YPD medium at 30°C. The total genomic DNA of the selected yeast transformants were isolated (Hoffman and Winston, 1987) and successful integration of either the *YHB1* or *SET5* overexpression cassette into the yeast genome was confirmed with PCR analyses using primers PGK1-L and YHB1-R or the SET-R primers for the relevant transformants. Yeast transformants thus possessed the native copy of *YHB1* and *SET5* plus one or more integrated copies of the gene under constitutive transcriptional regulation.

Table 2.2: Strains and plasmids utilized in this study

Yeast strain/plasmid	Abbreviated name	Relevant genotype	Source
Plasmids:			
pBKD1		*bla* δ-site *PGK1_P-PGK1_T kanMX* δ-site	(McBride et al., 2008)
pBDK1-*YHB1*		*bla* δ-site *PGK1_P-YHB1-PGK1_T kanMX* δ-site	This work
pBDK1-*SET5*		*bla* δ-site *PGK1_P-SET5-PGK1_T kanMX* δ-site	This work
Parental yeast strains:			
S. cerevisiae Y294:		α *leu2*-3,112 *ura3*-52 *his3 trp1*-289	ATCC 201160
(*fur1*::*LEU2* pMU1531)	Y294-Ref	*bla ura3/URA3 ENO1_P-XYNSEC-ENO1_T*	(Ilmen et al., 2011)
(*fur1*::*LEU2* pMI1529)	Y294-[cel7A]	*bla ura3/URA3-ENO1_p-CEL7A-ENO1_T*	(Ilmen et al., 2011)
Constructed yeast strains:			
S. cerevisiae Y294 (*fur1*::*LEU2* pMI1529)			
Y294_ *YHB1* overexpressed	Y294-[cel7A]-*YHB1*	*bla ura3/URA3 ENO1_P-CEL7A-ENO1_T kanMX PGK1_P-YHB1-PGK1_T*	This work
Y294_ *SET5* overexpressed	Y294-[cel7A]-*SET5*	*bla ura3/URA3 ENO1_P- CEL7A-ENO1_T kanMX PGK1_P-SET5-PGK1_T*	This work

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Table 2.3: Primers used in this study

Primer name	Oligonucleotide sequence (5'-3') (restriction site in bold)	Restriction sites
YHB1-L	GCAT**TTAATTAA**AAATGCTAGCCGAAAAAACCC	*Pac*I
YHB1-R	GCAT**GGCGCGCC**CTAAACTTGCACGGTTGACATC	*Asc*I
SET5-L	GCAT**TTAATTAA**AAATGACATTGACTATCAAAATAGGAAC	*Pac*I
SET5-R	GCAT**GGCGCGCC**TTATCTTTCATCCACTGCGACC	*Asc*I
PGK-L	CTAATTCGTAGTTTTTCAAGTTCTTAGATGC	
*kan*MX-L	CCGCGATTAAATTCCAACAT	
*kan*MX-R	CGATAGATTGTCGCACCTGA	
TFC1b-L	ACACTCCAGGCGGTATTGAC	
TFC1b-R	CTTCTGCAATGTTTGGCTCA	
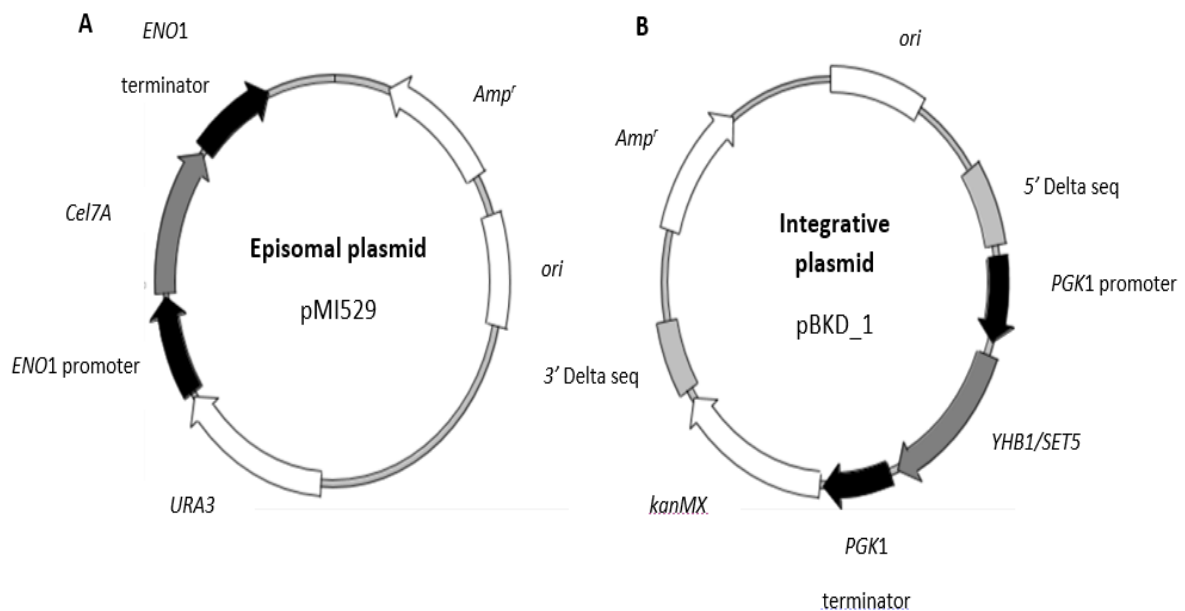
Figure 2.1: Schematic representation of plasmids used in this study. (A) Episomal plasmid that was originally transformed into the Y294 yeast strain carrying the gene encoding *T.e.cel7A*. (B) Delta integration vectors pBKD1-*YHB*1 or pBKD1-*SET*5 that were used to enhance *T.e.cel7A* activity. The stress related genes were cloned under the transcriptional control of the strong *PGK1* promoter/terminator system and the marker gene *kanMX* was used on integrative plasmid in all cases.

2.2   Media and culturing conditions

S. cerevisiae strains were routinely cultured in YPD broth (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose) medium at 30°C on an orbital shaker at 180 rpm. For the generation of yeast transformants, cells were selected on YPD plates containing 20 g/L agar and 200 µg/mL Genetecin G418-sulfate (Sigma). For stress related assays, strains were cultured in 10 mL YPD media that was supplemented singly with the following inhibitors: ethanol (7.5%, 8.0% and 8.5%), NaCl (1 M and 1.2 M), tunicamycin (0.5 µg/mL and 0.8 µg/mL), sodium orthovanadate (100 µg/mL and 200 µg/mL) and for heat stress grown at 35 and 37°C.

2.3   Enzyme activity assays

Transformants were initially screened after cultivation in 5 mL YPD grown at 30°C for 72 h on an orbital shaker at 180 rpm. Transformants with the highest normalised activity compared to the reference strains were subsequently assayed in triplicate. These transformant strains were cultured in 100 mL shake flasks containing 10 mL YPD for 72 h at 30°C shaking at 180 rpm. *p*-Nitrophenyl based assays were carried out using *p*-nitrophenyl-β-D-cellobioside (*p*NPC; Sigma) by cultivating the yeast strains and determining *T.e.cel7A* enzyme activity at 50°C for 3 h in liquid as previously described (Ilmen et al., 2011, Kroukamp et al., 2013). A *p*NP standard curve in the range of 1.5 mM to 3 mM was used. All volumetric values were normalised with dry cell weight (DCW) of the corresponding yeast cultures in g/L (Harkness and Arnason, 2014). Enzyme activities were expressed as units/g DCW, where one enzyme unit (U) was defined as the amount of enzyme required to produce 1 µmol *p*NP in one minute under assay conditions.

2.4   Growth curve analysis

Strains were inoculated in triplicate at a starting optical density (OD_{600nm}) of 0.1 into 20 mL YPD in 125 mL Erlenmeyer flasks. These flasks were incubated on a rotary shaker (180 rpm) at 30°C for the duration of the analysis. OD_{600nm} readings of samples were taken using a LKB ULTROSPEC II Spectrophotometer. 1 mL samples were taken every 2 hours for the first 18 hours, after which samples were taken at 3 h intervals with a final sample taken after 48 h, when growth had ceased or strains had reached stationary phase.

2.5   Screening for tolerance to bioethanol specific stressors

Yeast strains were cultivated in YPD medium at 30°C to an OD_{600nm} of 1. Ten-fold serial dilutions of cultures were spotted onto YPD agar plates containing the appropriate inhibitors to determine the tolerance capabilities of the strains. Cells were cultivated for 1-2 days at 30°C unless otherwise noted. The inhibitors used in this study include NaCl (1 M and 1.2 M), sorbitol (1.5 M and 2 M), hydrogen peroxide (1 mM and 2 mM) and tunicamycin (0.8 μg/mL and 1 μg/mL). For ethanol tolerance assays the cells were resuspended in 20% and 30% ethanol solutions, and incubated at room temperature for 10 min. Serial dilutions of the ethanol-stressed cells were spotted onto regular YPD plates to determine the relative survival rate of cells of the different strains. For heat shock the cells were resuspended in an equal volume of dH_2O at a temperature of 50°C. The cell suspensions were then incubated at this temperature for periods of 15 and 40 min, and subsequently plated out in ten-fold serial dilutions onto YPD agar plates.

2.6   Gene copy number determination

Real-time quantitative PCR (qPCR) was used to enumerate the *kanMX* antibiotic selection marker gene that had been used to facilitate the gene integrations allowing us to determine the copy numbers of each of the stress tolerance related gene expression cassettes. A single reference gene encoding transcription factor TFIIIC (*TFC1*) was selected to normalise the copy number of our genes of interest, as it is present as a single copy in the haploid complement *S. cerevisiae* genome (Teste et al., 2009). This method was performed as previously described

by Van Zyl et al., (2014). Strains were grown to saturation and DNA extraction was carried out using a method described by Hoffman (2001). Stock DNA concentrations ranged from 30 ng/µl to 0.01 ng/µl. The primers used are specific to the *TFC1* gene present on the yeast genome and the *kanMX* gene present on gene cassettes that were utilised. The qPCR analysis was carried out using the KAPA[TM] HRM Fast PCR Kit and the Applied Biosystems StepOne Real-Time PCR System and quantifications of gene copy number were determined using the relative standard curve method (Van Zyl et al., 2014). The efficiency of amplification for each primer set was determined from a plot of cycle threshold (C_t) values of serial dilutions of the template DNA. The efficiency of amplification of the qPCR analysis was based on the slope of the standard curve of the *kanMX* gene (119.26%) and of the *TFC1* gene (129%). The relative copy numbers of the gene cassettes and plasmids were determined relative to the *TFC1* and *kanMX* DNA concentrations.

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CHAPTER THREE

RESULTS AND DISCUSSION

3.1   Preliminary screening of pHO based plasmids

The development of *S. cerevisiae* for CBP requires the high level secretion of cellulases, particularly cellobiohydrolases (Ilmen et al., 2011). This high level of secretion is required for non-cellulolytic organisms such as *S. cerevisiae* to utilize crystalline cellulose substrates (La Grange et al., 2010). The difficulty of producing CBHs in sufficient quantities is considered as a major hurdle in the development of yeast as a CBP organism (Den Haan et al., 2007a, Lynd et al., 2005). The initial screening of the CBH yeast transformants that contained the stress tolerance related genes targeted for integration to the *HO* locus demonstrated a wide range of enzyme activity of which some were higher, lower or without change compared to the parental strain. The *HO* region was selected as a target for integration as it was previously shown to not have an effect on growth rate and nearly all laboratory strains have a mutation at the *HO* locus (Voth et al., 2001). Ten different colonies from each transformed strain was selected and inoculated into 5mL YPD and tested for *T.e.cel7A* activity. The colonies with the highest enzyme activity per gram dry cell weight (DCW) for each strain were selected for further study. The selected colonies were then grown in 10 ml YPD cultures for three days and were assayed in triplicate (**Fig. 3.1A)**. After 24 hours of growth in YPD media there was no significant increase in cellobiohydrolase activity compared to the parental. Only after 48 hours did the strains start showing increases in activity with the majority of the strains (pHO-*PPR1*, pHO-*YCR049C*, pHO-*YHB1*, pHO-*ATX1* and pHO-*SET5*) showing higher activity compared to the parental with activity increasing after 72 hours. The pHO-*YDJ1* strain had no significant change in activity when compared to the parental, while the pHO-*PRB1* strain had no significant changes after 72 hours.

Figure 3.1: Supernatant enzyme (pNPC) activities of recombinant *S. cerevisiae* Y294 strains harbouring the pMI529 episomal plasmid (*T. emersonii cel7A*). Parental strain (PAR) only contains the pMI529 episomal plasmid. Strains are indicated only by the native gene they over-expressed. (A) Initial assay of transformants. (B) Assay of transformants after several rounds of subcultivation, dotted line gives an indication of variation compared to (A). For both assays values are the mean activity values of three biological repeats and error bars indicate the standard deviation.

To asses reproducibility of those results these strains were then assayed again by first pre-culturing the strains in 5 mL YPD and then inoculating them in 10 mL cultures in triplicates (**Fig. 3.1B**). The enzyme activity changed dramatically compared to the first round of assays (**Fig. 3.1A and Fig. 3.1B**). A similar trend was observed in a BGL bearing strain (data not shown). The pHO-*PPR1* and pHO-*YCR049C* bearing strains maintained similar levels of activity while the parental strain showed increased levels of activity relative to assay and compared

to the other strains in assay B. The pHO-*YHB1* strain had higher levels of activity while the pHO-*YDJ1* strains activity levels was lower than previously. The most noticeable increase was with the pHO-*PRB1* and pHO-*SET5* strain (**Fig. 3.1B**).

After repeating the transformation, screening and assay experiments it was concluded that the transformants were unstable which could be due to incompatibility of the strain and vector. Preserving desirable characteristics obtained by molecular modification stable is an important consideration for industrial use of yeast strains (Zhang et al., 1996). Since genomic instability varies greatly between strains, it was important to determine the stability of the transformants. After sequencing the plasmids we found that several of the plasmids had a few base pairs missing and had mismatched nucleotide sequences when compared to the original sequence data. From these findings and based on previously results by Qingqing wan (Shanhai Jiao Tong University) it was decided to clone the native *YHB1* and *SET5* genes that demonstrated increased cellobiohydrolase activity, into pBDK integrative vectors which had previously been used in this type of study and in this yeast strain (Kroukamp et al., 2013).
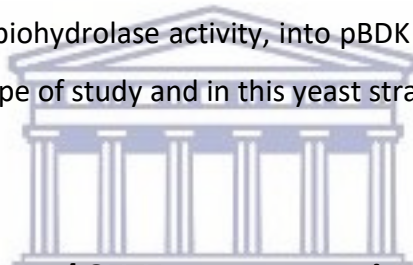
3.2   Screening of *YHB1 and SET5* overexpressing strains

We were able to successfully clone and verify the sequence of the *YHB1* and *SET5* genes into pBKD integrative vectors and subsequently transform these plasmids to Y294-[cel7A]. The *YHB1* or *SET5* overexpressing transformants were screened and those with the highest enzyme activity per gram dry cell weight were selected for further study. While screening there was a wide range of reporter enzyme activity observed in both sets of transformants with the same constitutively expressed gene, with some strains having lower values than the parental strain's enzyme activity (data not shown). This is an example of phenotypic variance between transformants which could be due to the gene copy number variation and position of integration into different delta sequences present in the host genome. The transformants with the highest activity were first screened using PCR to ensure that at least one of each gene was integrated into the respective strain (not shown). These transformants showed consistent enzyme activity after several rounds of screening compared to the pHO-based plasmids (**Fig. 3.1A and Fig. 3.1B**). The best *YHB1* and *SET5* overexpressing strains were grown in either 10 mL YPD or buffered SC media for 5 days and were assayed in triplicate. After 72 h

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of growth in YPD, the Y294-[*cel7A*]-*YHB*1 and Y294-[*cel7A*]-*SET5* had higher cellobiohydrolase activity compared to the parental strain per gram dry cell weight of 0.24 U/gDCW and 0.239 U/gDCW, respectively (**Fig. 3.2**). The enzyme activity of these strains continued to increase after 96 h while the parental strain activity remained relatively constant from 72 h to 120 h. The highest levels of improvement at 120 hours of cultivation compared to the parental was an improvement of 22% (Y294-[*cel7A*]-*SET5*) and 55% (Y294-[*cel7A*]-*YHB1*), respectively. When grown in SC media the Y294-[*cel7A*]-*SET5* and parental strain had similar levels of activity, while the Y294-[*cel7A*]-*YHB1* strain only had slight increases in activity (data not shown). SDS-PAGE analysis of supernatant of strains cultivated in SC-media showed that the heterologous CBH found in the supernatant of all strains had a similar size and glycosylation pattern (not shown). We therefore assumed that all observed differences in activity levels were due to differences in protein titer and not specific activity.
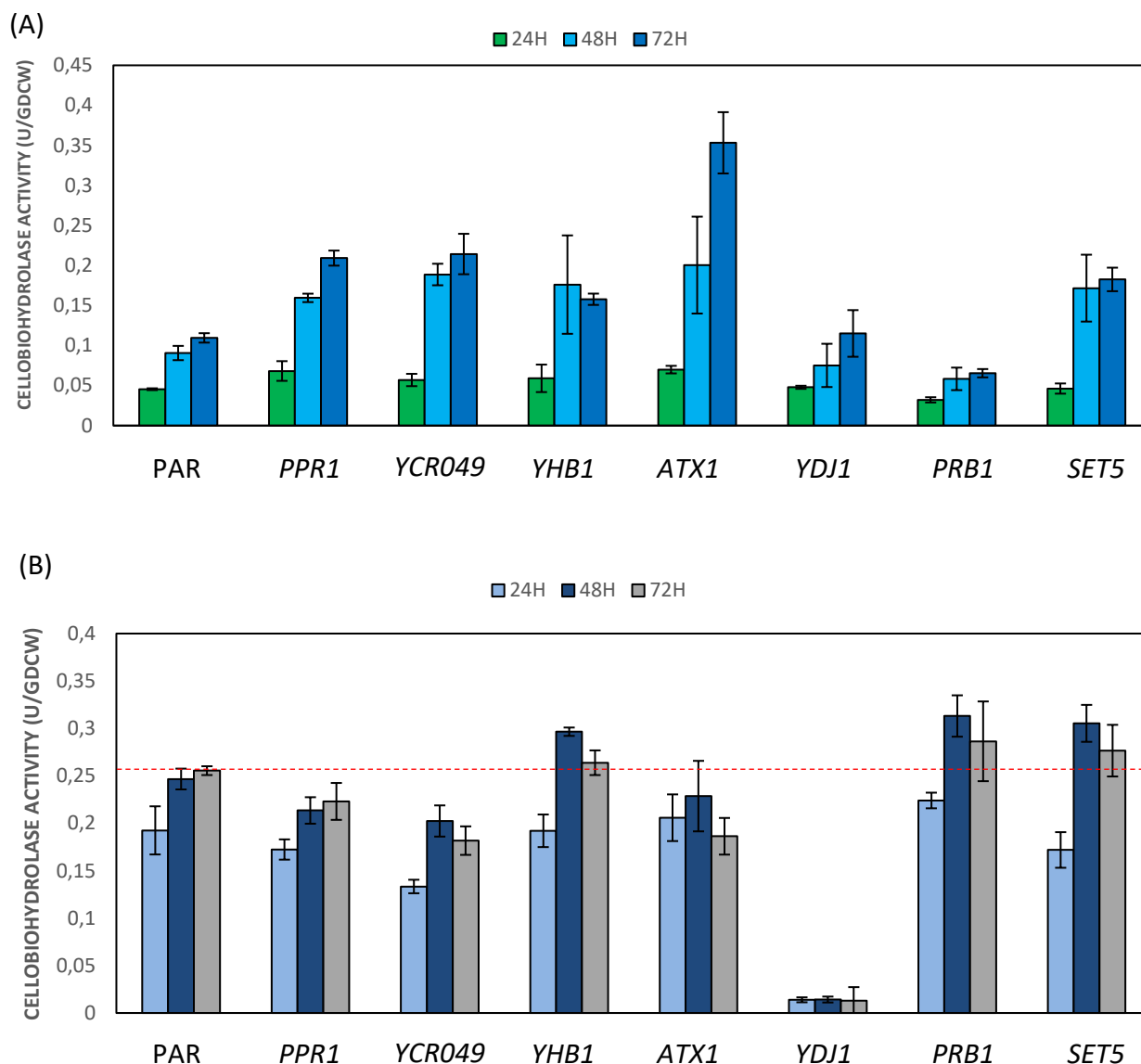

Figure 3.2: Supernatant cellobiohydrolase (pNP-C) activities of recombinant *S. cerevisiae* Y294 strains harbouring the pMI529 episomal plasmid (*T. emersonii cel7A*). The highest levels of improvement at 120 hours of cultivation is indicated as percentage improvement over the parental strain. Values are the mean activity values of three biological repeats and error bars indicate the standard deviation.

Experiments done by Qingqing wan (Shanhai Jiao Tong University-personal communication) using pHO-based plasmids demonstrated a similar result, where a *SET5*-overexpressing strain showed 30% higher CBH activity compared the parental strain.

Heterologous CBH production was previously shown to induce stress in yeast cells (Ilmen et al., 2011). Both *SET5* and *YHB1* have been linked to playing a role in oxidative stress (Khatun et al., 2017, Ter Linde and Steensma, 2002, Zeng et al., 2016). *SET5* has been linked to improved activities of antioxidant enzymes and generation of ATP (Zhang et al., 2015). Recent advances in stress responses demonstrated that reactive oxygen species (ROS) have been linked to ER stress and the UPR (Cao and Kaufman, 2014). These studies suggest that altered redox homeostasis in the ER is sufficient to cause ER stress, which could, in turn, induce the production of ROS in the ER and mitochondria. The increased secreted enzyme phenotype of these recombinant strains could thus be due to more efficient oxidative damage reduction. A similar study where *SOD1* was overexpressed resulted in an increase in endoglucanase activity (Kroukamp et al., 2013). The *SET5* gene encodes for a methyltransferase that is involved in the methylation of histone H4 Lys5, -8, -12 (Green et al., 2012). This could further suggest that *SET5* may play a role in the epigenetic control of genes that regulate stress responses involved in heterologous protein production. To our knowledge, this is the first reported case where *YHB1* and *SET5* overexpression in *S. cerevisiae* led to higher heterologous protein secretion.

3.3   The effects of inhibitors on the *T.e.cel7A* activity of selected strains

In addition to stress induced by heterologous protein secretion, yeasts may face numerous environmental stress factors (Gasch, 2003). When there is a sudden change in environment, cells must rapidly adjust their internal machinery to that required for growth in the new environment. For bioethanol production, the fermentation environment has very specific environmental challenges compared to the wild which include varying ethanol concentrations, high temperatures, osmotic stress and the presence of lignocellulosic-derived inhibitors (Mukherjee et al., 2014). Yeast cells are often exposed to these stresses in a sequential and multiple manner (Nicolaou et al., 2010). In order to identify if the genes we over-expressed endowed strains with increased tolerance towards secretion and environmental stresses we cultured the strains under various conditions and determined the supernatant enzyme activity (**Fig. 3.3).**

In the first experiment strains were grown in the presence of NaCl to mimic osmotic stress (**Fig. 3.3A)**. It is clear that the supernatant enzyme activity significantly decreased in the

presence of NaCl compared to the original enzyme activity data (**Fig. 3.2**). The Y294-[*cel7A*]-*YHB1* and Y294-[*cel7A*]-*SET5* strains had the higher cellobiohydrolase activity compared to the parental strain after 72 hr. This this could suggest that these genes also play a role in increasing tolerance to osmotic stress. These stress-tolerance related genes were identified in strains grown in very high gravity conditions (Liu et al., 2012a). It would therefore stand to reason that they would improve the osmotic tolerance in our recombinant strains

In the second experiment, strains were grown at 35°C and assayed after 48 and 72 h (**Fig. 3.3B**). The enzyme activity of all strains significantly increased when the temperature was increased by 5°C compared to when these strains were grown at 30°C. When the temperature was increased to 40°C no growth was observed (data not shown). The Y294-[*cel7A*]-*YHB1* strain had a slight decrease in activity compared to the parent, while the Y294-[*cel7A*]-*SET5* had a slight increase in activity. A similar result was seen in *S. cerevisiae* SEY2102 when the rate and secretion of recombinant invertase was tested in the temperature range of 25 - 45°C and showed maxima at 35°C (Marten et al., 1995). An increase in temperature is also linked to an increase in membrane fluidity (Laroche et al., 2001), which could have led to an increase secretion of the reporter protein. Furthermore, this result may indicate the role of heat shock proteins in improved CBH secretion in yeast. This aspect will be the subject of a subsequent study.

In the third experiment strains were cultured in the presence of the *N*-glycosylation inhibitor tunicamycin (**Fig. 3.3C**). Tunicamycin triggers endoplasmic reticulum stress response and inhibits efficient protein secretion in eukaryotes (Iwata et al., 2016).In this experiment it is evident that the presence of tunicamycin led to a decrease in activity in all strains compared to the original activity data (**Fig. 3.2**). The Y294-[*cel7A*]-*SET5* strain showed significantly higher activity compared to the parental and Y294-[*cel7A*]-*YHB1* strains after 72 h. This gives us an indication that *SET5* may play a role in alleviating ER stress.

Growth in the presence sodium orthovanadate is also linked to ER stress (**Fig. 3.3D**). The presence of sodium orthovanadate in the cultivation media didn't have a significant effect on the strains overexpressing the stress tolerance related genes. The parental strains activity was fairly similar when compared to the original enzyme activity data (**Fig. 3.2**), after 72 h.
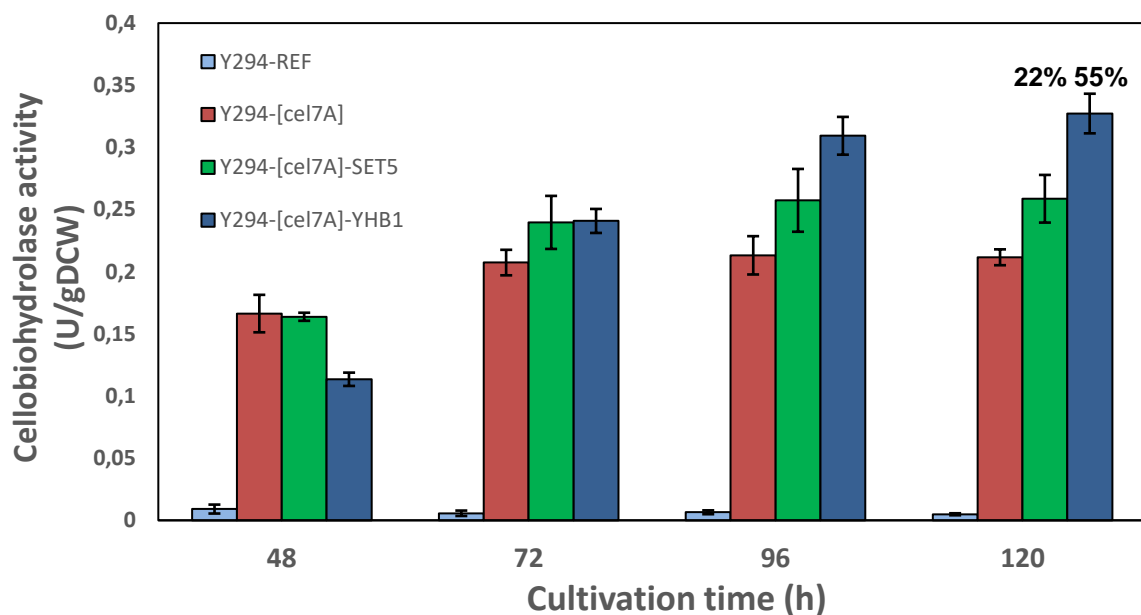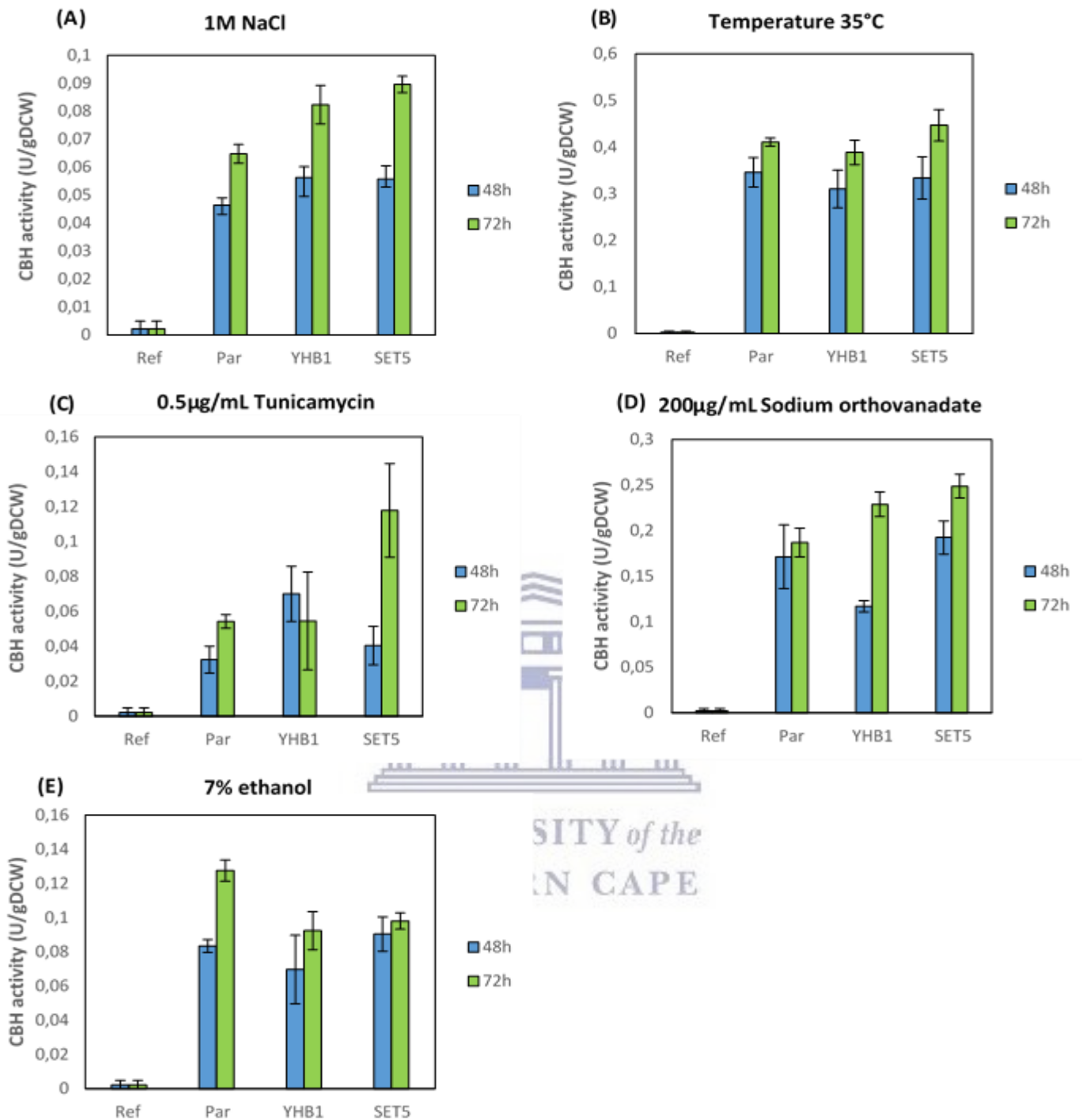
Figure 3.3: Supernatant cellobiohydrolase (pNPC) activities of recombinant *S. cerevisiae* Y294 strains harbouring *T. emersonii cel7A* and the stress tolerance related *SET5* or *YHB1* genes. Reference strain (Ref) contains an empty vector. Panels indicate different stress conditions included in the cultivation. (A) 1M NaCl, (B) cultivatiion at 35°C, (C) 0.5 μg/mL tunicamycin, (D) 200 μg/mL sodium orthovanadate and (E) 7% ethanol. All values represent mean values of assays done in triplicate with error bars indicating standard deviation.

A similar trend in activity was observed in the Y294-[*cel7A*]-*YHB1* strain when compared to the original enzyme activity data (Fig. 3.2), where there was a significant increase in activity from 48 h to 72 h.

When grown in the presence of ethanol (**Fig. 3.3E**), the parental strain had higher enzyme activity than the two strains overexpressing the stress tolerance related genes. Ethanol is an inhibitor of yeast growth at relatively low concentrations, inhibiting cell division, decreasing cell volume and specific growth rate, while high ethanol concentrations reduce cell vitality and increase cell death (Stanley et al., 2010). Ethanol also influences cell metabolism and macromolecular biosynthesis by inducing the production of heat shock-like proteins, lowering the rate of RNA and protein accumulation, altering metabolism, denaturing intracellular proteins and glycolytic enzymes and reducing their activity (Hu et al., 2007). The Y294-[*cel7A*]-*YHB1* and Y294-[*cel7A*]-*SET5* strains had similar OD values when compared to the parental (data not shown), indicating that the ethanol did not necessarily affect growth when compared to the parental, but other mechanisms involved in the secretion pathway may have been affected.
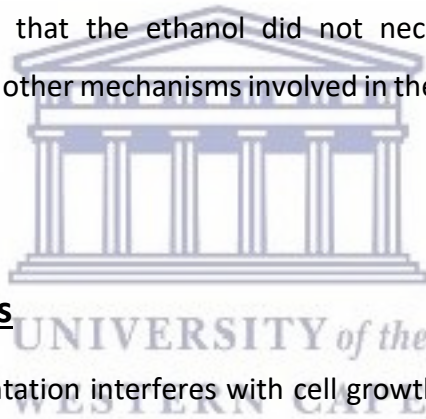
3.4   <u>Stress plate assays</u>

Stress associated with fermentation interferes with cell growth, internal secretory pathway mechanisms and the level of protein secreted in the medium (Kaufman, 1999, Bauer and Pretorius, 2000). It is therefore also important to focus on the effect of different environmental factors on recombinant strains and not just on engineering the host strain or protein of interest when developing CBP yeast strains.

Yeast strains were cultivated in YPD medium at 30°C to an OD_{600nm} of 1. Ten-fold serial dilutions of cultures were spotted onto YPD agar plates containing the appropriate inhibitors to determine the tolerance capabilities of the strains. A control plate containing YPD only was used to demonstrate the normal growth of these strains (**Fig. 3.4A**). No changes in colony growth or pigment were observed in all strains.
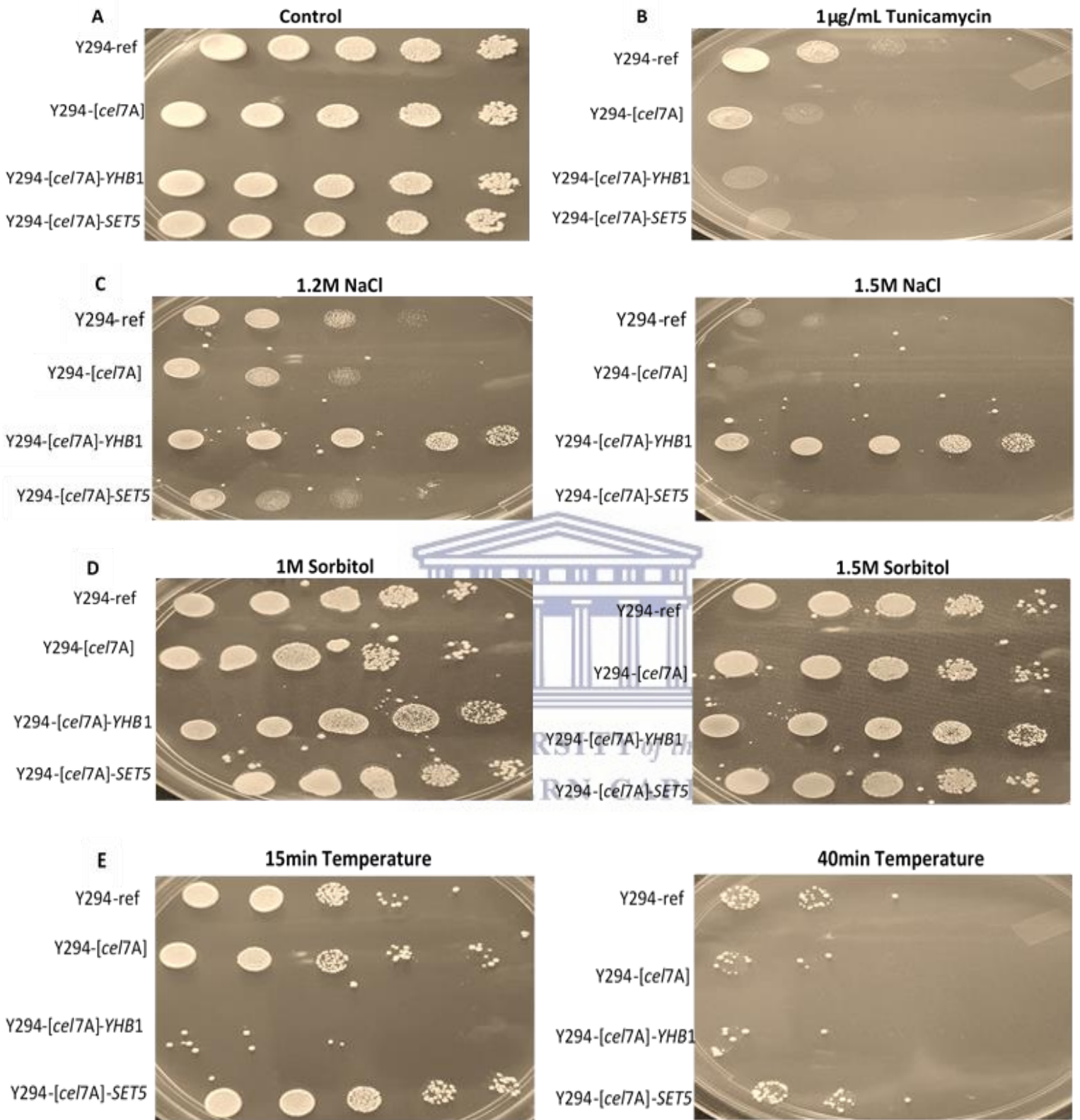
Figure 3.4: Stress plate assays of selected strains after 72 h cultivations **(A)** YPD (control), **(B)** ER stress, **(C)** hypersaline stress, **(D)** osmotic stress, **(E)** heat shock, **(F)** ethanol tolerance and **(G)** oxidative stress.

F

20% ethanol

Y294-ref
Y294-[cel7A]
Y294-[cel7A]-YHB1
Y294-[cel7A]-SET5

30% ethanol

Y294-ref
Y294-[cel7A]
Y294-[cel7A]-YHB1
Y294-[cel7A]-SET5

G

2mM H₂O₂

Y294-ref
Y294-[cel7A]
Y294-[cel7A]-YHB1
Y294-[cel7A]-SET5

3mM H₂O₂

Y294-ref
Y294-[cel7A]
Y294-[cel7A]-YHB1
Y294-[cel7A]-SET5
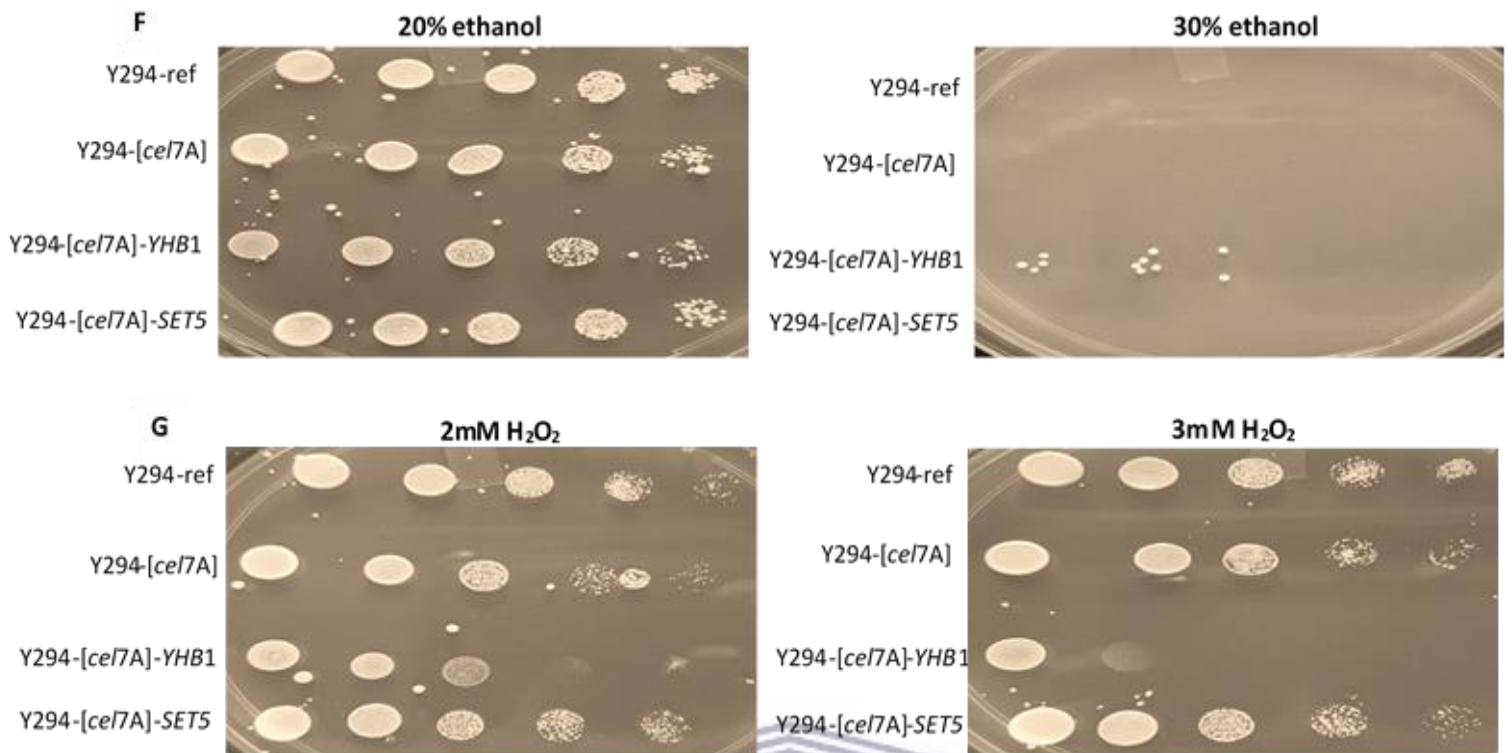
Figure 3.4 (continued)

Tunicamycin inhibits *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). When the strains were grown in the presence of tunicamycin (**Fig. 3.4B**), the two strains overexpressing the stress tolerance related genes had increased sensitivity to tunicamycin. This could be an indication that these genes could play a role in altering the cell wall as it strongly influences the release of heterologous proteins (Bartkeviciute and Sasnauskas, 2004). The control strain had a higher tolerance to tunicamycin as expected as it was not expressing any heterologous proteins and thus suffered less inherent UPR stress.

The recombinant yeasts' tolerance to increasing levels of osmotic and hypersalinity stress was determined, in order to establish whether the overexpression of stress tolerance related genes could lead to improved effects on the yeasts' growth capability (**Fig. 3.4C and Fig. 3.4D**). The Y294-[*cel7A*]-*YHB1* strain showed the highest resistance to increasing concentrations of NaCl and sorbitol. This makes sense since these genes were originally identified in strains grown under high gravity conditions (Liu et al., 2012a).

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Upon exposure to high temperature for 15 min (**Fig. 3.4E**), various tolerances were observed, with the Y294-[*cel7A*]-*SET5* strain proving to be the most tolerant. When the treatment time was increased to 40 min, the tolerance of all strains decreased with the reference and Y294-[*cel7A*]-*SET5* strain exhibiting similar levels of tolerance. Hou *et al.*, (2013) demonstrated that the heat shock response (HSR) improves heterologous protein production by relieving ER stress, suggesting a link between tolerance of stress caused by recombinant cellulolytic enzyme production and tolerance to environmental stresses. This can further be linked to the Y294-[*cel7A*]-*SET5* strain exhibiting higher enzyme activity when cultured at a higher temperature (**Fig. 3.3B**). During fermentation cells release a significant amount of energy in the form of heat, and this change in temperature is perceived as stress by the cell (Bauer and Pretorius, 2000). Increases in temperature as little as 2-3°C have been shown to negatively influence fermentation efficiency, therefore it is important to have a strain that can tolerate changes in temperature without compromising fermentation parameters such as ethanol productivity.

The effects of ethanol concentration and high temperature stresses are similar and mutually amplify cellular sensitivity (Piper et al., 1997). When these strains were incubated in 20% ethanol, all of them exhibited similar levels of tolerance (**Fig. 3.4F**). When the ethanol concentration was increased to 30% only the Y294-[*cel7A*]-*YHB1* strain showed observable survival. Ethanol stress, like temperature, also plays a role in changing properties of cellular membranes, particularly in increasing membrane permeability and changes in membrane fluidity (Bauer and Pretorius, 2000).

Oxidative stress occurs when there are toxic levels of oxygen-derived ROS (Jamieson, 1998)**.** Expression of recombinant proteins causes ER stress and the use of oxygen as a terminal electron acceptor during oxidative protein folding means that the ER is also a significant source of ROS (Tu and Weissman, 2004). All strains aside from Y294-[*cel7A*]-*YHB1,* demonstrated similar levels of tolerance to increasing concentrations of H_2O_2 (**Fig. 3.4G**). Tolerance of severe heat shock has been tightly linked to aerobic metabolism and oxidative stress (Morano et al., 2012). This statement holds true for the Y294-[*cel7A*]-*SET5* strain as it seems to be tolerant to oxidative stress (**Fig. 3.4G**) and had higher tolerance to temperature stress when compared to the parental (**Fig. 3.4E**). The Y294-[*cel7A*]-*YHB1* strain's sensitivity

to could be linked to increased internal ER stress from heterologous protein production as it is also a significant source of ROS (Tu and Weissman, 2004).

3.5   Growth rates of the transformants

S. cerevisiae is already well established for the production of a wide range of heterologous proteins which often impose a metabolic burden on the cells leading to a decrease in specific growth rate (van Rensburg et al., 2012). Differences in cellulolytic enzyme production and secretion may arise from differences in cell growth. Since both the Y294-[*cel7A*]-*YHB1* and Y294-[*cel7A*]-*SET5* strains demonstrated higher CBH activity (**Fig. 3.2**), the effect of overexpressing these native genes on growth kinetics was determined in comparison to the parental Y294-[*cel7A*] and a reference Y294 strain containing an empty vector (**Fig. 3.5**).
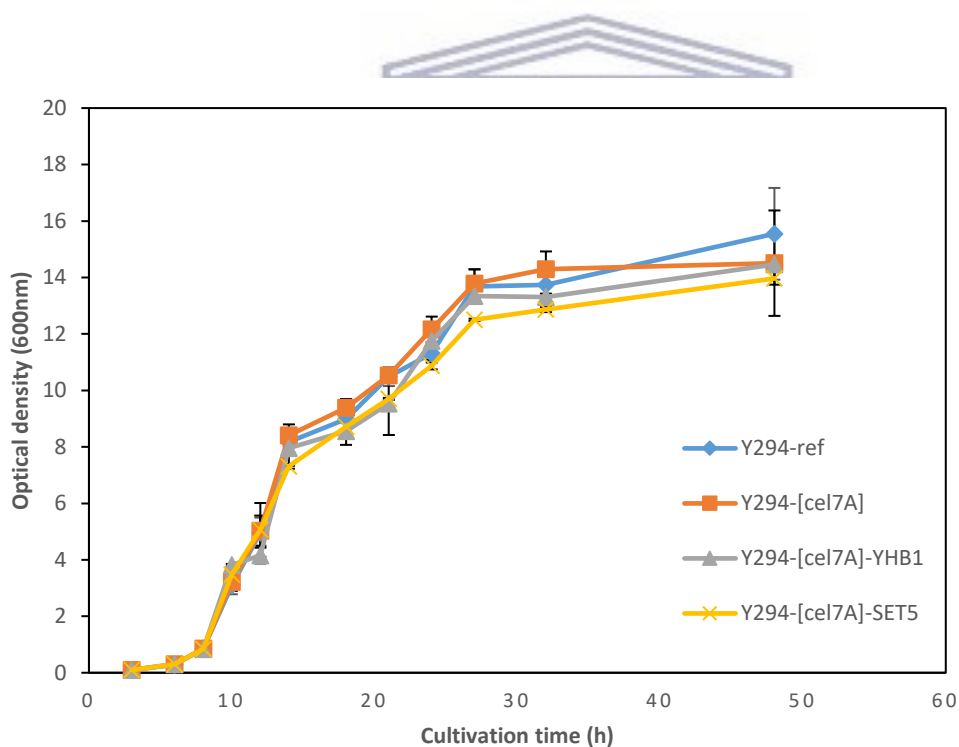
Figure 3.5: Growth curves of the parental yeast strains and transformants expressing stress tolerance related *YHB1* and *SET5* genes during the cultivation period in YPD. Absorbance was measured at 600 nm. Mean values from triplicate experiments are shown and error bars indicate the standard deviation from the mean.

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The growth performance of transformants and control strains was measured over a cultivation period of 48 h in YPD. Overexpressing the stress tolerance genes had no significant deleterious effects on the growth capability of the strains.

3.6 Integrated gene copy numbers

The relative copy numbers of the overexpressed stress tolerance genes (in addition to the native copy) were determined relative to the *TFC1* reference gene and results are depicted in **Table 3.1.**

Table 3.1: The quantification of native *YHB1* and *SET5* genes integrated into the genome of reference strain Y294. Standard deviation of triplicates is indicated with ± and rounded numbers are indicated in brackets.

Strain	Relative copy number
Y294[cel7A]-*YHB1*	1,01 ± 0,08 (1)
Y294[cel7A]-*SET5*	1,00± 0,33 (1)
YIII3-HO	1,23 ± 1,02 (1)

YIII3-HO was used as a reference strain. Quantitative real-time PCR analysis of the overexpressing strains revealed that all strains had only a single additional integrated copy. All samples were run in triplicate using three technical repeats. The varying reaction efficiencies meant that slight deviations from absolute values were expected. During the screening process, only the transformants with the highest activity were selected, which could have excluded strains that integrated a higher number of genes that could have ultimately led to derogatory effects on the secretion of the reporter protein. The influence of copy number of gene targets and expression levels have been investigated previously (Kroukamp et al., 2013, Van Zyl et al., 2014, Van Zyl et al., 2016). Results have suggested that the number of a specific gene being overexpressed did not proportionately lead to an increase in extracellular protein concentration. There are several other gene candidates that have been shown to influence protein secretion (Robinson et al., 1994, Ruohonen et al., 1997, Valkonen et al., 2003). According to our data, single integration of a particular stress tolerance related

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gene can lead to improvements in secretion. The influence of the number of cellulase genes expressed episomally or integrated into the genome have also been investigated (Teng et al., 2015, Davison et al., 2016). While some studies indicate that increasing gene copy numbers can increase some enzymatic activity, this is not true for all enzymes (Yamada et al., 2010a). Increased enzyme activity was reported to correlate with the DNA content of yeast cells and gene copy number with diploid genomic states had higher levels of protein production compared to haploid states, with even greater levels produced in tetraploid species.

CHAPTER FOUR

SUMMARY AND CONCLUDING REMARKS

Lignocellulosic biomass is an attractive source of fermentable sugars for conversion to bioethanol since it is inexpensive, abundant and can lessen the burden of adequate food production (Dashtban et al., 2009). A lot of research is currently focusing on utilizing cellulosic biomass for the production of bioethanol, and the creation and development of microorganisms capable of degrading cellulose into monomeric sugars which can be fermented into alcohols at high rates and yields (Kricka et al., 2014). *S. cerevisiae* in particular has many characteristics that make it appealing for industrial applications including high sugar consumption rate, tolerance of high osmolality and various other factors (Temnykh et al., 2000, Den Haan et al., 2007c, Gibson et al., 2007, Hasunuma et al., 2011, van Zyl et al., 2011a). Yeasts can generally secrete significant titers of recombinant proteins, and many studies have focused on engineering and enhancing secretion of cellulases in yeast for optimal second generation ethanol production (Goyal et al., 2011, Den Haan et al., 2007a, Yamada et al., 2011).

The expression of cellulases, particularly CBH, have been shown to induce stress by activating the UPR in *S. cerevisiae* (Ilmen et al., 2011). *S. cerevisiae* is one of the best established heterologous host systems in terms of genetic and physiological background and research has shown that stress situations of the host cells can largely influence the productivity of an expression system (Ghaemmaghami et al., 2003). Some of the stress encountered in a recombinant yeast strain arises from increasing heterologous gene copy numbers, codon usage of the expressed gene, transcription using strong promoters, translation signals, processing and folding in the ER and Golgi, and finally secretion out of the cell (Mattanovich et al., 2004). These stresses caused by the exploitation of the cellular system to produce a recombinant protein often hampers the final protein product due to the metabolic burden (van Rensburg et al., 2012). Aside from internal stresses caused by expression of these heterologous proteins, other external environmental factors also play a role in the secretion and robustness of the strain. For bioethanol production, the fermentation environment has very specific environmental challenges compared to the wild, which include varying glucose and ethanol concentrations, high temperatures and the presence of lignocellulosic-derived

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inhibitors (Mukherjee et al., 2014). Yeast cells are often exposed to these stresses in a sequential manner (Nicolaou et al., 2010).

The aim of this study was to ultimately determine whether recently identified stress-tolerance related genes would play a role in alleviating stresses caused both by recombinant protein production and environmental stresses that would typically be found in the fermentation environment. It was clear that two genes, *SET5* and *YHB1,* clearly played a role in increasing the heterologous enzyme activity and helped the strains cope better with certain fermentation stress factors. This increase in activity could be linked to an increase in secreted protein as these strains also demonstrated higher activity in the presence of the ER stressor tunicamycin. A future invertase assay would also give us an indication whether more native yeast protein is being secreted. It is important to note that differential cellulolytic activity has been observed when different background hosts were used and distinctly protein-specific effects were observed by Idiris et al., (2010); Kroukamp et al., (2013) and Van Zyl et al., (2016). Differential enzyme activity using different reporter proteins most likely results from differences in post-translational processing, size of the protein, glycosylation sites, gene copy number and protein stability. Here we demonstrate that the recombinant production of cellobiohydrolase could be increased with aid of strain engineering.

It has been previously described that a microorganism that produces a compound of interest efficiently are rarely also highly tolerant to acid, heat or similar environmental stresses (Remize et al., 1999). It was clear that the Y294-[*cel7A*]-*YHB*1 strain demonstrated multi-tolerant characteristics desirable in bioethanol production, i.e. high tolerance to osmotic stress, increased tolerance to secretion stress (tunicamycin) and high temperatures. Osmotic stress is of particular interest especially in high gravity fermentations, where initial sugar concentration in the media is over 250g/L, which reduce the cost and potential of contamination in 2nd generation bioethanol production (Liu et al., 2012b). The Y294-[*cel7A*]-*SET5* strain demonstrated the highest increase (55%) in enzyme activity and maintained higher activity levels under numerous tested stresses (NaCl, temperature, tunicamycin and sodium orthovanadate). It was also interesting to observe an increase in activity at 35°C across all strains, indicating that this temperature might be optimal for cellulase secretion and that over-production of heat shock proteins (or heat stress related proteins) may be another interesting target for engineering enhanced CBH secretion in yeast. Since tolerance to

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environmental stresses is a polygenic trait (Cubillos et al., 2011), controlled by the expression of multiple native genes, it is usually very difficult to insert tolerance to a specific stressor into a desirable host strain. A strain with innate tolerance to fermentation stress would have been a good comparison or reference. The effect of *YHB1* and *SET5* overproduction on stress tolerance should be investigated in a range of host strains in future.

The growth rate of these strains was not significantly affected. Since we used optical density as a proxy for cell density, differences in cell size and cells sticking together could not be accounted for and could also have interfered with differences in cell density (Smith et al., 2014). A further step in this study would be to analyse the effect of cell size on heterologous enzyme production and secretion.

Genes associated with genome plasticity, i.e. genes encoding proteins involved in amino acid biosynthesis and transport, sulphur and nitrogen assimilation, and protein degradation, play an important role in yeast for adaptation to new environments (Carreto et al., 2011). The two stress tolerance related genes used in this study show potential not only in increasing the secretion capacity of *S. cerevisiae* but also increasing its tolerance to certain environmental stresses. These results only give us limited information regarding the physiological properties of the strains and an-omics based approach would help us understand the underlying mechanisms of these genes. In conclusion, we have shown for the first time that overexpression of the *S .cerevisiae YHB1* and *SET5* genes could improve heterologous CBH production and stress tolerance in this host.

4.1   <u>**Future prospects**</u>

Due to time constraints, not all objectives of this study were met. Future work would include:

- monitoring the changes in gene expression that occur in the UPR through qPCR
- combining the two genes to see if further enhancements can be found
- overexpressing these genes in other cellulase bearing strains
- combing these genes with the *PSE1* gene as co-expression with *SOD1* showed enhanced in BGL activity (Kroukamp *et al*., 2013)
- Using various different background strains.

Transcriptome and proteomic analysis of the improved strains can be performed to further study the molecular mechanism underlying improved cellulase production and stress tolerance. Future research should also aim to understand how strains behave when confronted with multiple stresses simultaneously.

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