The *in vitro* detection and measurement of the unfolded protein response in *Saccharomyces cerevisiae*

Gillian Cedras

Thesis presented in fulfilment of the requirement for the degree of Masters of Science at the Faculty of Natural Science, Department of Biotechnology, University of the Western Cape, South Africa.

Supervisor: Dr. Riaan den Haan

Co-supervisor: Prof. Willem H. van Zyl

December 2018

Name: Gillian Cedras

Student number: 3357644

I declare that ''**The *in vitro* detection and measurement of the unfolded protein response in *Saccharomyces cerevisiae***'' 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 have been indicated and acknowledged by complete references.

Full name: Gillian Cedras

Signature:

Date: December 2018

TABLE OF CONTENTS

A. ACKNOWLEDGEMENTS

I would like to thank the following people that supported me throughout completion of this thesis:

- My supervisor, Dr. Riaan Den Haan, for his patience, guidance and support during my project and write up of this thesis. I will be forever grateful for having the opportunity to learn from you.

- Prof. Willem H. van Zyl for being co-supervisor on this project and supporting me financially.

- My fellow lab colleagues that were in the yeast lab during the past two years for their support and making this an enjoyable experience: Jarryd Lamour, Kim Freeman, Daylon Morgan, Philliza van Breda, Rafeeqah Thompson, Odwa Jacob, Michaela Bothma and Bronwyn Chetty.

- My mother, Rachel Cedras, and sisters, Carlise Cedras and Carla Daniels for their unconditional support and encouragement.

- My friends in the Biotechnology department for their encouragement, stimulating discussions and all the fun times we had: Junaid Mia, Lee-Ann Niekerk, Dr. Arun Gokul, Fahiem Carelse, Taskeen Simons and Po-ann Chen.

- The Biotechnology department for any advice given on this work.

- Dr. Leonardo van Zyl for his immense knowledge and the insightful comments he shared with me.

- The NRF for supporting me financially.

B. LIST OF ABBREVIATIONS

1G first generation

2G second generation

ATF6 activating transcription factor 6

BGL β-glucosidase

BiP immunoglobulin protein

CBH cellobiohydrolase (exoglucanase)

CBM carbohydrate-binding module

CBP consolidated bioprocessing

CTAB cetyl trimethyl ammonium bromide

DCW dry cell weight

DTT dithiothreitol

E95 95% ethanol

EG endoglucanase

eGFP codon optimized green fluorescent protein

ER endoplasmic reticulum

ERAD endoplasmic reticulum-associated protein degradation

ERAI endoplasmic reticulum stress-activated indicator

GFP green fluorescent protein

GHG greenhouse gas

Grp glucose-regulated protein

HA hemagglutinin protein

HAC1 histone acetyltransferase

ICEV internal combustion engine vehicles

Ire1p inositol-requiring enzyme

LB luria Bertani

MFI mean fluorescence intensities

mRNA messenger ribonucleic acid

OD optical density

PCR polymerase chain reaction

PERK protein ER kinase

QC quality control

qPCR quantitative polymerase chain reaction

r.t.qPCR reverse transcriptase quantitative polymerase chain reaction

SHF separate hydrolysis and fermentation

SSCF simultaneous saccharification and co-fermentation

SSF simultaneous saccharification and fermentation

TB terrific broth

Tm tunicamycin

U enzyme unit

UPR unfolded protein response

UPRE unfolded protein responsive element

YPD yeast extract, peptone, glucose

C. LIST OF FIGURES

D. LIST OF TABLES

E. ABSTRACT

Bioethanol is currently the most widely used biofuel and can be used as a direct replacement for current fossil fuel based transportation fuels. Consolidated bioprocessing (CBP), in which bioethanol is produced in a single reactor by a single microorganism, is a cost-effective way of producing bioethanol in a second generation process using lignocellulosic biomass as feedstock. The yeast *Saccharomyces cerevisiae* possesses industrially desirable traits for ethanol production and is able to produce heterologous cellulases, which are required for CBP. However, *S. cerevisiae* produces low titres of cellulases and one suspected reason for this is the stress caused by the heterologous proteins that induce the unfolded protein response (UPR). The UPR is a stress response pathway that will either lead to increased folding capacity within the ER or to degradation of these proteins and possibly apoptosis of the cell. It is thus beneficial to be able to determine when and to what extent the UPR is active during CBP organism development. Current methods of measuring the UPR include RNA and reverse transcriptase qPCR (r.t.qPCR) measurements, which can be cumbersome and expensive. The purpose of this study was to develop a vector based biosensor that will detect and quantify UPR activation. The vector consisted of either the *T.r.xyn2* or *eGFP* reporter genes under the control of the *S. cerevisiae HAC1*p or *KAR2*p promoters. *HAC1* and *KAR2* are important regulators of UPR as their activation allows the UPR to achieve its function. The eGFP reporter under the transcriptional control of *KAR2*p was shown to be the superior combination due to the improved dynamic range when the UPR was induced in transformed *S. cerevisiae* strains by the stress inducer, tunicamycin. This UPR biosensor also proved to be more sensitive when measuring UPR induction in cellulase producing strains, depicting significant differences, compared to previous r.t.qPCR based tests which were unable to detect these differences. We thus developed a UPR biosensor that has greater sensitivity for changes in UPR induction compared to RNA based methods and the first *KAR2*p based UPR biosensor plasmid that allowed for more accurate detection and measurement of the UPR in cellulase secreting *S. cerevisiae* strains. The ability to quantify UPR induction will assist in identifying candidate cellulase genes that do not greatly induce the UPR, making them ideal to use in developing CBP yeasts.

Keywords: Consolidated bioprocessing; Cellulases; *Saccharomyces cerevisiae*; Unfolded protein response; UPR biosensor; reporter gene; CBP yeast

CHAPTER 1: LITERATURE REVIEW

1.1 Bioenergy

1.1.1 Introduction

Fuel production from renewable sources is gaining more attention world-wide as the need for fossil fuel replacements increases (Tian et al., 2018). This is mainly due to the expected decrease, or shortage, in fossil fuels in the near future, as well as the negative environmental impact it has. Fossil fuel combustion has been a major contributor to the high levels of pollution that has taken place over the past number of decades (Sarkar et al., 2012, Galbe and Zacchi, 2012). The transport sector in particular is a major contributor to global greenhouse gas (GHG) emissions, and thus the replacement of these oil-derived fuels with more environmentally friendly biofuels can potentially decrease environmental impacts (Pereira et al., 2015). Emphasis is thus placed on fuel production from plants or organic waste as a way of providing more sustainable and environmentally friendly energy sources for consumer societies and industrial economies (Figure 1.1) (Naik et al., 2010).

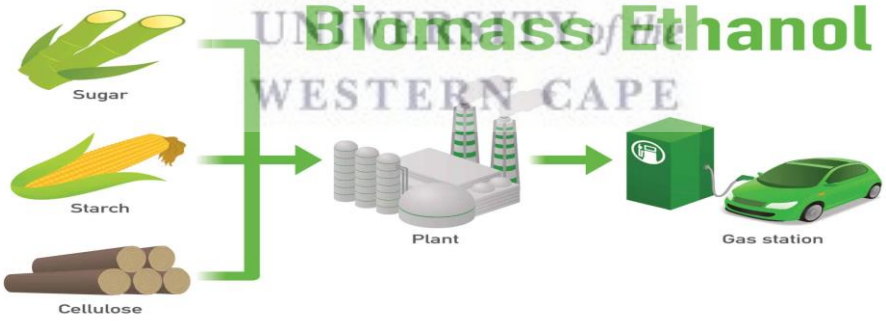

Figure 1.1: Bioethanol from biomass. Bioethanol can be produced from various types of biomass feedstocks (Volynets et al., 2017).

Bioenergy is defined as a form of renewable energy from living or non-living sources such as the energy derived from biological crops, wastes or residues (Sarkar et al., 2012, Volynets et al., 2017). Due to biomass being highly abundant and renewable, it has been identified as a

renewable and potentially sustainable alternative source of energy for the production of biofuels (Kricka et al., 2015). Biomass can be obtained from industrial, agricultural or forestry waste, as well as dedicated energy crops. These types of biomass can be used in the production of different types of biofuels including bioethanol, biodiesel and biohydrogen. Bioethanol is commercially the most widely utilized biofuel that can be produced through fermentation and thermochemical processes using sugars and starches (Fargione et al., 2009).

1.1.2 Bioethanol

Bioethanol is an attractive solution to replace existing fossil fuels since it is made from renewable sources and, due to its oxygenation, has the potential to cause a reduced amount of particulate emissions in compression ignition engines (Sun et al., 2016, Volynets et al., 2017). The fact that bioethanol can directly replace fossil fuels in modern engines either directly as a fuel or in a mixture with gasoline is another appealing trait. While a litre of ethanol only contains 66% of the energy that is normally provided by a litre of petrol, ethanol can improve the performance of petrol due to its higher octane level (Nigam and Singh, 2011). This mixture also decreases the sulphur content of the fuel and thus also decreases sulphur oxide emissions. The most common blends that are used is 10% ethanol and 90% gasoline, but modern internal combustion engine vehicles (ICEV) can use up to 85% ethanol and 15% gasoline (E85) or even 95% ethanol (E95) (Hamelinck et al., 2005, Khuong et al., 2017).

Biomass feedstocks used to produce bioethanol are sucrose–containing feedstocks (sugarcane, sweet sorghum, etc.), starch feedstocks (corn, wheat, etc.) and lignocellulosic biomass (grasses, straw, wood, etc.) (Naik et al., 2010, Kostas et al., 2016). Corn starch feedstocks are the primary source of bioethanol production in the United States, while cane sugar and molasses are commonly used in Brazil (Volynets et al., 2017). However, the type of biomass feedstock used will influence the process used to produce the bioethanol. Bioethanol can either be produced in a first or second generation process, and those terms are used to classify liquid biofuels.

1.2 Classification of bioethanol

1.2.1 First generation bioethanol

First generation (1G) bioethanol is a mature technology that is well established and used to produce the majority of bioethanol worldwide (Kostas et al., 2016). Most bioethanol is produced from corn glucose or sucrose through fermentation, and countries with a good agronomic-based economy are able to use 1G technology for fuel production. The sugars, which are easily extracted from the crops, are used as a carbohydrate source for fermentation, after which ethanol is distilled from the broth (Karakashev et al., 2007, Kricka et al., 2015). 1G bioethanol is mainly characterized by its ability to be used in existing combustion engines through blending with petroleum-based fuels, and thus distributed by means of existing infrastructure (Naik et al., 2010). The factors limiting 1G biofuel production in general are concerns over carbon and energy balances and environmental impacts, as well as the high production costs of the bioethanol. However, the food-versus-fuel debate remains the main disadvantage of 1G biofuels (Nigam and Singh, 2011). This debate revolves around the use of food crops (corn, sugarcane, soybean etc.) for fuel production, which has been one of the reasons for increases in food prices as 1G fuel production increases. This raises concerns that farmLand that could be used for production of crops consumed by humans, will be used for biofuel production. Therefore there has been a shift in recent years from edible to non-edible lignocellulosic biomass for the production of biofuel.

1.2.2 Second generation bioethanol

Second generation (2G) bioethanol production is the most favored alternative option for biofuel production due to the use of plant biomass, also known as lignocellulosic biomass, which can be either non-edible whole plant biomass or the non-edible residues left behind following food crop production (figure 1.2) (Nigam and Singh, 2011, Favaro et al., 2015). Lignocellulose is highly abundant and widely distributed, making it desirable in the fuel production industry. The biomass can be supplied in large scale from various low cost materials

such as agricultural residues, wood, municipal, and industrial wastes (Limayem and Ricke, 2012).

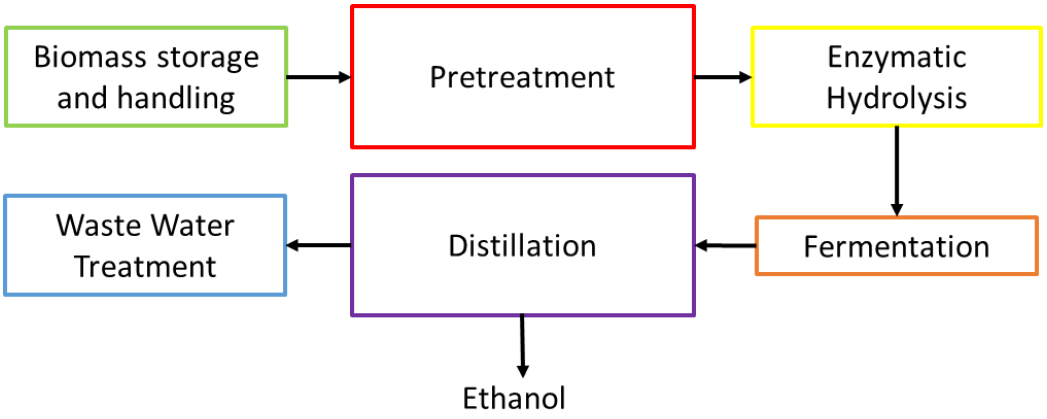

Figure 1.2: A simplified scheme for the production of ethanol from lignocellulosic biomass. Biomass undergoes various stages of processing before the final product, ethanol, is produced (Agbogbo and Coward-Kelly, 2008).

Lignocellulosic residues are produced in large amounts from industries such as the sugarcane industry, where it is generated during 1G ethanol and sugar production (Pereira et al., 2015). These residues include bagasse and trash such as green leaves, dry leaves and tops. 2G ethanol production would allow for utilization of these wastes, which is arguably its most desirable trait as this completely avoids the food versus fuel conflict. One of the downsides to using a complex structure such as lignocellulose for ethanol production is accessing cellulose and hemicellulose, which are the constituent polymers containing the sugars that are eventually fermented into bioethanol. This recalcitrant structure necessitates expensive pretreatment prior to hydrolysis, representing one of the technical barriers that needs to be overcome when using lignocellulosic biomass for fuel production (Kricka et al., 2015, Naik et al., 2010, Den Haan et al., 2013b). Despite this, 2G remains the preferred option for large-scale liquid biofuel production, with great potential to overcome concerns about energy security and environmental impacts.

1.3 Bioconversion of lignocellulosic biomass

1.3.1 Components of lignocellulosic biomass

Lignocellulose can be broken down into three main components namely cellulose, hemicellulose and lignin (Figure 1.3) (Galbe and Zacchi, 2012, Cortes-Tolalpa et al., 2017). Cellulose can be described as the main component of lignocellulose. It consists of linear chains of β-(1.4)-glycosidic bonded D-glucose residues that are associated with each other through hydrogen bonds, forming crystalline cellulose fibers that are insoluble in water and most organic solvents. This is the most desired component of the lignocellulose that will be most readily fermented into bioethanol if the component sugars are released. In order to access cellulose, hemicellulose must first be removed since this biopolymer covers most of the cellulose fibers. This removal will increase the digestibility of cellulose.

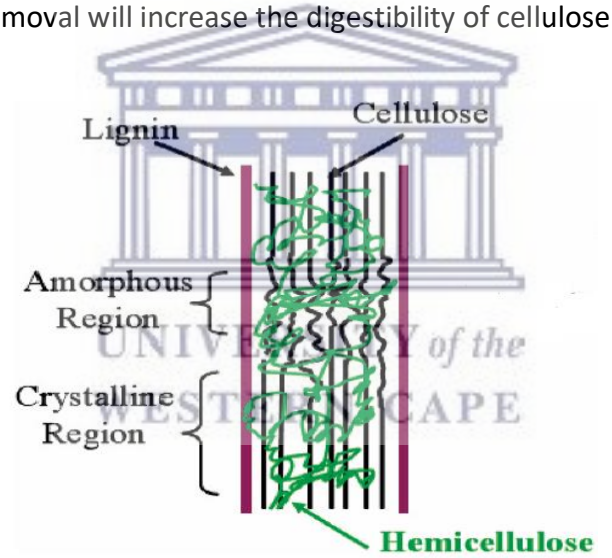

Figure 1.3: Schematic representation of the composition of lignocellulosic biomass. Lignocellulosic biomass consists of three main constituents: cellulose, hemicellulose and lignin (Schwietzke et al., 2009).

Hemicellulose can be described as a heterogeneous biopolymer that consists of hexoses, pentoses, and uronic acids (Madadi et al., 2017). When removing the hemicellulose, it should be noted that factors such as temperature and retention time must be controlled since hemicellulose can be sensitive to operation conditions and can easily degrade to form unwanted products. The third component, polyphenolic lignin, cannot be fermented but must be partially removed in order to enhance cellulose digestibility (Sun et al., 2016). Lignin is an

aromatic biopolymer that is synthesized from phenolpropanoid precursors. This component is often just recovered and burned for the purpose of providing heat and energy, though significant research is being done in order to valorize the component (Mood et al., 2013, Das et al., 2017).

1.3.2 Pretreatment of lignocellulose

The recalcitrant structure of lignocellulosic biomass protects the carbohydrates that are desired for ethanol production by preventing it from being degraded by enzymes and microorganisms (Tian et al., 2018). Thus, pretreatment is important to break the recalcitrance by removal of hemicelluloses and lignin, with the type of pretreatment used determining the extent to which the biomass will become more easily accessible to the enzymes (Madadi et al., 2017, Sindhu et al., 2016). Various pretreatment methods have been utilized for the breakdown of this recalcitrant structure. Pretreatment is important as it plays an important role in the subsequent saccharification steps (Rao et al., 2016). The pretreatment functions to break the matrix, reducing the degree of crystallinity of cellulose and ultimately exposing the amorphous cellulose, which is the main target for initial enzymatic attack (Figure 1.4) (Sarkar et al., 2012).

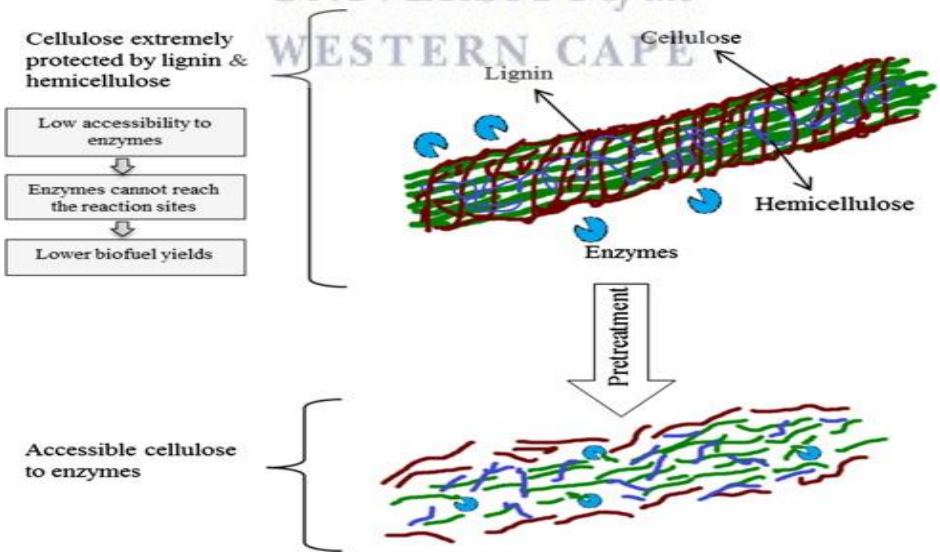

Figure 1.4: Schematic of the effect of pretreatment of lignocellulosic biomass. Pretreatment is a crucial step in the removal of lignin and hemicellulose to expose the desired cellulose (Tian et al., 2018).

Pretreatment of lignocellulosic biomass can be divided into physical, chemical and biological methods (Rao et al., 2016, Rabemanolontsoa and Saka, 2016). Physical pretreatment methods are used to increase the accessibility of enzymes or acids by disrupting the structure of the biomass. Methods used include reducing the size of the biomass through grinding chipping or milling, and irradiation through microwave, gamma rays or electron beam. Chemical pretreatment breaks down the polymerization and crystallinity of the biomass through methods using bases, acids, organic solvents and ionic liquids (Tian et al., 2018). Chemical methods are very effective but have disadvantages such as high costs, toxic product formation and loss of carbohydrate, among others. Biological or microorganism pretreatment methods uses microorganisms or their metabolites to break down the biomass. This method is considered a promising eco-friendly technology with many advantages but is hampered by very long retention times.

1.3.3 Cellulose hydrolysis

Since cellulose represents the main source of fermentable sugars from lignocellulose for ethanol production, it is important to be able to hydrolyze it completely (van Zyl et al., 2011, Kricka et al., 2015). However, since cellulose is in crystalline form, it requires three enzymatic activities, namely endoglucanases (EGs), cellobiohydrolases (exoglucanases) (CBHs) and β-glucosidases (BGLs) to work in synergy (Figure 1.5). These enzymes are collectively known as cellulases. EGs functions by acting on the non-crystalline amorphous regions of cellulose, producing free chain ends, cellobiose and cello-oligosaccharides as products (Madadi et al., 2017, Sindhu et al., 2016). CBHs act directly on the crystalline regions of cellulose, resulting in the formation of cellobiose as product. BGLs utilizes the cellobiose produced by EG and CBH, as well as the cello-oligosaccharides formed by EG, to produce glucose.

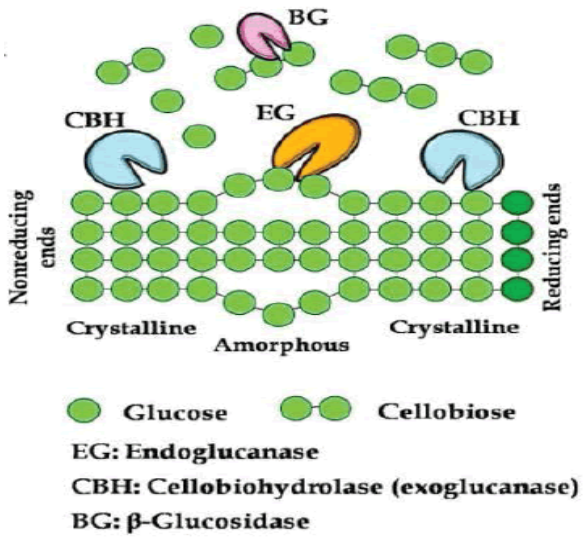

Figure 1.5: The sequential breakdown of lignocellulose by cellulases. The three classes of cellulases, endoglucanase, β-glucosidase and exoglucanases (CBHI and CBHII), working in synergy to break down lignocellulose (Madadi et al., 2017).

1.4 Integrated technologies for bioconversion of lignocellulosic biomass

1.4.1 Types of integrated technologies

Various process configuration technologies have been developed to provide for integrated substrate hydrolysis and fermentation in an attempt to cut costs when using lignocellulosic biomass feedstocks to produce ethanol (Figure 1.6) (Saini et al., 2015). A mature technology that is already employed at industrial scale, is separate hydrolysis and fermentation (SHF). This process uses separate chambers to perform substrate hydrolysis and fermentation, respectively, and has notable features such as optimal operating conditions for each of the steps involved. One main disadvantage of using SHF is that it can lead to end-product inhibition, which can be a limiting factor (Balat et al., 2008). This has thus led to the development of simultaneous saccharification and fermentation (SSF) technology, in which enzymatic hydrolysis and fermentation of the resulting hexose sugar product is now consolidated, overcoming the end-product inhibition that occurs in SHF. Despite the advantages of this process, such as lower costs and higher yields due to the fewer vessels used and removal of feedback inhibition, there is no optimal conditions for enzyme hydrolysis and

fermentation as these processes occur over widely varying temperature ranges (Hamelinck et al., 2005).

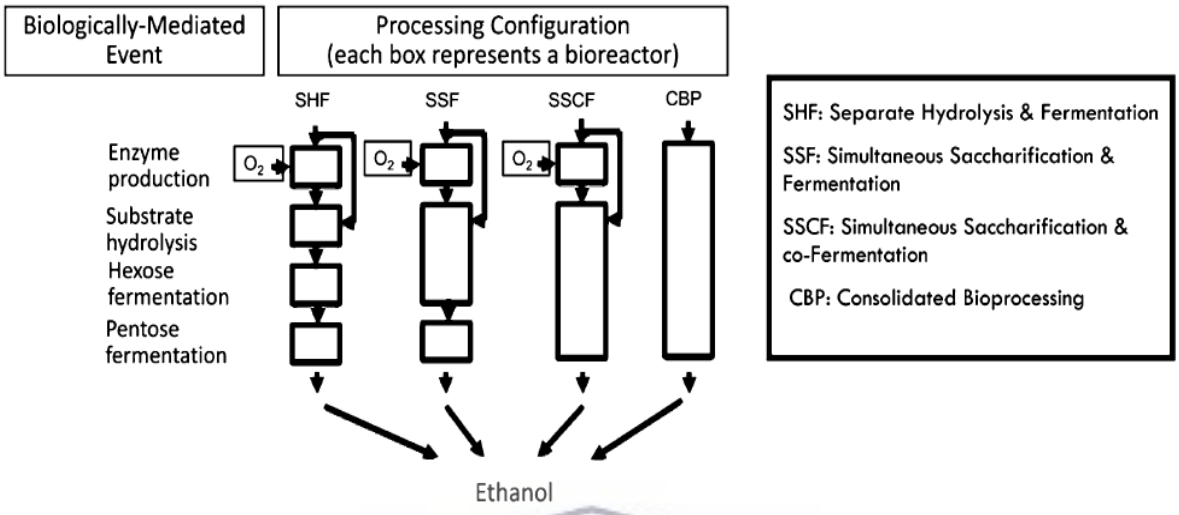

Figure 1.6: Processing configurations for lignocellulosic biomass conversion to ethanol. The different configurations available for enzyme hydrolysis and fermentation in the production of bioethanol from lignocellulose is depicted (Saini et al., 2015).

Simultaneous saccharification and co-fermentation (SSCF) was developed for the co-fermentation of hexose and pentose sugars with the use of microorganisms that are able to utilize both these types of sugars (Saini et al., 2015). The organism that is used should be compatible with required operating conditions. A major drawback to this process is the lack of microorganisms available that are able to ferment both pentoses and hexoses at rates required by industry. However, this can be overcome with the use of co-culture techniques, in which a combination of microorganisms are used to ferment hexoses and pentoses respectively. Consolidated bioprocessing (CBP) is performed in a single chamber by a single microorganism that is able to produce all the necessary enzymes, as well as ferment the resulting sugars into desired products (Den Haan et al., 2013b).

1.4.2 Consolidated bioprocessing (CBP)

CBP is a very attractive process in the biofuel industry for the production of bioethanol due to being more cost-effective when using lignocellulosic biomass as the feedstock (Saini et al.,

2015). The cut in costs is mainly due to the lack of separate enzyme production, and due to substrate hydrolysis and fermentation of resulting sugars being performed in a single reactor by a single microorganism or microbial consortium (Figure 1.7).

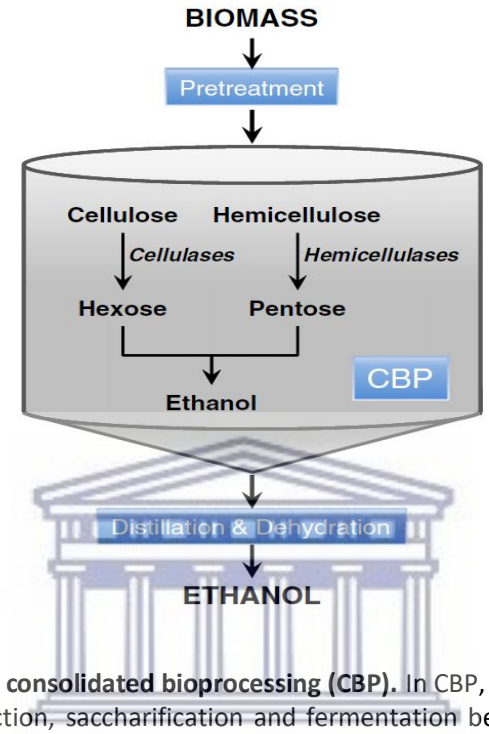

Figure 1.7: Diagram illustrating consolidated bioprocessing (CBP). In CBP, the ethanol is produced in a single reactor due to enzyme production, saccharification and fermentation being consolidated (Hasunuma and Kondo, 2012b).

Avoiding enzyme production is very important for cost reductions since cellulase production can have various factors associated with it, such as the substrate, raw materials capital costs and other utilities involved, which in this case is avoided (Hasunuma and Kondo, 2012b). CBP is also capable of producing bioethanol at a much higher efficiency compared to current processes that use dedicated cellulase production. Higher hydrolysis rates, smaller reactor volumes and reduced costs can also be obtained when achieving CBP with thermophilic organisms. CBP thus has exceptional potential in the biofuel industry if an ideal microorganism can be developed that is able to efficiently produce the cellulases that are required to break down the lignocellulosic biomass to fermentable sugars and subsequently convert it into ethanol at a high rate (Hong et al., 2014).

1.4.3 CBP microorganism development

Naturally occurring microorganisms that can be used for CBP might not exist, but there are microorganisms that possess some of the traits desired and that can be developed to be beneficial to the process (Van Zyl et al., 2007, Lynd et al., 2005). These microorganisms can be divided into two groups called the native cellulolytic microorganisms and recombinant cellulolytic microorganisms. Native cellulolytic microorganisms are capable of high saccharolytic activity but some lack the product formation properties required at industrial scale. Strategies for developing these microorganisms for CBP involve engineering in order to improve their ethanol production and to avoid by-product formation (Lynd et al., 2017). Native cellulolytic microorganisms include mostly anaerobic bacteria such as the *Clostridium* species, and various fungi such as *Trichoderma* species and *Fusarium oxysporum* that are capable of producing large amounts of saccharolytic enzymes. These microorganisms possess highly efficient and complex saccharolytic capabilities. One of the first such candidates for engineering was *Clostridium thermocellum* due to its fast growth rates on crystalline cellulose by making use of cellulosomes and it is still considered a good potential CBP organism (Hasunuma and Kondo, 2012b, Lynd et al., 2017). However, anaerobic bacteria are not ideal for the purpose of ethanol production as by-products formed during fermentation can limit the ethanol yield and they tend to have low ethanol tolerance (Van Zyl et al., 2007). Filamentous fungi in turn are slow degraders of cellulose and generally produce low ethanol yields. Candidates for the recombinant cellulolytic microorganism strategy are selected to naturally produce high yields of a desired product. However, they lack the saccharolytic ability found in native cellulolytic organisms and strategies for developing these microorganism for CBP would thus include engineering of a heterologous cellulase system to allow for cellulose utilization (Hasunuma and Kondo, 2012b, Den Haan et al., 2015). Recombinant cellulolytic microorganisms, which have been engineered to heterologously express cellulases and hemicellulases, are primarily bacterial hosts such as *Escherichia coli*, *Klebsiella oxytoca* and *Zymomonas mobilis,* but also include the yeasts *Saccharomyces cerevisiae* and the xylose-fermenting strains *Pachysolen tannophilus*, *Candida shehatae*, and *Pichia stipits* (Hasunuma and Kondo, 2012b).

1.5 Saccharomyces cerevisiae

1.5.1 Introduction

Apart from being the worlds foremost industrial ethanologen used in potable and industrial ethanol production, the yeast *S. cerevisiae* is also one of the most extensively studied hosts for the production of heterologous proteins (Tang et al., 2015, Wang et al., 2017). It is widely used to produce heterologous proteins that are of medical and industrial interest. It has desirable traits for this purpose as it is able to perform post translational modifications and is thus capable of proper protein folding. However, *S. cerevisiae* produces low levels of secreted heterologous proteins (Kroukamp et al., 2018). Various strain engineering attempts have been made to improve heterologous protein secretion in *S. cerevisiae*, with significant interest due to properties that would enable strains to be beneficial for production of fuels and chemicals from renewable feedstocks. *S. cerevisiae* is thus expected to be a prominent host in the future of plant biomass conversion into commodity products (Ilmén et al., 2011). This yeast is already used in industries that produce starch- and sugar-based 1G ethanol and several successful attempts have been made to develop xylose fermenting strains of the yeast as the wild-type cannot utilize pentose sugars (Diao et al., 2013). Despite this, developing *S. cerevisiae* for the rapid conversion of pretreated lignocellulose to ethanol remains a formidable task (Hasunuma and Kondo, 2012a, Wang, 2015, Hong et al., 2014).

1.5.2 *S. cerevisiae* for CBP

When it comes to industrial ethanol production, *S. cerevisiae* is usually considered to be the first choice primarily due to its many advantages for the process such as high ethanol productivity, high ethanol yield from glucose and its inherent resistance to various inhibitors that are found in the lignocellulosic hydrolysate compared to bacteria (Hasunuma and Kondo, 2012b, Wang et al., 2017). However, using *S. cerevisiae* for ethanol production from lignocellulosic biomass in CBP, would require engineering to allow it to express cellulases that would break down the biomass into fermentable sugars (Figure 1.8) (Favaro et al., 2015, Kroukamp et al., 2018). On an industrial scale, CBP strains of *S. cerevisiae* would be expected to

be able to ferment both pentose and hexose sugars, have minimum nutritional requirements and be able to function under inhibitory conditions caused by pretreatment-derived inhibitors. As an industrial strain it should furthermore secrete high levels of heterologous hydrolases to ensure sustainable hydrolysis and fermentation of cellulose for ethanol production. It can thus prove to be very challenging to develop *S. cerevisiae* for this purpose.

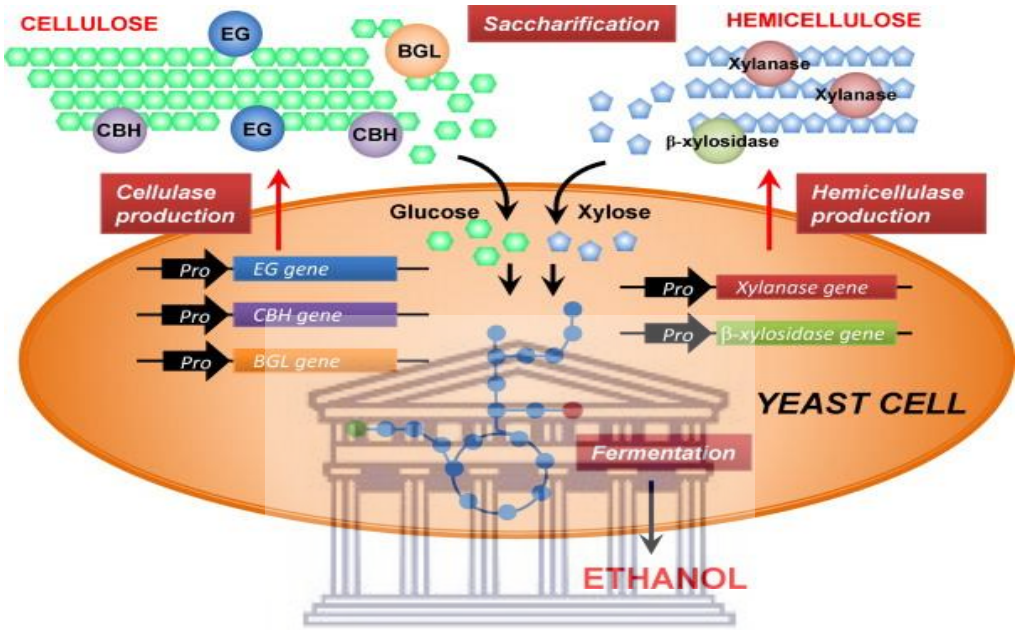

Figure 1.8: Recombinant yeasts for CBP. CBP yeasts should be able to secrete cellulases (BGL, CBH and EG) and hemicellulases (Xylanase and β-xylanase) to break down the biomass feedstock and produce bioethanol from the resulting glucose and xylose (Hasunuma and Kondo, 2012a).

The heterologous expression and secretion of cellulases remains the most important part of developing *S. cerevisiae* for CBP (Figure 1.8) (Den Haan et al., 2015). The complete enzymatic digestion of crystalline cellulose to expose the fermentable sugars in lignocellulosic biomass, requires EG, CBH and BGL to work in synergy, as mentioned before. The functional secretion of CBH is known to be crucial for its function in CBP and it is the most important enzyme for crystalline cellulose degradation. Recombinant *S. cerevisiae* strains have previously been shown to be able to successfully produce and secrete CBHs, with the glycosylated heterologous CBH1 even having a specific activity that was similar to the native enzyme produced by *Trichoderma reesei* (Den Haan et al., 2007a). However, it was expressed at very low titers, which can prove to be the most significant challenge towards successful CBP by recombinant

yeast (Den Haan et al., 2013b). EG expression on the other hand, has been more successful in *S. cerevisiae* as both fungal and bacterial EGs have been produced by the yeast (Den Haan et al., 2007b). This is mainly due to the much higher specific activity of EGs compared to CBHs on synthetic and amorphous cellulose substrates, making detection and measurement much easier even at small amounts. Despite heterologous EGs often being highly hyperglycosylated, this didn't always have a negative effect on their specific activity. *S. cerevisiae* was also successfully engineered to express BGLs at levels that were sufficient for the yeast to sustain growth on cellobiose, the disaccharide product of CBH action on cellulose (Van Zyl et al., 2007). The recombinant yeast was able to use cellobiose as sole carbon source at rates that were comparable to glucose.

Expression of individual cellulases, with the exception of CBH, is thus not a significant problem for CBP yeast development, but the expression of more than one could prove to be a bigger challenge due to the limited cellulase secretion capacity of *S. cerevisiae* (Van Zyl et al., 2014). Studies have been performed focusing on expression of multiple cellulases with the aim of recreating a fully cellulolytic system (Ilmén et al., 2011, Hasunuma et al., 2013) and although these studies were successful in some regard, CBHs remain the most complicating factor in all cases. High level secretion of CBH is required for the successful hydrolysis of crystalline cellulose, and ultimately for CBP of crystalline cellulose to ethanol (Kroukamp et al., 2018). However, it was shown that significant production of heterologous CBHs caused stress to yeast cells and in many cases induced a response termed the unfolded protein response (UPR) in an attempt to re-establish cellular homeostasis (Ilmén et al., 2011). It is thus important to understand how protein secretion works in yeast to identify where the bottlenecks are in order to alleviate them.

1.5.3 The protein secretion pathway of *S. cerevisiae*

Protein secretion is one of the most important processes in all living organisms (Figure 1.9) (Delic et al., 2013). Unlike bacteria which secrete proteins that function either in the cell wall or the exterior space, eukaryotes also secrete many proteins that function in the intracellular compartments. In eukaryotes, the protein secretion pathway is initiated by the translocation of

proteins across the endoplasmic reticulum (ER) membrane, by way of a signal peptide at the N-terminus of nascent polypeptides. Secretion then generally continues from the ER to the Golgi pathway. The ER, an organelle making up approximately 30% of the proteome in yeast, is the site where proteins undergo most of their post-translational processing (Kupsco and Schlenk, 2015). The ER is thus essential as large amounts of proteins are sent there for processing.

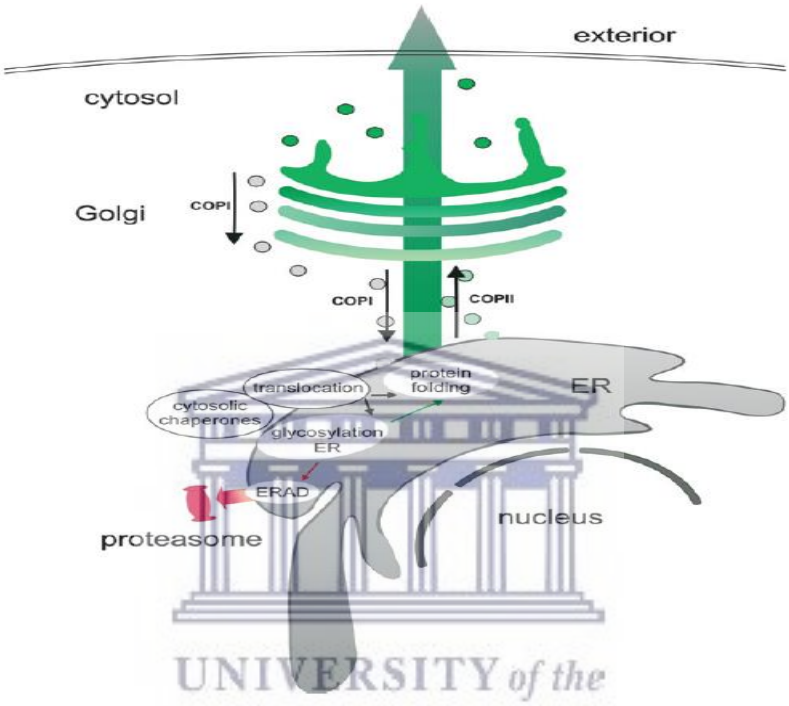

Figure 1.9: The protein secretion pathway of yeast. An overview of the protein secretion pathway in yeast from the initial synthesis in the cytosol to secretion of the glycosylated protein (Delic et al., 2013).

At the ER, the Sec61 translocon is responsible for the translocation of post translational nascent proteins to the ER lumen (Anelli and Sitia, 2008). The ER is the main organelle that is involved in protein folding and also facilitates the quality control of incorrectly folded proteins. In the ER, the nascent proteins will be folded into their native structures by the binding immunoglobulin protein (BiP), which is an ER-resident chaperone protein of the HSP70 family. Calnex, a lectin chaperone that is responsible for folding and initial glycosylation of glycoproteins, will bind to the nascent glycoprotein for correct folding and N-glycan processing. Covalent modifications such as N-glycosylation, disulphide bond formation, signal sequence processing, glycosyl-phosphatidyl-inositol addition, sorting and degradation are sustained in

the yeast ER. Correctly assembled and folded proteins will be transported to the Golgi apparatus. In the Golgi apparatus, proteins that pass through are modified and sorted as they are prepared for transportation to their specified destination in the cell. Misfolded proteins are targeted for degradation through ER-associated protein degradation (ERAD) (Yoshida, 2007). This is achieved through the quality control (QC) system as the binding of misfolded proteins to the BiP complex results in their transportation to the cytosol where they are eventually degraded.

1.5.4 Heterologous protein expression in yeast

Yeasts have a reputation as a good host for the production of heterologous proteins, accounting for about 20% of the current biopharmaceutical drug portfolio (Delic et al., 2013). *S. cerevisiae* is one of the preferred hosts for heterologous protein production due to its ability to perform post-translational modifications such as folding, glycosylation, etc., as well as being able to secrete these proteins in their native functional form and being generally regarded as safe (Figure 1.10) (Hou et al., 2012).

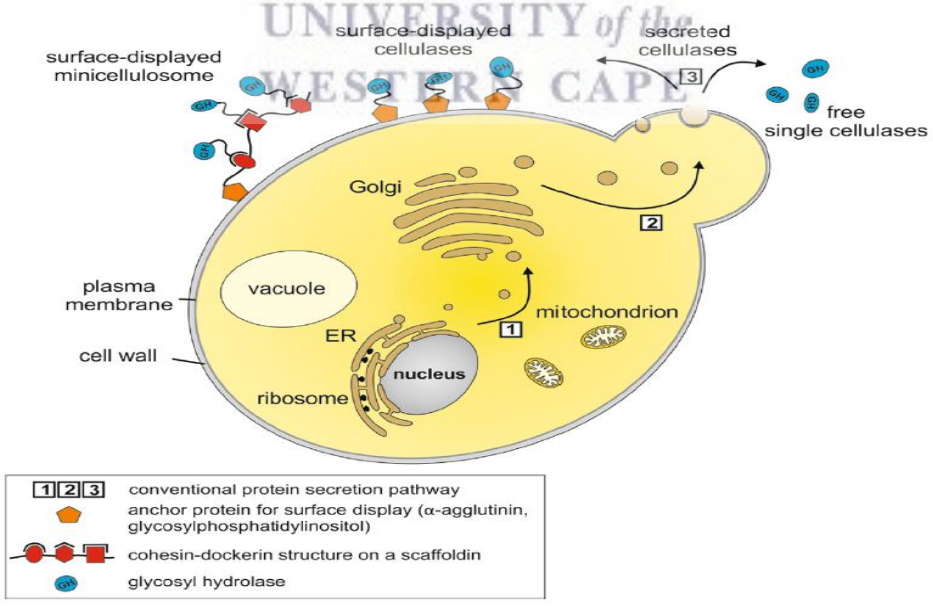

Figure 1.10: Illustration of the different components involved in the yeast heterologous protein secretion and the three different cellulase secretion strategies used for CBP (Lambertz et al., 2014).

Despite the secretion superiority of *S. cerevisiae*, heterologous protein secretion is not always very efficient and is produced at low levels (Kroukamp et al., 2018). This can be due to various factors such as the properties of the proteins, the expression vector system used, the leader sequences and also the folding and secretion capacity of the cell that can affect the protein titer. The limited folding capacity of *S. cerevisiae* was shown to be the reason for low levels of protein production, as unfolded proteins that enters the ER lumen are either folded or degraded, with both of these having an impact on the cell and protein production (Hampton, 2000, Den Haan et al., 2013a). When disruption of the ER function occurs, it can result in a state known as ER stress which is characterized by an accumulation of unfolded proteins (Kupsco and Schlenk, 2015). When there is an overload of unfolded or misfolded proteins, the cell will react by activating the UPR pathway (Delic et al., 2013).

1.6 Unfolded protein response (UPR)

1.6.1 Introduction

There has been increased interest in understanding how ER stress pathways work and how it can be controlled, as this is seen as a potential way of treating human diseases such as cancer and neurodegenerative disorders (Hetz and Papa, 2017, Moore and Hollien, 2012). However, understanding the UPR goes beyond this, as this pathway is important for the normal functioning and development of the ER in various organisms. The UPR was first characterized in budding yeast, and it remains a key model organism for studies that focus on understanding ER stress and the various mechanisms controlling the response to it (Zhang and Kaufman, 2006, Yu et al., 2015).

The UPR can be described as a highly conserved signaling pathway that is capable of altering gene expression and protein translation to assist with correct folding of proteins during ER stress (Kupsco and Schlenk, 2015). The UPR is thus able to provide the nucleus and cytosol information about the protein folding state in the ER lumen, which then buffer the changes that occur in the unfolded protein load (Hetz, 2012, Back et al., 2005). When ER stress is

irreversible, the UPR will remove the damaged cells through apoptosis. The responsibility of the UPR is thus to reestablish homeostasis in the ER.

1.6.2 First discovery of the UPR

The glucose-regulated protein (Grp) 78, a mammalian ER chaperone, played a major role in the discovery of the UPR (Ma and Hendershot, 2001, Howell, 2017). Grp78p was discovered by its upregulation in RNA tumor virus transformed cells as a result of glucose depletion from the medium of rapidly growing tumor cells. Changes in the ER environment caused by various conditions and agents were also found to increase their expression. This discovery, as well as an independent study performed by Haas and Wabl (1983) that identified Grp78p as an ER-localized protein known as BiP, led to the classification of Grp78/BiP as the first ER molecular chaperone. BiP was found to prevent the transport of many different unfolded proteins in the ER by binding to them. This was demonstrated in a study by Gething and Sambrook (1992) in which an unfolded variant of influenza hemagglutinin protein (HA) was overexpressed, and resulted in the induction of BiP and Grp94 expression. Eventually this signaling pathway being classified as the unfolded protein response (UPR).

1.6.3 The UPR in yeast

1.6.3.1 Mechanisms of action in yeast

In yeast, the UPR is a linear signaling pathway consisting of a histone acetyltransferase (*HAC1*) pathway that is regulated by inositol-requiring enzyme (Ire1p), with the aim of regulating the transcription of UPR target genes encoding for foldases, chaperones, and proteins involved in lipid metabolism and glycosylation (Figure 1.11) (Yu et al., 2015). The Hac1p transcription factor and the Ire1p kinase/RNase domains are important components in the UPR pathway in yeast (Guerfal et al., 2010). The ER chaperone BiP/Kar2p associates with an inactive Ire1p under normal, non-stressed conditions. BiP is encoded by the *KAR2* gene and functions to maintain the functional state of the translocational machinery in the ER lumen by assisting in the folding and assembly of proteins that are translocated across the ER membrane (Mori et al., 1992, Zhang et al., 2006).

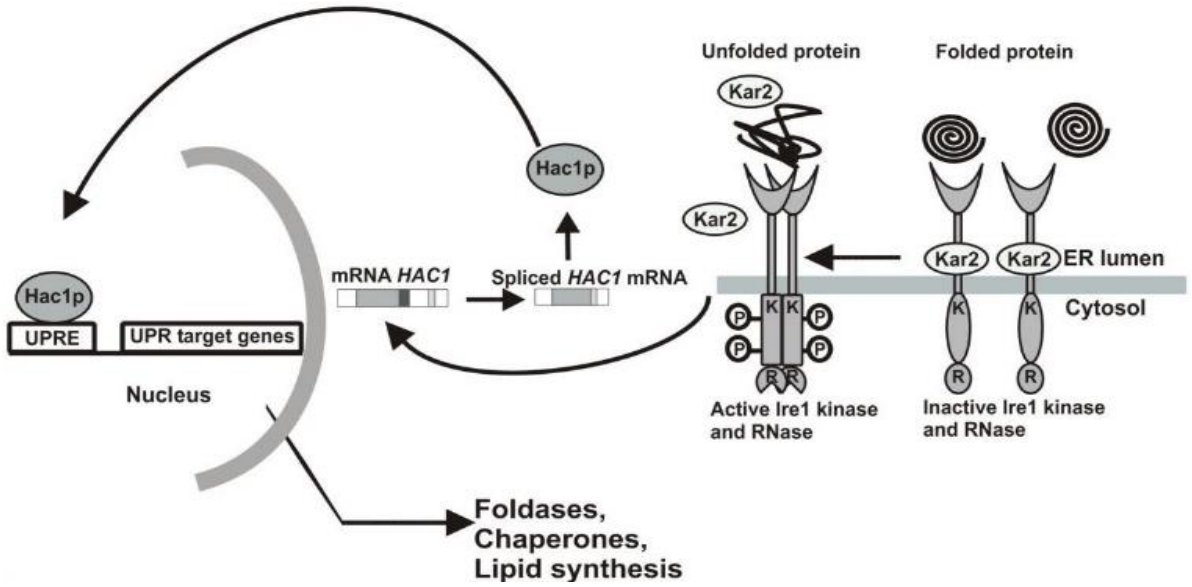

Figure 1.11: Diagram of the mechanism of the unfolded protein response in *S. cerevisiae* (Guerfal et al., 2010).

Under stress conditions, such as the accumulation of unfolded proteins, BiP dissociates from the Ire1p complex, causing Ire1p to cluster and directly interact with unfolded proteins (Guerfal et al., 2010). This clustering results in the transautophosorylation of the Ire1p complex which activates its RNase function. This activation of the Ire1p complex leads to the unconventional splicing of *HAC1* mRNA, cleaving it at positions that are not normally recognized by the spliceosome (Navarro-Tapia et al., 2018). The *HAC1* mRNA is constitutively expressed at basal levels, but due to the presence of its introns, it does not encode for a protein until splicing occurs. The translation block of the mRNA is released when the introns are removed, allowing it to encode the Hac1p transcription factor, which will bind to the unfolded protein responsive element (UPRE) found in the promoters of UPR target genes after translocation to the nucleus (Guerfal et al., 2010).

1.6.3.2 UPR regulation in yeast by *IRE1* and *HAC1*

Since the UPR in yeast is a linear pathway, it is clear that the main components that regulates the UPR are the Ire1p and the Hac1p transcription factor (Guerfal et al., 2010). The only ER stress sensor that has been identified in yeast is the Ire1p, which uses an ER luminal stress-

sensing domain to monitor ER homeostasis and is thus able to use an RNase domain and cytoplasmic kinase to activate the UPR (Figure 1.11) (Chen and Brandizzi, 2013). This is in contrast to mammals, which have two additional ER-stress sensors known as protein ER kinase (PERK) and activating transcription factor 6 (ATF6), to help cope with the complex situations that the UPR is faced with (Hetz, 2012). During ER stress, the RNase domain of the Ire1p is activated through higher-order oligomerization, autophosphorylation and conformational changes. BiP negatively regulates the Ire1p as its association with the complex inactivates it, however the dissociation of BiP is not sufficient for activation of the Ire1p (Kimata et al., 2007). Inactivation of the Ire1p through Ptc2 phosphatase can lead to the partial attenuation of the UPR (Montenegro-Montero et al., 2015).

It is known that during ER stress BiP binds to Ire1p at the luminal domains and dissociates, suggesting that BiP is either triggered by the release of misfolded proteins or these proteins compete for it to allow the sensors to be oligomerized and activated (Moore and Hollien, 2012). However, the variants of Ire1p in yeast that do not bind to BiP have been found to respond normally to ER stress. Therefore it has been proposed that in order for correct activation of the Ire1p, BiP needs to bind and release It (Guerfal et al., 2010). The current direct model in *S. cerevisiae* for the activation of Ire1p is that oligomerization is mediated when Ire1p binds to misfolded proteins that accumulates during stress. This is supported by the luminal domain of the Ire1p in yeast that has a groove that is similar to the histocompatibility complex peptide binding site (Moore and Hollien, 2012). Through this binding groove, the Ire1p in yeast is able to bind to carboxypeptide Y (misfolded form), leading to the oligomerization of Ire1p and thus backing the direct binding model. Since the UPR in yeast is mainly a stress response, it thus makes sense that Ire1p is activated by misfolded proteins.

Despite *HAC1* being known to be constitutively expressed at low levels in yeast, it is unable to produce any protein due to the secondary structure of its introns and thus remains unspliced (Guerfal et al., 2010). This translation block is released once its introns is removed, allowing it to produce the Hac1p, which will eventually bind to the UPRE in UPR target genes, allowing the UPR to achieve its function. The best characterized UPRE's have been found in the *HAC1* gene,

as well as in the UPR target gene *KAR2* (Guerfal et al., 2010). Since the *HAC1* gene contains an UPRE on its promoter, it is able to upregulate its own transcription. Hac1p is capable of upregulating over 400 genes and cause the secretory pathway to be remodeled in a way that increases the capacity of the ER, resulting in decreased misfolding and increased membrane synthesis to accommodate increased ER function (Moore and Hollien, 2012).

1.6.4 The role of UPR in protein secretion

As stated above, the UPR plays an important role in the secretory pathway, especially during an ER stress state, as it will enable the pathway to deal with the stress of accumulated misfolded proteins (Lindholm et al., 2017). The UPR is able to do this due to its ability to regulate many different classes of target genes. The UPR is able to upregulate genes and functions that would increase folding, post-translational processing and degradation rates, resulting in a decrease in the concentration of unfolded proteins in the ER (Young et al., 2011, Walter and Ron, 2011). The UPR thus not only monitors activities in the ER, but also the entire secretion pathway, allowing it to detect ER events that occur due to secretory defects. The response can therefore be an upregulation of one or more steps in the secretory pathway that might prove to be rate-limiting. Nonetheless, the UPR target genes should function in ways that comprise of the physiological outputs that makes the UPR vital for the survival of ER stress (Hetz and Papa, 2017). This can be observed for one of the classes of genes that are involved in ERAD. Many of the components of ERAD that allows it to detect and prevent progression of misfolded proteins in the secretion pathway are induced by the UPR, thus making the UPR essential for ERAD to achieve its function.

1.6.5 Manipulation of *HAC1* and *KAR2* for enhanced protein secretion

Several lines of research have been focusing on ways to improve heterologous production of secreted proteins by activating the UPR (Yu et al., 2015). The capacity of the ER can be improved to handle more unfolded proteins and the capacity of the cell for heterologous protein production by co-expression of their encoding genes with the *HAC1* gene may allow for manipulation of the UPR signal pathways. Endogenous enzyme production in *S. cerevisiae* could be significantly enhanced by over-expression of the *HAC1* gene (Payne et al., 2008).

Mature *HAC1* mRNA was over-expressed for the induction of molecular chaperone production, which enhanced the secretory production of heterologous proteins such as α-amylase by 20% (Valkonen et al., 2003). It can thus be said that the efficiency of heterologous protein production was determined by the efficiency of protein folding and processing in the ER.

The overexpression of ER chaperones and foldases in *S. cerevisiae* have been tested to try and reduce the accumulation of heterologous proteins and facilitate their secretion (Harmsen et al., 1996, Zhang et al., 2006). Kar2p was overproduced and stimulated bovine prochymosin secretion 26-fold but it did not affect plant thaumatin secretion. When *KAR2* and *PDI* were coexpressed they acted synergically and were able to increase single-chain antibody fragment secretion (Shusta et al., 1998). Overexpression of *PDI*, was shown to enhance the secretion of human growth factor B by 10-fold and *Schizosaccharomyces pombe* acid phosphatase by 4-fold. However, it was not always beneficial for production of foreign protein to increase *KAR2/*BiP or *PDI*, as overexpressed *PDI* was found to cause a decrease in recombinant protein production as well as the accumulation of proteins in mammalian cells (Davis et al., 2000). Overexpressing chaperones and foldases will thus not always aid in overcoming the secretion block and these effects need to be tested on a case-by-case basis for different heterologous genes. The secreted protein's physical characteristics and adjustment of the expression level of folding factors will be important in designing a method for improved heterologous protein production by *S. cerevisiae* (Valkonen et al., 2003, Kroukamp et al., 2013).

1.6.6 ER stress and UPR detection

Most of the ER stress sensors and UPR detection methods that have been applied in the past are based on the Ire1p-mediated unconventional splicing of *HAC1* mRNA to allow production of the Hac1p transcription factor (Back et al., 2005, Leber et al., 2004). This is a sensible route to follow since Hac1p is known to induce the expression of various genes involved in the UPR to alleviate ER stress. Splicing of the yeast *HAC1* mRNA and the mammalian *XBP1* mRNA have thus become the standard markers of ER stress. The most common methods used to detect *HAC1* mRNA splicing include Northern analysis and reverse transcription-quantitative polymerase chain reaction (r.t.qPCR). The r.t.qPCR is done by using primers that are specific to

HAC1/*XBP1* mRNA and will be able to detect whether it is spliced or unspliced (Samali et al., 2010). This method is considered very cumbersome and due to its low sensitivity it is unable to detect UPR activation in live cells. Tunicamycin and DTT have been previously reported to induce *HAC1* mRNA splicing and activate the UPR (Rüegsegger et al., 2001), but r.t.qPCR was not sensitive enough to detect the splicing of *HAC1* mRNA when induced by low tunicamycin and DTT concentrations (Fang et al., 2015).

Fluorescent reporter constructs have also been developed by fusing the *HAC1/XBP1* promoter sequences to a variant of green fluorescent protein (GFP) (Pendin et al., 2016). When ER stress occurs, a frame shift will occur in the chimeric *HAC1/XBP1*-FP mRNA due to splicing, resulting in the translation of a fusion protein that will allow detection of ER stress by monitoring fluorescence. Other biosensors that were developed such as the luciferase reporter systems (Fang et al., 2015, Hikiji et al., 2015) mentioned below are more sensitive to *HAC1* mRNA splicing and thus allows for continuous and live detections of the UPR. Due to the simplicity of a biosensor reporter system, it is ideal to use this approach for UPR detection in yeast.

An example of a fluorescent construct is the ER stress-activated indicator (ERAI) that was developed by Iwawaki et al. (2004) with the aim of analyzing ER stress *in vivo* in human cells. ERAI was constructed by fusing the gene encoding venus, a variant of GFP, as a reporter downstream of the human *XBP1* (*HAC1*), as well as a 26-nt ER stress-specific intron (Figure 1.12). During ER stress, splicing of *XBP1* mRNA will result in the removal of the 26 nt intron, causing a frame shift that will produce a fusion protein of Xbp1p and venus in the cells. This will allow for detection of these cells by monitoring fluorescence activity of venus. When tested in transgenic mice, the stress indicator was found to be very specific and sensitive to ER stress and worked in an Ire1p-dependant manner.

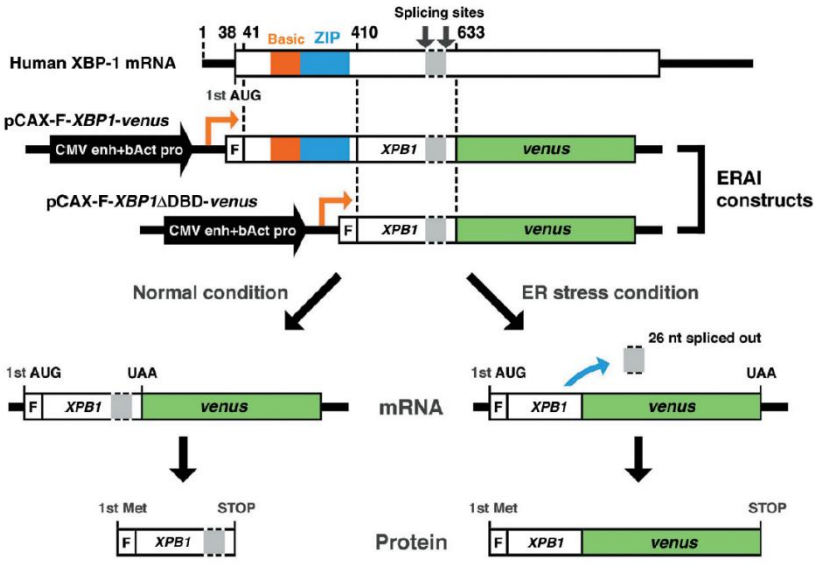

Figure 1.12: Diagram of the ER stress-activated indicator (ERAI). ERAI consists of the venus gene as a reporter downstream of the human *XBP1* (analogous to *HAC1*), as well as a 26-nt ER stress-specific intron. During ER stress, splicing of *XBP1* will result in the removal of the 26 nt intron, causing a frame shift that will produce a fusion protein of *XBP1* and venus in the cells, allowing fluorescent detection (Iwawaki et al., 2004).

A stress indicator developed by Hikiji et al. (2015) utilized NanoLuc to create an assay of Ire1p activity that was highly sensitive in human cells (figure 1.13). NanoLuc is a small luciferase that has a higher activity than firefly luciferase, which has also been used in reporter constructs for ER stress sensors in the past. The NanoLuc-based Ire1p assay system consisted of the NanoLuc gene, containing a Myc/His-epitope at the C-terminus, fused with the *XBP*1 splice region from unspliced *XBP1*. During ER stress when *XBP1* mRNA is spliced, it will result in the production of dXBP1-NanoLuc and luciferase activity can then be measured. Due to this assay being highly sensitive, it is possible, in some cases, to evaluate changes in luciferase activity continuously within a culture medium.

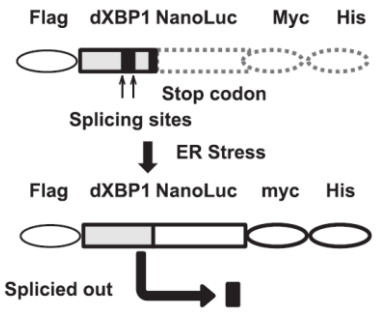

Figure 1.13: Diagram of the highly sensitive NanoLuc-based Ire1p assay system for UPR detection in human cells. The system consists of the NanoLuc gene, containing a Myc/His-epitope at the C-terminus, fused with the *XBP1* splice region from unspliced *XBP1* (Hikiji et al., 2015).

A dual-luciferase reporter gene system was developed by Fang et al. (2015) that is highly sensitive to allow UPR activation to be monitored in live cells of *S. cerevisiae* (Figure 1.14). The reporter vector consisted of a 21 bp *HAC1* 5′ UTR sequence that was placed between a Renilla luciferase gene and a firefly luciferase gene. Firefly luciferase gene translation is blocked by binding to the *HAC1* intron that is located downstream of the firefly luciferase, and it can be removed by Ire1p-mediated splicing during activation of the UPR. The activity of the two luciferases, from the same vector, can thus be used to monitor the activation of the UPR in *S. cerevisiae*. Increased sensitivity was obtained through the addition of a *GAL10* promoter to control expression of UPR reporter genes.

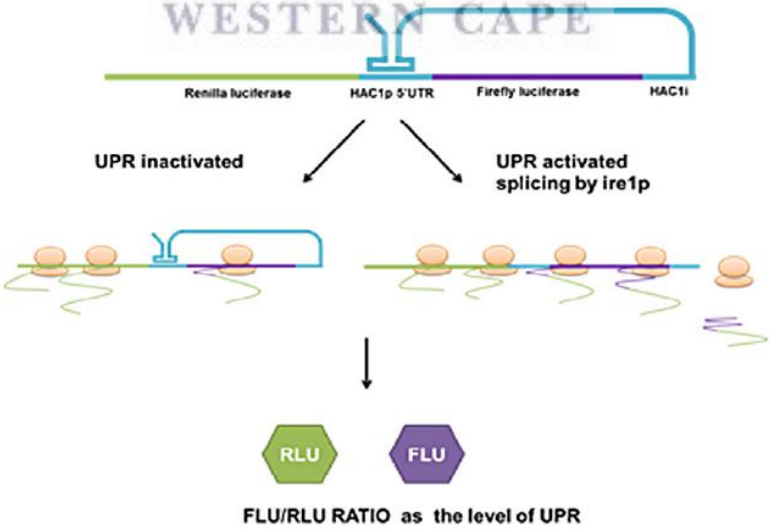

Figure 1.14: The dual luciferase reporter system for UPR detection. The system consists of a Renilla luciferase and a firefly luciferase separated by *HAC1* 5' UTR. During inactive UPR, translation of Firefly luciferase is blocked by the *HAC1* intron, which is removed during UPR activation and thus the ratio of the luciferases is used to determine level of UPR induction (Fang et al., 2015)

1.7 Objectives of this study

The UPR, as established in the above literature review, is a stress response pathway that can be induced by heterologous protein secretion, which will either lead to increased folding capacity within the ER or to degradation of these proteins and apoptosis of the cell. In order to develop superior industrial recombinant organisms producing various heterologous proteins, it would be beneficial to be able to determine when the UPR is activated by the production of these proteins and to what extent. The purpose of this study was to develop a vector based biosensor that will detect and measure the level of UPR induction in the yeast *Saccharomyces cerevisiae*. The current methods that are used to measure UPR induction through *HAC1* mRNA splicing can prove to be cumbersome and expensive. Thus the use of a reporter gene system can prove to be a simple detection test that allows for accurate measurement at a high sensitivity as observed with the dual-luciferase reporter gene system that was developed by Fang et al. (2015). This biosensor can then be used to help identify candidate cellulase genes that do not greatly induce the UPR, making them ideal to use in developing CBP yeasts. It can also help to identify gene targets for strain engineering that allow for improved secretion of heterologous proteins by lowering ER-stress. We used a low copy number centromeric vector and tested the applicability of green fluorescent protein (detected through flow cytometry) and a highly secreted xylanase with a simple assay for their applicability as a UPR biosensor. Some of the objectives included:

- Creating a reporter gene system for the detection of UPR induction through a simple assay or plate screen.
- Cloning known UPR induced gene promoters (*HAC1*p or *KAR2*p) into a vector containing reporter genes (*T.r.xyn2* or *eGFP*).
- Transforming the vectors into *S. cerevisiae* strains in which UPR induction at various levels have been confirmed.
- Testing *T.r.*xyn2 on liquid and plate assays, and eGFP using fluorescent assays.
- Testing the linearity of the biosensors at different levels of induction.

- Testing if quantitative differences are comparable with known UPR induction differences of cellulase producing *S. cerevisiae* strains.

The following chapter will cover the materials and methods that was used in this study. This will be directly followed by the results and discussion chapter.

CHAPTER 2: MATERIALS AND METHODS

2.1 Media and culturing conditions

The *S. cerevisiae* strain Y294 (ATCC201160) was the background strain for all strain derivatives used in this study. All yeast strains were cultured at 30 °C on YPD medium (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose), supplemented with 100 µg/mL of the appropriate antibiotic (Geneticin (G418) or Hygromycin B), purchased from Sigma (Darmstadt) when required for selection of transformants. Y294 transformants were screened for xylanase activity on AZCL-xylan SC^{-URA} medium plates (1.7 g/L yeast nitrogen base w/o amino acids and ammonium sulfate (Difco), 5 g/L ammonium sulfate, 20 g/L glucose, 15 g/L agar, 1 g/L AZCL-xylan (Sigma) and supplemented with required amino acids) and incubated for 24 hours at 30 °C. For activity assays and eGFP fluorescence measurements, Y294 transformants were cultured in 10 mL YPD medium (10 g/L yeast extract, 20 g/L peptone and supplemented with 2% glucose) in 100 mL flasks containing 100 µg/mL of the appropriate antibiotic for 120 hours at 30°C while shaking at 180 rpm on a rotary shaker.

2.2 Microbial strains and plasmids

Descriptions of the plasmids and yeast strains that were constructed and used in this study, are summarized in **Table 2.1** and **Table 2.2** respectively. The *S.c.*yENO1, *S.c.*BGL1, *S.c.T.r.*cbh1, *S.c.T.e.*cbh1 and *S.c.T.e.*cbh1-CCBM strains were obtained from Ilmén et al. (2011) and Den Haan et al. (2007a), and all other strains listed in the table containing the plasmids were constructed in this study.

2.3 *E. coli* and yeast transformation

E. coli XL Gold was used for plasmid propagation and was cultivated in terrific broth (TB) containing 100 µg/mL ampicillin at 37 °C overnight. Plasmid DNA was extracted using the cetyl trimethyl ammonium bromide (CTAB) method (Del Sal et al., 1988) and plasmids or ligation mixtures were added to *E. coli* competent cells before incubation on ice for 30 minutes. The cells were then heat shocked at 42 °C for 20 seconds and spread plated on Luria Bertani (LB)

medium plates (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl and 20 g/L agar) containing 100 µg/mL ampicillin.

Yeast genomic DNA was isolated using the quick yeast mini prep method (Hoffman and Winston, 1987). Yeast transformations were carried out using the LiOAc/DMSO protocol (Hill et al., 1991). Following an overnight expression step in YPD at 30 °C, transformants were plated on YPD medium plates containing the appropriate antibiotic. Total genomic DNA of the yeast transformants were extracted (Hoffman and Winston, 1987) and the successful transformation of the yeasts with the plasmids were confirmed with PCR analysis using primers listed in **Table 2.3** with New England Biolabs Taq polymerase as recommended by the manufacturer.

Table 2.1: Plasmids used in this study.

Plasmids	Description	Source
pRDH182	*$ENO1_p$*-Trichoderma reesei xyn2 (xynIIA) (native)-$ENO1_T$, Amp*	(Brevnova et al., 2011)
pHK211	*$PGK1_p$-eGFP- $CYC1_T$kanMX*	H. Kroukamp
pHK212	*$PGK1_p$-eGFP- $CYC1_T$hph*MX	H. Kroukamp
pGC1 (pHK211- *HAC1p-xyn2*)	*$HAC1_p$-xyn2- $CYC1_T$kanMX*	This study
pGC2 (pHK211-*KAR2p-xyn2*)	*$KAR2_p$-xyn2- $CYC1_T$kanMX*	This study
pGC3 (pHK212-*HAC1p-xyn2*)	*$HAC1_p$-xyn2- $CYC1_T$hph*MX	This study
pGC4 (pHK212-*KAR2p-xyn2*)	*$KAR2_p$-xyn2- $CYC1_T$hph*MX	This study
pGC5 (pHK211-*HAC1p-eGFP*)	*$HAC1_p$-eGFP- $CYC1_T$kanMX*	This study
pGC6 (pHK211-*KAR2p-eGFP*)	*$KAR2_p$-eGFP- $CYC1_T$kanMX*	This study

*Subscript P denotes promoter and subscription T denotes terminator.

Table 2.2: *S. cerevisiae* strains used in this study.

Strain name	Abbreviation used	Description
***S. cerevisiae* Y294 yENO1**	*S.c.*yENO1	*S. cer. fur1::LEU2 with $ENO1_P$- $ENO1_T$ (Reference strain)*
***S. cerevisiae* Y294 BGL1**	*S.c.*BGL1	*S. cer. fur1::LEU2 with $PGK1_P$-Saccharomycopsis fibuligera bgl1* (*cel3A*)-$PGK1_T$
***S. cerevisiae* Y294 *T.r.*cbh1**	*S.c.T.r.*cbh1	*S. cer. fur1::LEU2 with $ENO1_P$-Trichoderma reesei cbh1 (T.r.cbh1)-$ENO1_T$*
***S. cerevisiae* Y294 *T.e.*cbh1**	*S.c.T.e.*cbh1	*S. cer. fur1::LEU2 with $ENO1_P$-Talaromyces emersonii cbh1 (T.e.cbh1)-$ENO1_T$*
***S.cerevisiae* Y294 *T.e.*cbh1-CCBM**	*S.c.T.e.*cbh1-CCBM	*S. cer. fur1::LEU2 with $ENO1_P$-Talaromyces emersonii cbh1-CCBM (T.e.cbh1)-$ENO1_T$*
***S. cerevisiae* Y294 yENO1 with pGC1**	*S.c.*yENO1 pGC1	*S. cer. fur1::LEU2 with $ENO1_P$- $ENO1_T$ with pGC1**
***S. cerevisiae* Y294 yENO1 with pGC2**	*S.c.*yENO1 pGC2	*S. cer. fur1::LEU2 with $ENO1_P$- $ENO1_T$ with pGC2*
***S. cerevisiae* Y294 yENO1 with pGC3**	*S.c.*yENO1 pGC3	*S. cer. fur1::LEU2 with $ENO1_P$- $ENO1_T$ with pGC3*
***S. cerevisiae* Y294 yENO1 with pGC4**	*S.c.*yENO1 pGC4	*S. cer. fur1::LEU2 with $ENO1_P$- $ENO1_T$ with pGC4*
***S. cerevisiae* Y294 yENO1 with pGC5**	*S.c.*yENO1 pGC5	*S. cer. fur1::LEU2 with $ENO1_P$- $ENO1_T$ with pGC5*
***S. cerevisiae* Y294 yENO1 with pGC6**	*S.c.*yENO1 pGC6	*S. cer. fur1::LEU2 with $ENO1_P$- $ENO1_T$ with pGC6*
***S. cerevisiae* Y294 BGL1 with pGC2**	*S.c.*BGL1 pGC2	*S. cer. fur1::LEU2 with $PGK1_P$-S.f.bgl1* (*cel3A*)-$PGK1_T$ *with pGC2*
***S. cerevisiae* Y294 BGL1 with pGC6**	*S.c.*BGL1 pGC6	*S. cer. fur1::LEU2 with $PGK1_P$-S.f.bgl1* (*cel3A*)-$PGK1_T$ *with pGC6*
***S. cerevisiae* Y294 *T.r.*cbh1 with pGC2**	*S.c.T.r.*cbh1 pGC2	*S. cer. fur1::LEU2 with $ENO1_P$-T.r.cbh1 (T.r.cbh1)-$ENO1_T$ with pGC2*
***S. cerevisiae* Y294 *T.r.*cbh1 with pGC6**	*S.c.T.r.*cbh1 pGC6	*S. cer. fur1::LEU2 with $ENO1_P$-T.r.cbh1 (T.r.cbh1)-$ENO1_T$ with pGC6*
***S. cerevisiae* Y294**	*S.c.T.e.*cbh1 pGC2	*S. cer. fur1::LEU2 with $ENO1_P$-T.e.cbh1 (T.e.cbh1)-$ENO1_T$*

***T.e*.cbh1 with pGC2**		*with pGC2*
***S. cerevisiae* Y294 *T.e*.cbh1 with pGC6**	*S.c.T.e*.cbh1 pGC6	*S. cer. fur1::LEU2 with $ENO1_p$-T.e.cbh1 (T.e.cbh1)-$ENO1_T$ with pGC6*
***S. cerevisiae* Y294 *T.e*.cbh1-CCBM with pGC2**	*S.c.T.e*.cbh1-CCBM pGC2	*S. cer. fur1::LEU2 with $ENO1_p$-T.e.cbh1-CCBM (T.e.cbh1)-$ENO1_T$ with pGC2*
***S. cerevisiae* Y294 *T.e*.cbh1-CCBM with pGC4**	*S.c.T.e*.cbh1-CCBM pGC4	*S. cer. fur1::LEU2 with $ENO1_p$-T.e.cbh1-CCBM (T.e.cbh1)-$ENO1_T$ with pGC4*
***S. cerevisiae* Y294 *T.e*.cbh1-CCBM with pGC6**	*S.c.T.e*.cbh1-CCBM pGC6	*S. cer. fur1::LEU2 with $ENO1_p$-T.e.cbh1-CCBM (T.e.cbh1)-$ENO1_T$ with pGC6*

*See table 2.1 for descriptions of the plasmid components.

2.4 UPR biosensor construction

DNA manipulations were performed using standard protocols (Sambrook and Russell, 2001). Restriction endonuclease-digested DNA was eluted from 1% agarose gels with the freeze and squeeze method (Sambrook and Russell, 2001). Restriction endonucleases and T4 DNA ligase were purchased from ThermoScientific (Waltham, MA USA) and used as recommended by manufacturer. The Phusion DNA polymerase that was used for PCR was also purchased from ThermoScientific and used as recommended by manufacturer with an Applied Biosystems 2720 Thermal cycler. The primers used in this study are listed in **Table 2.3**. The CloneJET PCR Cloning Kit that was used for initial cloning of putative promoters was purchased from ThermoFisher and used as recommended by the manufacturer.

Table 2.3: Primers used in this study. Restriction sites are shown in bold.

Primer	Oligonucleotide sequence (5'- 3')	Restriction sites
HAC1p-L	ATGC**GAATTC**CCTCTTTCATAGCCTGAGATCCG	EcoRI
HAC1p-R	ATGC**TTAATTAA**AGTGGCGGTTGTTGTCGTAGG	PacI
KAR2p-L	ATGC**GAATTC**TTACATAAAAGTATCTCAGCAGCATTATTC	EcoRI
KAR2p-R	ATGC**TTAATTAA**GGTATGTTTGATACGCTTTTTCCC	PacI
XYN2-L	GATC**TTAATTAA**AATGGTCTCCTTCACCTCC	PacI

XYN2-R	GTAC**GGCGCGCC**CTCCCTTTAGCTGACGGTG	AscI
eGFP-L	ATGGTTTCATTTACATCTTTATTAGCCG	
eGFP-R	TTATTTGTACAATTCATCCATACCATGG	

The UPR biosensors that were developed in this study consisted of the putative promoters of the *HAC1* and *KAR2* UPR genes as predicted by Yeastract software (www.yeastract.com). The respective putative UPR promoters were amplified from the extracted genomic DNA of the *S. cerevisiae* 288C strain by PCR using primers HAC1p-L and HAC1p-R, or KAR2p-L and KAR2p-R primers. The endonuclease restriction sites for EcoRI (GAATTC) and PacI (TTAATTAA) were included at the 5'-ends of the left and right primers respectively. The putative UPR promoter fragments (*HAC1*p ~600 bp and *KAR2*p ~800 bp) were eluted from a 1% agarose gel and cloned into a pJET1.2 cloning vector using the CloneJET PCR Cloning Kit. The ligation mixture was then transformed into *E. coli* XL Gold heat shock competent cells. The promoters were removed from the pJET1.2 cloning vector, after confirming that the ligation was successful, using the EcoRI and PacI restriction endonucleases and cloned into the EcoRI and PacI sites of the pHK211, to create plasmids pGC5 and pGC6 (donated by Dr. Heinrich Kroukamp, Macquarie University, Australia) (Table 2.1). The resulting plasmids contain a codon optimized green fluorescent protein (*eGFP*) reporter gene downstream of the putative UPR promoters (Figure 3.1). To create the UPR biosensors with the *Trichoderma reesei* xyn2 (*T.r.xyn2*) reporter gene, pRDH182 plasmid DNA was extracted and *T.r.xyn2* was amplified using PCR with primers XYN-L and XYN2-R (Table 2.3). The endonuclease restriction sites for PacI (TTAATTAA) and AscI (GGCGCGCC) were included at the 5'-ends of the left and right primers, respectively. The ~700 base pair (bp) *T.r.xyn2* amplicon was eluted after agarose gel electrophoresis and ligated into the PacI and AscI sites of the pHK211 and pHK212 plasmids downstream of the putative UPR promoters, replacing the eGFP reporter genes with *T.r.xyn2*. Subsequently the promoters in those plasmids were replaced with EcoRI/ PacI fragments of *Hac1*p or *Kar2*p in order to create plasmids pGC1, pGC2, pGC3 and pGC4 (Figure 3.1). These reporter genes will allow for the direct measurement of the promoter activation in response to UPR stress, through increased fluorescence or xylanase secretion. Successful plasmid construction was confirmed using

restriction enzyme digestion and sequences were confirmed using Sanger sequencing (Central Analytical Facility, Stellenbosch). These tests showed that plasmid construction was correct as planned (not shown).

2.5 Enzyme activity assays

The *S. cerevisiae* Y294 transformants with the pGC1, pGC2, pGC3 and pGC4 UPR biosensors (Table 2.2) were initially screened on AZCL-xylan plates to confirm xylanase activity. These transformants were then cultured in 100 mL flasks containing 10 mL YPD growth media and 100 µg/mL of appropriate antibiotic (G418 or Hygromycin B). To evaluate tunicamycin (Tm) response, required volumes of a 1 mg/mL Tm stock was used to obtain 0.2, 0.5 and 2 µg/mL Tm. Xylanase activity in the culture supernatant was measured using 1% (w/v) Beechwood xylan (Sigma) resuspended in 0.05 M sodium acetate buffer (pH 5) as previously described (La Grange et al., 2001). Samples were diluted as required to determine the optical density (OD_{600nm}) of the cultures using a LKB ULTROSPEC II Spectrophotometer. Dry cell weight (DCW) was estimated from OD_{600nm} readings and used to normalize the volumetric values of the yeast cultures (Van Zyl et al., 2014). Xylanase activities were determined as units/g DCW in which one enzyme unit (U) was defined as the amount of enzyme needed to release one µmol of reducing sugar equivalent per minute. All assays were performed in triplicate and measured at 48, 72, 96 and 120 hours, except for the Tm test where activity was measured at 0, 6, 24 and 48 hours.

2.6 Fluorescence microscopy

The *S. cerevisiae* Y294 transformants with the pGC5 and pGC6 UPR biosensors (Table 2.2) and the appropriate control strains were initially screened though fluorescence microscopy using a Zeiss Axioplan 2 imaging fluorescence microscope to confirm *eGFP* expression using excitation and emission of 495 nm and 530 nm, respectively. Fluorescence images were captured using a Zeiss AxioCam HRm camera and the AxioVision software (Zeiss) was used to create false colour (Green) images.

2.7 Flow cytometry

S. cerevisiae Y294 transformants were cultured in 100 mL flasks containing 10 mL YPD growth media and 100 µg/mL G418. For Tm tests, concentrations were added in the same way as described in the enzyme activity assays above. Relative eGFP fluorescence was measured with a BD FACS Aria III flow cytometer using the 100 µM nozzle and BD sheath fluid. The machine was calibrated using CS&T beads as recommended by manufacturer (BD Biosiences). The FITC channel (488nm) was used for measuring GFP fluorescence signals. The number of events recorded for each sample was 500 000. The software used for collecting data was FACS Diva version 8.0.2 (BD Biosciences) and files were exported as FCS 3.1. The fluorescence of all strains were measured in triplicates. Florescence intensities of the eGFP were measure at 24, 48 and 72 hours, except for the Tm test in which it was recorded at 0, 24 and 48 hours. The mean fluorescence intensities (MFI) were determined using Flowjo software version 10 (www.flowjo.com) and all values were normalized by dividing arbitrary fluorescence units by lowest MFI obtained for each result to determine relative fluorescence levels.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Construction of UPR biosensors and yeast strains

The two plasmids used to create the UPR biosensors, pHK211 and pHK212 (Figure 3.1 A and D), are low copy number centromeric plasmids that contain the *kanMX* (G418 resistance) or *hphMX* (hygromycin B resistance) selection markers, respectively. The plasmids contain the *eGFP* reporter under the transcriptional control of the constitutive *S. cerevisiae PGK1* promoter. The UPR biosensors were developed by removing the *PGK1* promoters through restriction enzyme digestions and cloning the putative UPR promoters *HAC1*p or *KAR2*p in its place, upstream of the *eGFP* reporter genes to create plasmids pGC5 and pGC6 (Figure 3.1 C). The pGC1, pGC2, pGC3 and pGC4 UPR biosensors consisted of the *T.r.xyn2* reporter gene cloned downstream of the putative UPR promoters, in place of the *eGFP* reporter gene (Figure 3.1 B and E). Plasmid constructions were confirmed through restriction enzyme digests and sequences were confirmed using Sanger sequencing (results not shown). The UPR biosensors were subsequently transformed into *S. cerevisiae* Y294 strains (Table 2.2). Transformants were confirmed through PCR analysis (data not shown) using putative UPR promoter and reporter gene primers (Table 2.3). The *S. cerevisiae* yENO1 reference strain was the strain most often used in this study as it was the strain in which the UPR was uninduced and was thus used to compare UPR induction levels of recombinant cellullase secreting *S. cerevisiae* Y294 strains. The *S. cerevisiae* Y294 CBH and BGL producing strains were previously described by Ilmén et al. (2011) as well as Van Zyl et al. (2014), and Van Zyl et al. (2016) respectively.

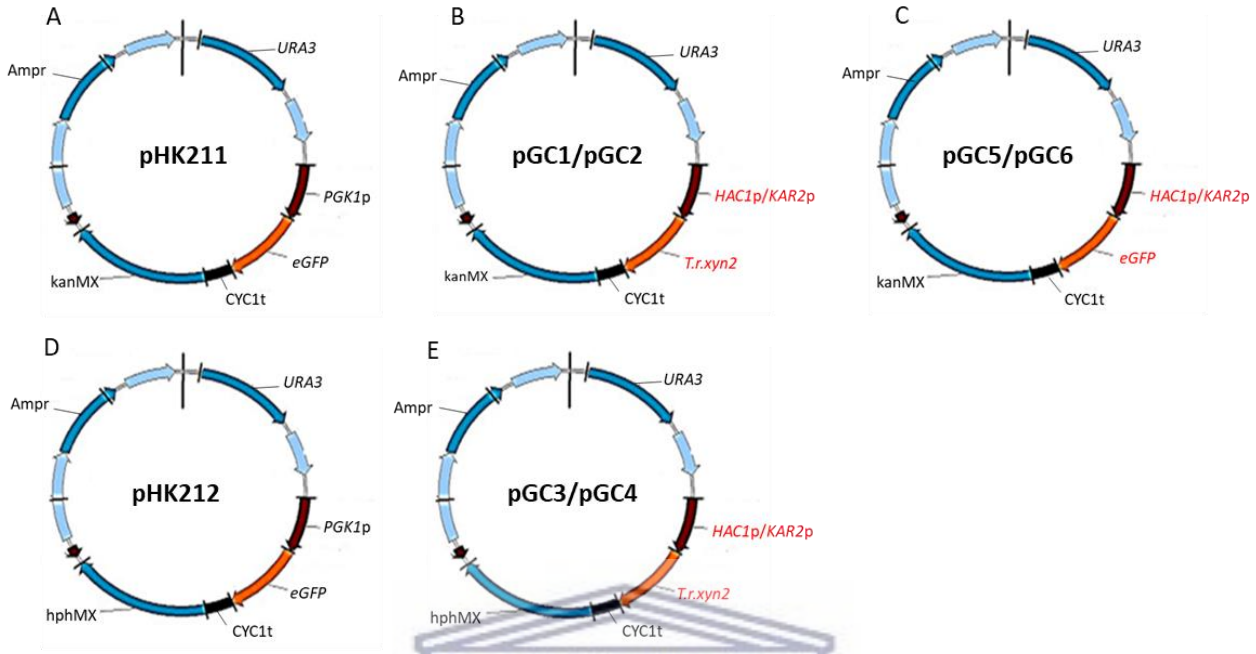

Figure 3.1: Schematic representation of the plasmids used in this study. A) The base pHK211 vector that was used to clone the desired promoters and genes. B) The pGC1 biosensor plasmid with *HAC1* promoter or pGC2 with *KAR2* promoter, with both containing the *T.r.xyn2* reporter gene and *kanMX* selection marker. C) The pGC5 biosensor plasmid with *HAC1* promoter or pGC6 with *KAR2* promoter, both containing the *eGFP* reporter gene and *kanMX* selection marker. D) The base pHK212 vector that was used to clone the desired promoters and genes when hygromycin resistance was required. E) The pGC3 biosensor plasmid with *HAC1* promoter or pGC4 with *KAR2* promoter, both containing the *T.r.xyn2* reporter gene and *hphMX* selection marker.

The biosensors constructed in this study differ in promoter (*HAC*1p and *KAR2*p), reporter gene (*T.r.xyn2* and *eGFP*) and selection marker (Geneticin G418 and Hygromycin B) (Figure 3.1). When cells display activation of the components of the UPR, they are generally described as undergoing ER stress and two inherent factors of the response are the splicing of *HAC1* mRNA and upregulation of Kar2p protein production (Kimata and Kohno, 2011). *HAC1* and *KAR2* are thus important regulators of the UPR as their activation allows the UPR to achieve its function and the promoters of these genes are thus ideal for developing a biosensor. The UPR upregulates *KAR2*p for the production of the ER chaperone BiP in order to enhance secretory protein folding (Lajoie et al., 2012). Similarly, it is the production of the Hac1p transcription

factor by expression of the spliced *HAC1* mRNA that is responsible for the upregulation of many genes involved in the UPR, including *KAR2* and *HAC1* itself, that increase the cell's folding capacity and allow the UPR to achieve its function of assisting cells with correct folding of proteins during ER stress (Hetz, 2012, Guerfal et al., 2010). *HAC1*p and *KAR2*p levels will thus always be overexpressed when ER stress occurs.

The reporters for the UPR biosensor, *T.r.xyn2* and *eGFP*, were selected due to the minimal effect that they had on the yeast cells and their ability to be easily be assayed. *T.r.*Xyn2 was selected due to it being a relatively small, easily secreted protein that is easy to assay (La Grange et al., 2001). The GFP variant that was used in this study is known for its fluorophore that can spontaneously form intracellularly without requiring cofactors and thus provides emitted fluorescence intensities that are a direct representation of the GFP expression (Ducrest et al., 2002). The GFP was codon optimized for *S. cerevisiae*. A previous study by Kaishima et al. (2016) comparing codon-optimized GFPs with those not codon-optimized found that the codon optimized eGFP had a 22 fold higher MFI in *S. cerevisiae*. GFP fluorescence or xylanase activity in the culture supernantant, was thus measured using flow cytometry or DNS assays, respectively, and the results were observed as a measure of the level of expression of the putative UPR promoters. A UPR sensor developed by Lajoie et al. (2012) consisting of GFP-tagged KAR2/BiP was found to express fluorescence levels that directly correlated with UPR activity and the GFP was thus found to be a robust reporter when doing high throughput analysis. Ducrest et al. (2002) compared a GFP and luciferase reporter system and showed that the GFP system was as sensitive as an enzyme-based system but more advantageous as its expression could be quantified within single cells by flow cytometry.

3.2 Screening of *S. cerevisiae* strains carrying the UPR biosensors

One of the aims of the biosensor that was developed in this study was to be able to indicate whether the UPR was active through screening of the respective reporter genes using a simple screening method. When the UPR is active in the cell, the *HAC1* and *KAR2* gene promoters are able to sense that and express the reporter genes producing either xylanase (*T.r.xyn2*) or GFP fluorescence (*eGFP*). Xylanase can be detected on AZCL-xylan containing SC^{-URA} medium plates

(Guo et al., 2017). These yeast strains were able to grow on the SC^{-URA} medium plates due the presence of the *URA3* selection marker on the plasmids used for the biosensors. AZCL xylan plates provides a good initial plate screen to determine if the UPR is active in the cell by means of indicating the presence of *T.r.*Xyn2, which can be observed by blue halos around the colonies (Figure 3.2). Positive transformants containing the pGC1, pGC2, pGC3 and pGC4 biosensor plasmids were spot plated on AZCL-xylan containing YPD medium plates and grown overnight at 30 °C before analysis.

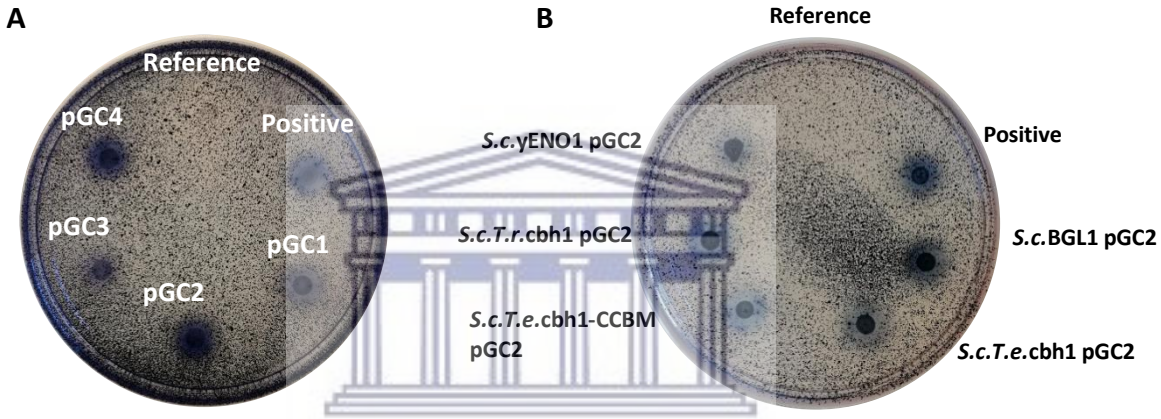

Figure 3.2: Yeast strains cultivated on 0.2% AZCL Birchwood - Xylan SC^{-URA} media plates. The plates confirm the presence of *T.r.xyn2* in the yeast transformants by the presence of the halo around the colonies. A) Screening of pGC1, pGC2, pGC3 and pGC4 in the control *S. cerevisiae* Y294 yENO1 reference strain. B) Screening of cellulase producing Y294 strains containing the pGC2 plasmid. Plates were incubated at 30 °C for 24 hours.

The AZCL-xylan plates (figure 3.2) confirmed xylanase activity for the strains indicated by the formation of dark zones around the colonies. It is clear by the zones that were obtained, that xylanase was present in each of the *S.c.*yENO1 reference strains containing the biosensors (Figure 3.2 A). This would indicate that the *HAC1*p and *KAR2*p was expressed at basal levels during uninduced conditions. Similarly, zones were observed for each of the cellulase producing *S. cerevisiae* Y294 strains that contained the UPR biosensor pGC2 (Figure 3.2 B). No significant differences between the zones were observed that would allow for quantification of the level of expression of these genes, but it can be used as a way of readily revealing the

presence of xylanase as shown in a previous study by Guo et al. (2017) confirming successful transformation.

Initial detection for the presence of the *eGFP* reporter was done through fluorescence microscopy (Kaishima et al., 2016). Colonies of the positive transformants containing the pGC5 or pGC6 biosensors were submerged in buffer on a microscopic slide and viewed using a Zeiss Axioplan 2 imaging fluorescent microscope and the images were captured using a Zeiss AxioCam HRm camera (Figure 3.3).

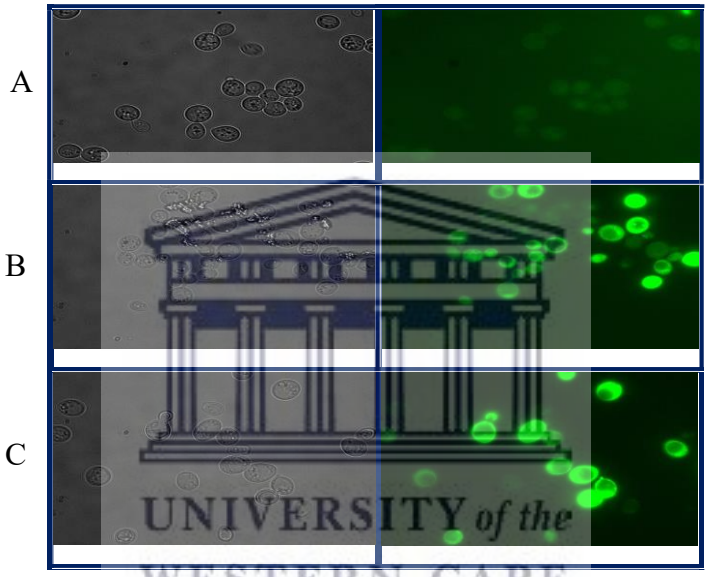

Figure 3.3: Zeiss Axioplan 2 imaging fluorescent microscope images using Zeiss AxioCam HRm camera. A) Parental Y294 control strain without a plasmid containing the eGFP gene. B) Strain containing the pGC5 plasmid. C) Strain containing the pGC6 plasmid.

The strains containing the two biosensors both showed fluorescence when viewed under the fluorescence microscope (Figure 3.3 B and C). The strains were compared to an untransformed *S.c.*yENO1 reference strain, which showed that Y294 had low background fluorescence (Figure 3.3 A). Fluorescence microscopy was thus able to show that the *eGFP* reporter gene was expressed but does not provide information on the amount of fluorescing cells or a way of quantifying the intensity of fluorescence and does thus not provide a means of knowing the level of UPR present in the cells. A study by Kaishima et al. (2016) comparing the expression of

various GFPs in *S. cerevisiae*, used fluorescence microscopy as an initial screen to compare the fluorescence intensities between the GFPs. However, this method is not appropriate to differentiate between levels produced by the same variant of GFP using different promoters.

Although screening of the reporter genes used in this study could be done using basic methods such as the AZCl-xylan plate screen and fluorescence microscopy, determining the relative level of UPR induction will require more complex quantitative assays. However, these screening methods provide an adequate initial screen to know if the biosensor is present in the yeast transformants and switched on.

3.3 Determination of best promoter and selection marker combination

In order to select the best combination of promoters, selectable markers and reporter proteins for the UPR biosensor, these components had to be directly compared. The differences between the two selection markers yielding resistance to G418 and hygromycin, was tested as well as the amount of background activity observed for either reporter in the control strains for *HAC1*p and *KAR2*p. It is important for the promoter in the UPR biosensor to have little or no background activity in uninduced conditions, as that will provide a better depiction of the level of UPR induction and allow for more accurate measurement of the stress response pathway. The *S.c.*yENO1 strain was used for comparison of the two promoters due to the strain not expressing any heterologous genes, despite carrying a reference plasmid and therefore providing an uninduced environment. Transformant strains carrying biosensor plasmids with different promoters and selection markers were cultivated under the same conditions for a period of 48 hours after which DNS assays were performed in 24 hour intervals up to 120 hours.

Higher xylanase activities per gram dry cell weight were observed in the control strain with the *HAC1*p containing biosensor (Figure 3.4 A). As this was observed in uninduced conditions, it thus shows that *HAC1*p is active in yeast cells at a higher basal level compared to *KAR2*p. *HAC1*p is known to be constitutively expressed at low levels in *S. cerevisiae* and studies have shown the effect that this has on heterologous secreted proteins (Guerfal et al., 2010). It would

thus be more ideal to measure the *HAC1* mRNA splicing event when using *HAC1* to measure the level of UPR induction as was reported previously (Dragosits et al., 2010). The strain expressing *T.r.xyn2* under the *KAR2*p yielded much lower xylanase activity (Figure 3.4 A). Since this was in an uninduced state, the lower levels of background xylanase expression suggests that *KAR2*p is a good candidate for the biosensor. Since *KAR2*p is a stress sensor in the UPR pathway, it is expected to be highly active when there is an accumulation of unfolded proteins such as those caused by heterologous protein expression in yeast (Ilmén et al., 2011). *KAR2*p was previously found to be expressed in high levels when yeasts were exposed to chemical agents that interfere with folding as well as in cells overexpressing heterologous proteins that induce the UPR (Guerfal et al., 2010), and thus this result is a good representation of the uninduced control strain. In addition, we observed that there was no significant increase in xylanase production between 48 hours and 120 hours.

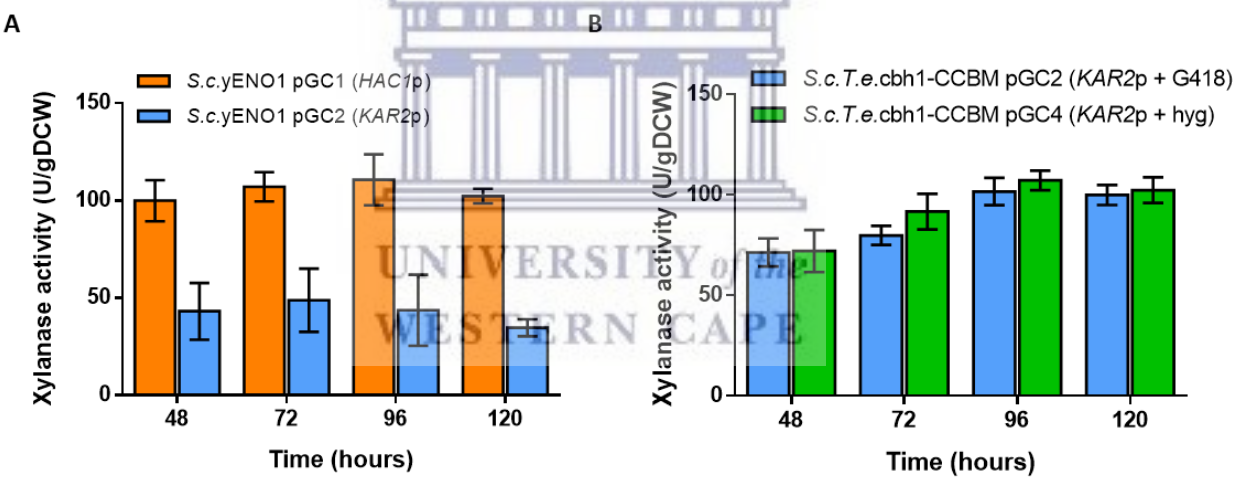

Figure 3.4: Comparison of the influence of promoters and selection markers used in this study. A) Xylanase activity of the control strains containing *HAC1*p and *KAR2*p were compared for determination of the best promoter for the UPR biosensor. B) Xylanase activity of CBH producing strains containing plasmids with different selection markers were compared for determination of best selection marker for the UPR biosensor. The activities represent the means of biological triplicates and error bars represent the standard deviation.

The selection marker is an important part of the biosensor as this will be used to select positive transformants during transformation (Bhalla et al., 2016). It is thus important that the selection

marker does not have a negative effect on the strain, UPR promoter or reporter gene and therefore does not affect the activity levels. However, it would be useful to create biosensor plasmids with a variety of selectable markers to compensate for different selectable markers already used to create the recombinant strains whose UPR induction we might want to determine. The comparison between the selection markers was done in a *S. cerevisiae* Y294 strain with CBH induction of the UPR (*T.e*.cbh1-CCBM producing strain) (Ilmén et al., 2011) containing the biosensor under control of the same promoter (*KAR2*p). The strains containing the biosensors were cultivated in YPD media that contained the corresponding selection marker and grown for a period of 120 hours. There were no significant differences between the xylanase activities per gram dry cell weight of the strains with the different selection markers that were used (figure 3.4 B) as determined by a two-tailed T-test assuming unequal variance. The xylanase activity followed a similar trend under both selections. The selection markers thus did not have an effect on the performance of the biosensors or the growth of the strains. The pGC2 (*KAR2*p with G418) biosensor was then selected for subsequent studies with the xylanase reporter protein.

3.4 Testing the dynamic range of the UPR biosensors with Tunicamycin

It was subsequently important to test the sensitivity and range of *KAR2*p in the biosensor as this will be an indication of how the biosensor will react when subjected to various levels of stress. The UPR can be activated through various chemicals that are able to induce ER stress, such as tunicamycin (Tm) and dithiothreitol (DTT) (Oslowski and Urano, 2011, Iwata et al., 2016). Tm functions by inhibiting the initial glycoprotein biosynthesis in the ER, causing an accumulation of unfolded glycoproteins which will result in ER stress. Although Tm treatment can cause cell death, a previous study by Dungeon et al. (2008) showed that this only occurs in cells that were grown in low osmolyte YPD media and not synthetic media. However it is still an ideal way to test the sensitivity range of the biosensors. The pGC2 (*KAR2p* with *T.r.xyn2*) and pGC6 (*KAR2p* with *eGFP*) biosensor containing strains were thus subjected to Tm in the uninduced conditions of the control strain to determine the sensitivity of the reporter genes to various levels of stress. There is no set concentration and duration for Tm treatment that will

induce ER stress as each system is different and should thus be determined independently (Oslowski and Urano, 2011). Therefore, we created a Tm gradient of 0, 0.2, 0.5, and 2 µg/mL for both xylanase activity assays and *eGFP* fluorescence assays (Figure 3.5). The relevant strains were grown in YPD media supplemented with Tm for a period of 48 hours.

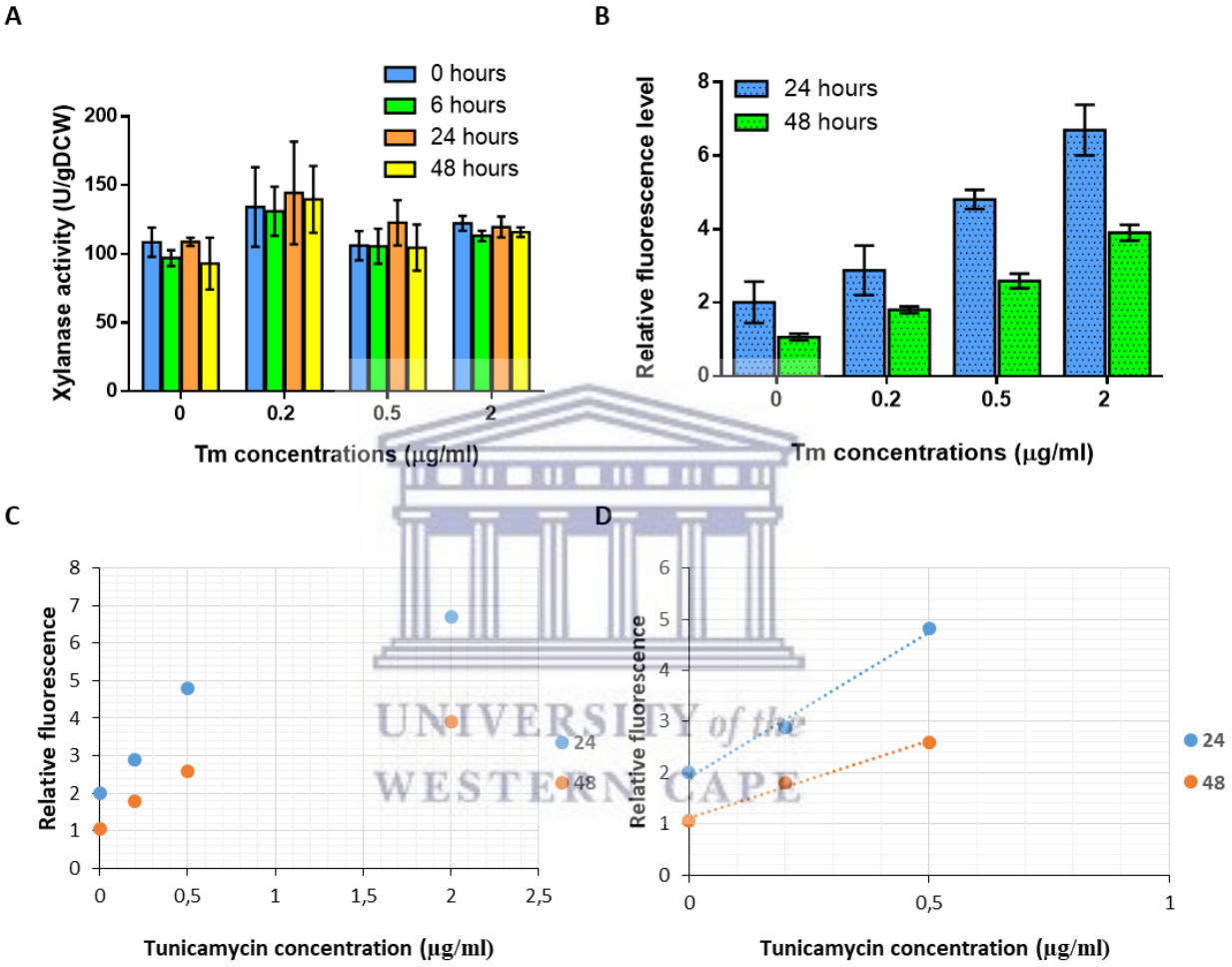

Figure 3.5: Xylanase activity and relative fluorescence levels of biosensors induced by Tunicamycin in S.c.yENO1 pGC2 and S.c.yENO1 pGC6 strains. A) Xylanase activity was measured at 0, 6, 24 and 48 hours and induced with 0.2, 0.5 or 2 µg/mL Tm. The activities represent the means of three biological repeats. B) Relative fluorescence levels were measured at 0, 24 and 48 hours and induced with 0.2, 0.5 and 2 µg/mL Tm. C) Relative fluorescence of the eGFP response against the increased Tm concentrations. D) Relative fluorescence against the increased Tm concentrations shows the linearity of the eGFP response over 0, 0.2 and 0.5 µg/mL Tm. Arbitrary fluorescence values were normalized against the lowest obtained fluorescence value (0 Tm, 48 hours). Error bars for both graphs represent the standard deviation.

No significant differences were observed across the Tm concentrations used for the pGC2 (*KAR2*p-*xyn2*) biosensor (Figure 3.5 A). All activity levels obtained were comparable to the uninduced (0 μg/mL Tm induction) *S.c*.yENO1 strain, which would indicate that the concentrations of Tm used did not have a notable effect on the activity. These quantitative xylanase results was similar to the AZCL-xylan plate screen results as it depicted more what the plate screen already showed, namely a notable background activity. However, the pGC6 biosensor displayed an increase in the relative eGFP fluorescent levels with an increase in Tm concentration after 24 hours (Figure 3.5 B). A notable linearity was observed for the first 0.5 μg/mL at the different Tm concentrations at the low end of the scale (Figure 3.5 C and D). The results also indicated that the best eGFP expression measurement can be made after 24 hours, which is most likely due to the short half-life of the eGFP. The GFP has previously been shown to have a half-life of approximately 8 hours (Pincus et al., 2010) and thus the rapid decrease we observed likely indicates that there was no more accumulation of GFP. The eGFP levels that were observed after 48 hours still displayed linearity in terms of the Tm level but the relative fluorescence that was observed had decreased by 50%. This result also indicates the sensitivity of detecting fluorescence using flow cytometry for determining fluorescence levels compared to the DNS assay for measuring the xylanase activity. Tm concentrations of 2.5 – 5 μg/mL are usually sufficient for induction of the UPR in most cell types (Oslowski and Urano, 2011). Based on this, the observation that an increase in the eGFP fluorescent levels were detected from a concentration of 0.2 μg/mL, indicates the sensitivity of the eGFP used in the pGC6 biosensor. Since the xylanase does not seem to be a good indicator of the range of levels of UPR induction that the cells may be experiencing, it makes the *eGFP* the better reporter gene choice for measuring the UPR as it was shown to be more sensitive. However, this observation could be due to various factors and therefore these biosensors should be tested in recombinant yeast strains, in which the UPR was previously shown to be induced by heterologous cellulase expression (Ilmén et al., 2011), for further analysis.

3.5 UPR induction by cellulases

The purpose of developing this UPR biosensor was to be able to determine when the UPR is active within yeast, as well as being able to measure the level of UPR that is being induced by heterologous protein expression. In *S. cerevisiae*, inefficient secretion has always been one of the main factors that inhibit efficient heterologous production of cellulases when developing yeast strains that are capable of utilizing lignocellulosic biomass for bioethanol production, which can be caused by a decrease in the rate of folding (Young et al., 2011). This is known to cause unfolded proteins to accumulate and thus induce the UPR (Yu et al., 2015, Ilmén et al., 2011). Therefore, it will be advantageous to know the level to which heterologous cellulases are inducing the UPR in *S. cerevisiae* as this will allow for development of more robust recombinant *S. cerevisiae* strains for CBP. In this way the biosensor can help us identify heterologous gene candidates more suitable to construct CBP yeast strains. The pGC2 and pGC6 biosensor plasmids were transformed into recombinant cellulase secreting *S. cerevisiae* Y294 strains with the purpose of measuring the level of UPR (Table 2.2) (Ilmén et al., 2011, Van Zyl et al., 2014, Van Zyl et al., 2016). These strains were compared to the *S.c.*yENO1 reference strain. The UPR induction data of the strains used in this study are known through previous RNA measurements (Ilmén et al., 2011, Van Zyl et al., 2014, Van Zyl et al., 2016). This result would thus be a comparison of the biosensors to the RNA data of these strains, which will allow for comparison of the UPR biosensors created in this study to the currently used RNA methods for determining UPR induction. For the purpose of this study, we tested heterologous BGL and CBH induction of the UPR. DNS-based xylanase assays were performed for a period of 120 hours and fluorescence levels were measured using flow cytometry for a period of 72 hours due to the short half-life of eGFP.

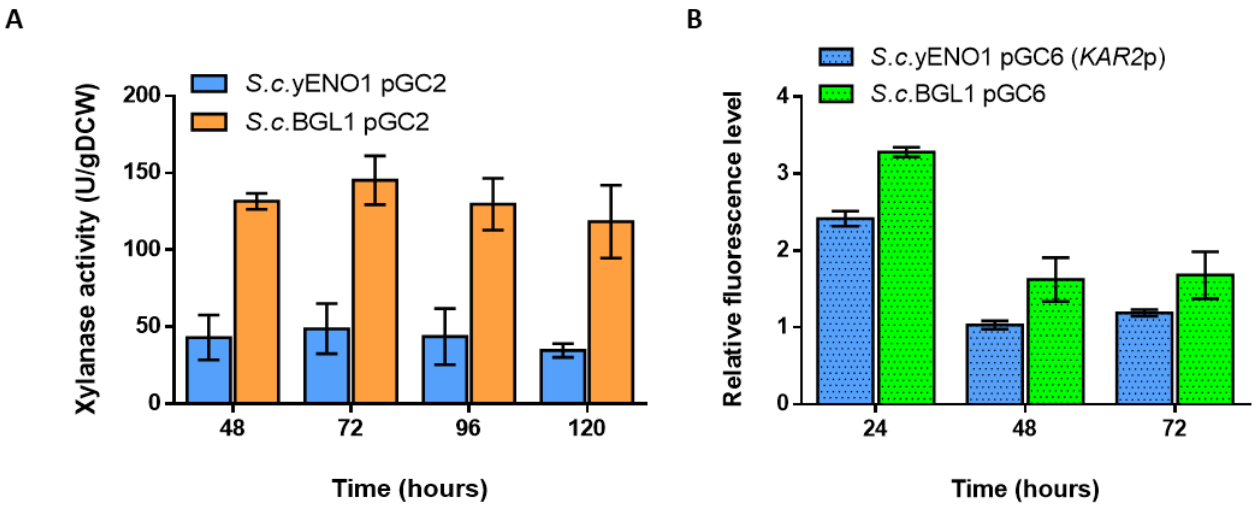

Figure 3.6: Induction of UPR by BGL expression in *S. cerevisiae* Y294 transformants. A) Comparison of the xylanase activity of a *S. cerevisiae* Y294 yENO1 strain and the BGL1 producing strain containing the pGC2 plasmid. The activities represent the means of biological triplicates. B) Relative fluorescence levels of the *S. cerevisiae* Y294 yENO1 reference strain and the BGL1 producing strain containing the pGC6 plasmid. Arbitrary fluorescence values were normalized against the lowest obtained fluorescence value (*S.c.*yENO1 pGC6, 48 hours). Error bars for both graphs represent the standard deviation of three biological repeats.

An appropriately 3-fold increase was observed for the xylanase activity in the *S.c.*BGL1 strain compared to the control at all corresponding time points (Figure 3.6 A). The highest activity was observed after 72 hours. The difference in the xylanase activity was determined to be highly significant ($p < 0.0001$) using the T-test. This increase is much less in the eGFP fluorescent strains (Figure 3.6 B). Based on the relative eGFP fluorescence of the strains, eGFP expression was highest after 24 hours after which fluorescence halved before leveling off. This is due to the short half-life of the GFP as mentioned before. The T-test showed that eGFP levels differed significantly only at the 24 hour mark, though the increase in fluorescence was only ~36%. Decreases in xylanase activity after 120 hours could be attributed to the changes brought by the UPR that allowed for a return to homeostasis but can also be as a result of cell death caused by failure to of the UPR to adapt cells to the conditions (Hetz and Soto, 2006, Lajoie et al., 2012) or due to cells entering the late stationary phase. These results do show that the heterologous expression of BGL1 caused a significant impact in the level of UPR compared to uninduced conditions in the *S.c.*yENO1 pGC2 strains and the results are in

agreement with the results shown previously using r.t.qPCR (Van Zyl, 2015). The difference between *xyn2* and *eGFP* gene expression was very significant, however, it should be noted that due to the xylanase itself being a heterologously secreted protein it is possible that it could result in an increase in the level of UPR induction and thus contribute to the increase in xylanase activity that was observed after 72 hours. Furthermore, the xylanase was previously shown to be a stable protein that can accumulate significantly over time (La Grange et al., 2001).

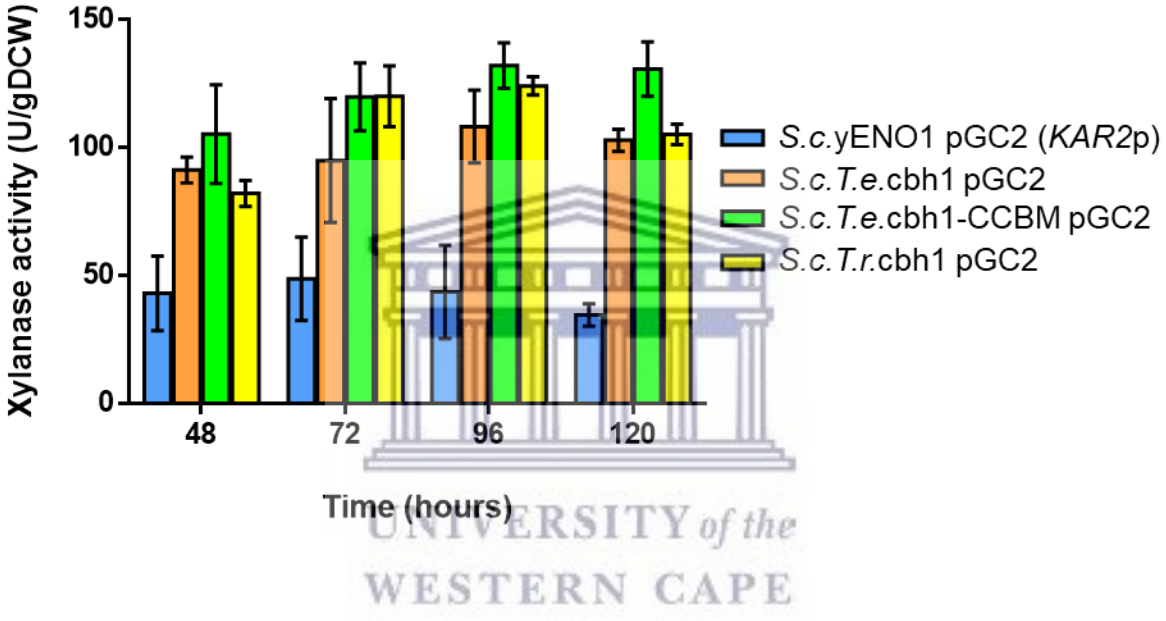

Figure 3.7: Induction of UPR by CBH expression in *S. cerevisiae* Y294 transformants. Comparison of xylanase activities of recombinant Y294 CBH producing strains containing the UPR biosensor pGC2. Activities represent the means of three biological repeats. Error bars represent the standard deviation.

The results obtained from the CBH induction of the UPR depicted clear differences between the CBH strains that were compared. The previous RNA work that was done on these strains showed *T.e.cbh1* to have low UPR induction, *T.e.cbh1*-CCBM to have medium UPR induction and *T.r.cbh1* to have high UPR induction (Ilmén et al., 2011). Across the 120 hours of cultivation for the CBH producing strains with the pGC2 biosensor, the highest xylanase activity levels for each strain were observed after 96 hours for each strain though the differences were not statistically significant in all cases (Figure 3.7). It was clear that the *S.c.T.e.cbh1* strain had the

lowest xylanase activity per gram dry cell weight at 96 hours, and can thus be said to have the lowest level of UPR induction. This would indicate that expression of the *T.e.cbh1* cellulase caused the least amount of stress on the secretory pathway, corresponding with the RNA measurements of the UPR for this strain (Ilmén et al., 2011). Despite this, activity levels were still more than 2-fold higher than the *S.c.*yENO1 pGC2 control strain, and therefore the stress levels were still significantly higher than what we would describe as normal in this study. The *T.e.cbh1*-CCBM (132.8 U/gDCW) and the *S. c T.r.*cbh1 (124.21 U/gDCW) strains were comparable in xylanase activity levels obtained and was higher than the *S.c.T.e.*cbh1 (108.37 U/DCW) xylanase activity that was obtained at 96 hours. These strains do not show the same level of induction that was observed by Ilmén et al. (2011), although they did respond the same as was reported in that study over the first 48 hours. Comparing the *T.e.*cbh1 strains, we noticed that the CBH, which had the carbohydrate-binding module (CBM), had higher UPR induction. The CBM was previously shown to cause an increase in the complexity of CBH production yeast, and it was also suggested that this fusion protein results in higher ER stress (Ilmén et al., 2011, Den Haan et al., 2013b). This addition of the CBM is thus likely the cause of the increased activity that was observed here.

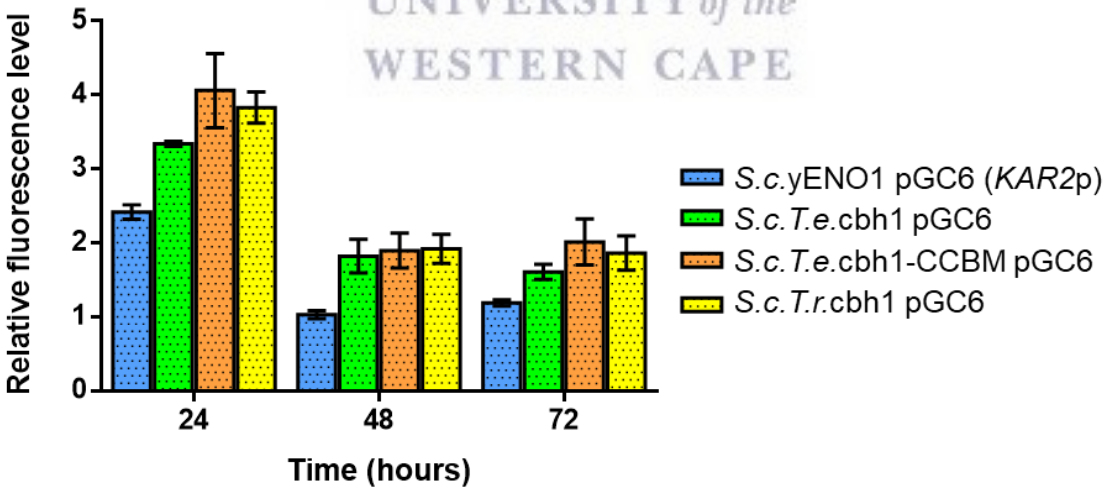

Figure 3.8: Induction of UPR by CBH expression in *S. cerevisiae* Y294 transformants. Relative fluorescence levels of recombinant Y294 CBH producing strains containing the pGC6 UPR biosensor. Arbitrary fluorescence values was normalized against the lowest obtained fluorescence value (*S.c.*yENO1 pGC6, 48 hours). Error bars for both graphs represent the standard deviation of three biological repeats.

The eGFP fluorescence results for the pGC6 biosensor that were obtained were similar to the xylanase activities in terms of the *S.c.T.e.*cbh1-CCBM strain having the highest UPR induction after 24 hours (67.98%) and *S.c.T.e.*cbh1 the lowest induction (38,26%) (Figure 3.8) compared to the control strain. The primary difference between the two results, was that the relative fluorescence levels of the CBH strains producing their peak fluorescence after 24 hours, and then decreased in relative fluorescence by 50% after 24 hours before leveling off. This, as mentioned before is as a result of the eGFP that has a relatively short half-life. In comparison, the recombinant *T.r.xyn2* xylanase was shown to be very stable, surviving in the growth media for over 144 hours (La Grange et al., 2001). This result thus shows that when measuring UPR levels with the pGC6 biosensor, it must be done within 24 hours for the most accurate result.

In the results obtained for cellulase induction of the UPR for both the *T.r.*xyn2 and eGFP, it was clear that the UPR induction was present from the early stages of cultivation and was observed to level off over time. The high levels of UPR induction in these recombinant cellulase producing *S. cerevisiae* Y294 strains were likely as a result of the general challenges that are faced in the recombinant yeast such as the incorrect folding, and the secretory mechanisms not being able to cope with heterologous protein production (Idiris et al., 2010, Glick et al., 2010). Therefore these results should take into consideration that any type of heterologous protein production can induce the UPR in yeast. It was clear in the results obtained that there were small differences between the two biosensors but they detected similar levels of UPR stress in recombinant cellulase producing yeast strains. In the Tm test, the pGC2 biosensor did not react to the concentration gradient that was used. However, it reacted similarly to the pGC6 biosensor when testing the level of UPR in cellulase producing strains. All xylanase activity that was present was readily available after 48 hours after which no further accumulation was observed. This is in contrast to relative eGFP fluorescence, in which the 24 hour reading was observed to be the best reading to measure due to the half-life of the eGFP. Future tests should include readings at 8 hours and 16 hours to test whether these give a different indication of UPR induction compared to the 24 hour observations.

It is important to note that CBHs have generally been reported to have low secretion titres with reports stating that it can vary from 0.002 to >1% of the total cell protein (Den Haan et al., 2013b, Van Rensburg et al., 2012, Ilmén et al., 2011). Despite this, CBH expression induces UPR levels that are similar to, or higher than those induced by BGL-expression, depending on the assay method used. Based on the results obtained in this study, it would seem that the low secretion titres could be due to the burden that CBH production causes on the secretory pathway. The eGFP fluorescence results was shown to be a more accurate depiction of the UPR activation due to its greater sensitivity. Based on these results of the cellulase induction, as well as the result for the Tm induction, it can be concluded that xylanase can only be used as a qualitative measure and conclusions drawn on the non-quantitative data should be avoided. Due to the better linearity that was observed in the Tm test for eGFP expression, the pGC6 biosensor would be better for quantitative assays of UPR induction. The eGFP tends to lose resolution after 24 hours which is most likely due to normalization due to the UPR returning homeostasis in the cell or due to eGFP degradation. For this reason, the eGFP measurements are best to be done within the first 24 - 48 hours.

CHAPTER 4: SUMMARY AND CONCLUSION

Second generation bioethanol production using lignocellulosic biomass as feedstock, has great potential to displace currently utilized petroleum based liquid fuels (Nigam and Singh, 2011). However, the complex structure of biomass necessitates expensive pretreatment prior to hydrolysis and is therefore one of the technical barriers that needs to be overcome (Kricka et al., 2015, Naik et al., 2010, Den Haan et al., 2013b). CBP is arguably one of the more cost effective ways of producing bioethanol from lignocellulosic biomass in a second generation process, but no ideal natural microorganism exists that is fit for this purpose (Saini et al., 2015). *S. cerevisiae* has many desirable traits for CBP such as high ethanol productivity, high ethanol yield and increased resistance to inhibitors found in the lignocellulosic hydrolysate compared to bacteria (Hasunuma and Kondo, 2012b, Wang et al., 2017). *S. cerevisiae* is also an extensively studied host for heterologous protein production and has thus been the subject of much research to engineer and enhance the expression of necessary cellulases to accomplish the ultimate goal of being able to break down lignocellulose for second generation production of bioethanol (Tang et al., 2015, Wang et al., 2017, Den Haan et al., 2007a, Van Zyl et al., 2014, Van Zyl et al., 2016). Despite significant success achieved in cellulase expression in yeast, the secretion titres of CBHs have been relatively low, and one of the reasons for this phenomenon is the induction of the UPR (Den Haan et al., 2013b, Ilmén et al., 2011).

Heterologous protein secretion is known to cause the accumulation of unfolded proteins in the ER, inducing the UPR and the severity of this response differs with different heterologous genes (Ilmén et al., 2011). ER stress caused by accumulation of unfolded proteins is sensed by UPR regulators, Ire1p and Kar2p, which uses the transcription factor Hac1p to activate the expression of over 400 genes (Guerfal et al., 2010). The UPR generally starts with the dissociation of the Kar2p from the Ire1p complex, activating it, which in turn results in the splicing of *HAC1* mRNA that encodes Hac1p. *HAC1*p and *KAR2*p are thus important regulators of UPR as their activation allows the UPR to achieve its function. Significant amounts of research has focused on manipulating genes involved in the UPR with the aim of enhancing

protein secretion (Yu et al., 2015, Payne et al., 2008, Harmsen et al., 1996, Zhang et al., 2006, Valkonen et al., 2003).

It is important to know when the UPR is induced by heterologous protein expression in order to identify gene candidates for strain engineering that will allow for improved secretion of heterologous proteins by lowering ER-stress. ER stress sensors and UPR detection methods that have been developed and applied in the past were based on the Ire1p-mediated unconventional splicing of *HAC1* mRNA introns and makes use of Northern analysis or r.t.qPCR (Back et al., 2005, Leber et al., 2004). These methods can prove to be cumbersome and expensive, and thus more recently fluorescence reporter constructs and enzyme based reporter systems have been reported (Pendin et al., 2016, Hikiji et al., 2015, Fang et al., 2015). These methods are more sensitive than r.t.qPCR and allows for continuous and live detections of the UPR. Due to the simplicity of a biosensor reporter system, it is ideal to use this approach for UPR detection in yeast. No biosensor to detect UPR in heterologous cellulase producing strains has previously been described.

The aim of this study was to develop a UPR biosensor for the *in vitro* detection and measurement of the UPR in *S. cerevisiae* independent of RNA-based methods. We compared the putative UPR biosensors based on *HAC1*p and *KAR2*p, which are promoter regions of two key regulators and sensors of the UPR. *KAR2*p was identified as the superior promoter for the biosensor due to its much lower expression in uninduced conditions, which is what we require from a UPR sensor as this would give a more accurate depiction of changes in ER-stress that might occur over time within the cell.

A good UPR biosensor should also contain an easily assayable reporter protein. This was a further objective of this study, to allow for detection of the UPR in a manner that is neither cumbersome nor expensive. Both xylanase and eGFP reporters were easy to screen on AZCL-xylan plates and fluorescence microscopy, respectively, for initial detection of *KAR2*p-based expression and can be used as an initial indication of whether the UPR was switched on or not. The *eGFP* coupled with *KAR2*p (pGC6) was subsequently shown to have better dynamic range in response to Tm, compared to xylanase. Tm concentrations of 2.5 – 5 µg/mL has previously

been shown to be sufficient for induction of the UPR in most cell types (Oslowski and Urano, 2011), however the pGC6 biosensor was able to detect Induction differences at concentrations as low as 0.2 µg/mL Tm illustrating the sensitivity of this UPR biosensor. However, this was not was not the case with xylanase as 2 µg/mL Tm had no notable impact on secreted xylanase activity. It was clear that the xylanase reporter in the pGC2 UPR biosensor plasmid could only be used for qualitative measurements of the UPR and that the eGFP reporter in the pGC6 UPR biosensor plasmid should be used for quantitative analysis of UPR induction. However it should be noted that the eGFP tended to lose resolution after 24 hours due to its shorter half-life, thus measurements of UPR induction are best to be done within the first 48 hours.

As mentioned, low CBH secretion titres has been one of the main obstacles when developing *S. cerevisiae* for CBP and CBH expression was shown to induce the UPR at levels that were equal to or higher than UPR induction by BGL expression, which was ascribed to the heavy burden that CBHs causes on the secretory mechanisms of the cell (Ilmén et al., 2011). Studies by Van Zyl (2015) showed that, when measuring *HAC1* splicing using r.t.qPCR measurements of UPR induction, there was no induction differences between control *S. cerevisiae* Y294 and CBH producing Y294 strains. The UPR biosensor developed in this study was able to detect differences in UPR induction between different CBH producing Y294 strains which shows the increased sensitivity of measuring *KAR2*p induction compared to measuring *HAC1* splicing. This was the first time that a plasmid based UPR biosensor was shown to successfully measure UPR induction in cellulase producing *S. cerevisiae* strains and corroborated the results of Ilmén et al. (2011) who used a different RNA-based approach. In conclusion, we developed a UPR biosensor that was shown to have greater sensitivity for changes in UPR induction than the currently applied r.t.qPCR method and will thus allow for more accurate detection and measurement of UPR induction in cellulase secreting *S. cerevisiae* strains. This tool will allow us to identify the best cellulase gene-candidates for CBP strain engineering that yield the required activity while not inducing high levels of secretion-based stress. Furthermore we can also investigate whether or not enhanced secretion phenotypes observed in engineered strains were partly due to decreased levels of UPR induction (Kroukamp et al., 2017, Van Zyl et al., 2014, Van Zyl et al., 2016, Davison et al., 2016).

4.1 Future prospects

Future work on this project would include:

- Performing growth analysis and tolerance (osmo-, heat- and ethanol tolerance) phenomena to determine changes in strain physiology as a result of the biosensor.
- Validating the pGC6 UPR biosensor with qPCR measurement of UPR induction using Tm and heterologous gene induction strategies.
- Testing the pGC6 UPR biosensor in various strains to determine the robustness of the tool.
- Testing strains engineered by our research group to have enhanced secretion phenotypes, to determine if they display lower UPR induction due to the strain engineering.
- Testing if "core promoter" regions or other UPR induced genes that are not constitutively expressed at low levels like *HAC1* or *KAR2* would yield superior biosensors.

REFERENCES

AGBOGBO, F. K. & COWARD-KELLY, G. 2008. Cellulosic ethanol production using the naturally occurring xylose-fermenting yeast, *Pichia stipitis*. *Biotechnology letters,* 30**,** 1515-1524.

ANELLI, T. & SITIA, R. 2008. Protein quality control in the early secretory pathway. *The EMBO journal,* 27**,** 315-327.

BACK, S. H., SCHRÖDER, M., LEE, K., ZHANG, K. & KAUFMAN, R. J. 2005. ER stress signaling by regulated splicing: IRE1/HAC1/XBP1. *Methods,* 35**,** 395-416.

BALAT, M., BALAT, H. & ÖZ, C. 2008. Progress in bioethanol processing. *Progress in energy and combustion science,* 34**,** 551-573.

BHALLA, N., JOLLY, P., FORMISANO, N. & ESTRELA, P. 2016. Introduction to biosensors. *Essays in biochemistry,* 60**,** 1-8.

BREVNOVA, E., MCBRIDE, J. E., WISWALL, E., WENGER, K. S., CAIAZZA, N., HAU, H., ARGYROS, A., AGBOGBO, F., RICE, C. F. & BARRETT, T. 2011. Yeast expressing saccharolytic enzymes for consolidated bioprocessing using starch and cellulose. Google Patents.

CHEN, Y. & BRANDIZZI, F. 2013. IRE1: ER stress sensor and cell fate executor. *Trends in cell biology,* 23**,** 547-555.

CORTES-TOLALPA, L., SALLES, J. F. & VAN ELSAS, J. D. 2017. Bacterial synergism in lignocellulose biomass degradation–complementary roles of degraders as influenced by complexity of the carbon source. *Frontiers in microbiology,* 8**,** 1628.

DAS, L., KOLAR, P., SHARMA-SHIVAPPA, R., CLASSEN, J. J. & OSBORNE, J. A. 2017. Catalytic valorization of lignin using niobium oxide. *Waste and Biomass Valorization,* 8**,** 2673-2680.

DAVIS, R., SCHOOLEY, K., RASMUSSEN, B., THOMAS, J. & REDDY, P. 2000. Effect of PDI overexpression on recombinant protein secretion in CHO cells. *Biotechnology progress,* 16**,** 736-743.

DAVISON, S. A., DEN HAAN, R. & VAN ZYL, W. H. 2016. Heterologous expression of cellulase genes in natural *Saccharomyces cerevisiae* strains. *Applied microbiology and biotechnology,* 100**,** 8241-8254.

DEL SAL, G., MANFIOLETTI, G. & SCHNEIDER, C. 1988. A one-tube plasmid DNA mini-preparation suitable for sequencing. *Nucleic acids research,* 16**,** 9878.

DELIC, M., VALLI, M., GRAF, A. B., PFEFFER, M., MATTANOVICH, D. & GASSER, B. 2013. The secretory pathway: exploring yeast diversity. *FEMS microbiology reviews,* 37**,** 872-914.

DEN HAAN, R., KROUKAMP, H., MERT, M., BLOOM, M., GÖRGENS, J. F. & VAN ZYL, W. H. 2013a. Engineering *Saccharomyces cerevisiae* for next generation ethanol production. *Journal of chemical technology and biotechnology,* 88**,** 983-991.

DEN HAAN, R., KROUKAMP, H., VAN ZYL, J.-H. D. & VAN ZYL, W. H. 2013b. Cellobiohydrolase secretion by yeast: current state and prospects for improvement. *Process Biochemistry,* 48**,** 1-12.

DEN HAAN, R., MCBRIDE, J. E., LA GRANGE, D. C., LYND, L. R. & VAN ZYL, W. H. 2007a. Functional expression of cellobiohydrolases in *Saccharomyces cerevisiae* towards one-step conversion of cellulose to ethanol. *Enzyme and Microbial Technology,* 40**,** 1291-1299.

DEN HAAN, R., ROSE, S. H., LYND, L. R. & VAN ZYL, W. H. 2007b. Hydrolysis and fermentation of amorphous cellulose by recombinant *Saccharomyces cerevisiae*. *Metabolic Engineering,* 9**,** 87-94.

DEN HAAN, R., VAN RENSBURG, E., ROSE, S. H., GÖRGENS, J. F. & VAN ZYL, W. H. 2015. Progress and challenges in the engineering of non-cellulolytic microorganisms for consolidated bioprocessing. *Current opinion in biotechnology,* 33**,** 32-38.

DIAO, L., LIU, Y., QIAN, F., YANG, J., JIANG, Y. & YANG, S. 2013. Construction of fast xylose-fermenting yeast based on industrial ethanol-producing diploid *Saccharomyces cerevisiae* by rational design and adaptive evolution. *BMC biotechnology,* 13**,** 1.

DRAGOSITS, M., STADLMANN, J., GRAF, A., GASSER, B., MAURER, M., SAUER, M., KREIL, D. P., ALTMANN, F. & MATTANOVICH, D. 2010. The response to unfolded protein is involved in osmotolerance of *Pichia pastoris*. *Bmc Genomics,* 11**,** 1.

DUCREST, A. L., AMACKER, M., LINGNER, J. & NABHOLZ, M. 2002. Detection of promoter activity by flow cytometric analysis of GFP reporter expression. *Nucleic acids research,* 30**,** e65-e65.

FANG, Z., KUANG, X., ZHANG, Y., SHI, P. & HUANG, Z. 2015. A novel *HAC1*-based dual-luciferase reporter vector for detecting endoplasmic reticulum stress and unfolded protein response in yeast *Saccharomyces cerevisiae*. *Plasmid,* 79**,** 48-53.

FARGIONE, J. E., COOPER, T. R., FLASPOHLER, D. J., HILL, J., LEHMAN, C., TILMAN, D., MCCOY, T., MCLEOD, S., NELSON, E. J. & OBERHAUSER, K. S. 2009. Bioenergy and wildlife: threats and opportunities for grassland conservation. *Bioscience,* 59**,** 767-777.

FAVARO, L., VIKTOR, M. J., ROSE, S. H., VILJOEN-BLOOM, M., VAN ZYL, W. H., BASAGLIA, M., CAGNIN, L. & CASELLA, S. 2015. Consolidated bioprocessing of starchy substrates into ethanol by industrial *Saccharomyces cerevisiae* strains secreting fungal amylases. *Biotechnology and bioengineering,* 112**,** 1751-1760.

GALBE, M. & ZACCHI, G. 2012. Pretreatment: the key to efficient utilization of lignocellulosic materials. *Biomass and bioenergy,* 46**,** 70-78.

GETHING, M.-J. & SAMBROOK, J. 1992. Protein folding in the cell. *Nature,* 355**,** 33.

GLICK, D., BARTH, S. & MACLEOD, K. F. 2010. Autophagy: cellular and molecular mechanisms. *The Journal of pathology,* 221**,** 3-12.

GUERFAL, M., RYCKAERT, S., JACOBS, P. P., AMELOOT, P., VAN CRAENENBROECK, K., DERYCKE, R. & CALLEWAERT, N. 2010. The *HAC1* gene from *Pichia pastoris*: characterization and effect of its overexpression on the production of secreted, surface displayed and membrane proteins. *Microbial cell factories,* 9**,** 1.

GUO, Z.-P., DUQUESNE, S., BOZONNET, S., NICAUD, J.-M., MARTY, A. & O'DONOHUE, M. J. 2017. Expressing accessory proteins in cellulolytic *Yarrowia lipolytica* to improve the conversion yield of recalcitrant cellulose. *Biotechnology for biofuels,* 10**,** 298.

HAAS, I. G. & WABL, M. 1983. Immunoglobulin heavy chain binding protein. *Nature,* 306**,** 387.

HAMELINCK, C. N., VAN HOOIJDONK, G. & FAAIJ, A. P. 2005. Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle-and long-term. *Biomass and bioenergy,* 28**,** 384-410.

HAMPTON, R. Y. 2000. ER stress response: getting the UPR hand on misfolded proteins. *Current Biology,* 10**,** R518-R521.

HARMSEN, M., BRUYNE, M., RAUE, H. & MAAT, J. 1996. Overexpression of binding protein and disruption of the *PMR1* gene synergistically stimulate secretion of bovine prochymosin but not plant thaumatin in yeast. *Applied microbiology and biotechnology,* 46**,** 365-370.

HASUNUMA, T. & KONDO, A. 2012a. Consolidated bioprocessing and simultaneous saccharification and fermentation of lignocellulose to ethanol with thermotolerant yeast strains. *Process Biochemistry,* 47**,** 1287-1294.

HASUNUMA, T. & KONDO, A. 2012b. Development of yeast cell factories for consolidated bioprocessing of lignocellulose to bioethanol through cell surface engineering. *Biotechnology advances,* 30**,** 1207-1218.

HASUNUMA, T., OKAZAKI, F., OKAI, N., HARA, K. Y., ISHII, J. & KONDO, A. 2013. A review of enzymes and microbes for lignocellulosic biorefinery and the possibility of their application to consolidated bioprocessing technology. *Bioresource technology,* 135**,** 513-522.

HETZ, C. 2012. The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nature reviews Molecular cell biology,* 13**,** 89.

HETZ, C. & PAPA, F. R. 2017. The unfolded protein response and cell fate control. *Molecular cell*.

HETZ, C. A. & SOTO, C. 2006. Stressing out the ER: a role of the unfolded protein response in prion-related disorders. *Current molecular medicine,* 6**,** 37-43.

HIKIJI, T., NORISADA, J., HIRATA, Y., OKUDA, K., NAGASAWA, H., ISHIGAKI, S., SOBUE, G., KIUCHI, K. & OH-HASHI, K. 2015. A highly sensitive assay of IRE1 activity using the small luciferase NanoLuc: Evaluation of ALS-related genetic and pathological factors. *Biochemical and biophysical research communications,* 463**,** 881-887.

HILL, J., DONALD, K. & GRIFFITHS, D. 1991. DMSO-enhanced whole cell yeast transformation. *Nucleic Acids Res,* 19**,** 5791.

HOFFMAN, C. S. & WINSTON, F. 1987. A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformaion of Escherichia coli. *Gene,* 57**,** 267-272.

HONG, J., YANG, H., ZHANG, K., LIU, C., ZOU, S. & ZHANG, M. 2014. Development of a cellulolytic *Saccharomyces cerevisiae* strain with enhanced cellobiohydrolase activity. *World Journal of Microbiology and Biotechnology,* 30**,** 2985-2993.

HOU, J., TYO, K. E., LIU, Z., PETRANOVIC, D. & NIELSEN, J. 2012. Metabolic engineering of recombinant protein secretion by *Saccharomyces cerevisiae*. *FEMS yeast Research,* 12**,** 491-510.

HOWELL, S. H. 2017. When is the unfolded protein response not the unfolded protein response? *Plant Science,* 260**,** 139-143.

IDIRIS, A., TOHDA, H., KUMAGAI, H. & TAKEGAWA, K. 2010. Engineering of protein secretion in yeast: strategies and impact on protein production. *Applied microbiology and biotechnology,* 86**,** 403-417.

ILMÉN, M., DEN HAAN, R., BREVNOVA, E., MCBRIDE, J., WISWALL, E., FROEHLICH, A., KOIVULA, A., VOUTILAINEN, S. P., SIIKA-AHO, M. & LA GRANGE, D. C. 2011. High level secretion of cellobiohydrolases by *Saccharomyces cerevisiae*. *Biotechnology for biofuels,* 4**,** 1.

IWATA, Y., HAYASHI, N., TABARA, K., MISHIBA, K.-I. & KOIZUMI, N. 2016. Tunicamycin-induced inhibition of protein secretion into culture medium of Arabidopsis T87 suspension cells through mRNA degradation on the endoplasmic reticulum. *Bioscience, biotechnology, and biochemistry,* 80**,** 1168-1171.

IWAWAKI, T., AKAI, R., KOHNO, K. & MIURA, M. 2004. A transgenic mouse model for monitoring endoplasmic reticulum stress. *Nature medicine,* 10**,** 98.

KAISHIMA, M., ISHII, J., MATSUNO, T., FUKUDA, N. & KONDO, A. 2016. Expression of varied GFPs in *Saccharomyces cerevisiae*: codon optimization yields stronger than expected expression and fluorescence intensity. *Scientific reports,* 6**,** 35932.

KARAKASHEV, D., THOMSEN, A. B. & ANGELIDAKI, I. 2007. Anaerobic biotechnological approaches for production of liquid energy carriers from biomass. *Biotechnology letters,* 29**,** 1005-1012.

KHUONG, L., MASJUKI, H., ZULKIFLI, N., MOHAMAD, E. N., KALAM, M., ALABDULKAREM, A., ARSLAN, A., MOSAROF, M., SYAHIR, A. & JAMSHAID, M. 2017. Effect of gasoline–bioethanol blends on the properties and lubrication characteristics of commercial engine oil. *RSC Advances,* 7**,** 15005-15019.

KIMATA, Y., ISHIWATA-KIMATA, Y., ITO, T., HIRATA, A., SUZUKI, T., OIKAWA, D., TAKEUCHI, M. & KOHNO, K. 2007. Two regulatory steps of ER-stress sensor Ire1 involving its cluster formation and interaction with unfolded proteins. *The Journal of cell biology,* 179**,** 75-86.

KIMATA, Y. & KOHNO, K. 2011. Endoplasmic reticulum stress-sensing mechanisms in yeast and mammalian cells. *Current opinion in cell biology,* 23**,** 135-142.

KOSTAS, E. T., WHITE, D. A., DU, C. & COOK, D. J. 2016. Selection of yeast strains for bioethanol production from UK seaweeds. *Journal of applied phycology,* 28**,** 1427-1441.

KRICKA, W., FITZPATRICK, J. & BOND, U. 2015. Challenges for the production of bioethanol from biomass using recombinant yeasts. *Advances in applied microbiology.* Elsevier.

KROUKAMP, H., DEN HAAN, R., LA GRANGE, D. C., SIBANDA, N., FOULQUIÉ-MORENO, M. R., THEVELEIN, J. M. & VAN ZYL, W. H. 2017. Strain breeding enhanced heterologous cellobiohydrolase secretion by *Saccharomyces cerevisiae* in a protein specific manner. *Biotechnology journal,* 12**,** 1700346.

KROUKAMP, H., DEN HAAN, R., VAN WYK, N. & VAN ZYL, W. H. 2013. Overexpression of native *PSE1* and *SOD1* in *Saccharomyces cerevisiae* improved heterologous cellulase secretion. *Applied Energy,* 102**,** 150-156.

KROUKAMP, H., DEN HAAN, R., VAN ZYL, J. H. & VAN ZYL, W. H. 2018. Rational strain engineering interventions to enhance cellulase secretion by *Saccharomyces cerevisiae*. *Biofuels, Bioproducts and Biorefining,* 12**,** 108-124.

KUPSCO, A. & SCHLENK, D. 2015. Chapter One-Oxidative Stress, Unfolded Protein Response, and Apoptosis in Developmental Toxicity. *International review of cell and molecular biology,* 317**,** 1-66.

LA GRANGE, D. C., PRETORIUS, I. S., CLAEYSSENS, M. & VAN ZYL, W. H. 2001. Degradation of xylan to d-xylose by recombinant *Saccharomyces cerevisiae* coexpressing the *Aspergillus*

niger β-xylosidase (*xlnD*) and the *Trichoderma reesei* xylanase II (*xyn2*) genes. *Applied and environmental microbiology,* 67**,** 5512-5519.

LAJOIE, P., MOIR, R. D., WILLIS, I. M. & SNAPP, E. L. 2012. Kar2p availability defines distinct forms of endoplasmic reticulum stress in living cells. *Molecular biology of the cell,* 23**,** 955-964.

LAMBERTZ, C., GARVEY, M., KLINGER, J., HEESEL, D., KLOSE, H., FISCHER, R. & COMMANDEUR, U. 2014. Challenges and advances in the heterologous expression of cellulolytic enzymes: a review. *Biotechnology for biofuels,* 7**,** 1.

LEBER, J. H., BERNALES, S. & WALTER, P. 2004. IRE1-independent gain control of the unfolded protein response. *PLoS biology,* 2**,** e235.

LIMAYEM, A. & RICKE, S. C. 2012. Lignocellulosic biomass for bioethanol production: current perspectives, potential issues and future prospects. *Progress in Energy and Combustion Science,* 38**,** 449-467.

LINDHOLM, D., KORHONEN, L., ERIKSSON, O. & KÕKS, S. 2017. Recent insights into the role of unfolded protein response in ER stress in health and disease. *Frontiers in cell and developmental biology,* 5**,** 48.

LYND, L. R., LIANG, X., BIDDY, M. J., ALLEE, A., CAI, H., FOUST, T., HIMMEL, M. E., LASER, M. S., WANG, M. & WYMAN, C. E. 2017. Cellulosic ethanol: status and innovation. *Current opinion in biotechnology,* 45**,** 202-211.

LYND, L. R., VAN ZYL, W. H., MCBRIDE, J. E. & LASER, M. 2005. Consolidated bioprocessing of cellulosic biomass: an update. *Current opinion in biotechnology,* 16**,** 577-583.

MA, Y. & HENDERSHOT, L. M. 2001. The unfolding tale of the unfolded protein response. *Cell,* 107**,** 827-830.

MADADI, M., TU, Y. & ABBAS, A. 2017. Recent status on enzymatic saccharification of lignocellulosic biomass for bioethanol production. *Electron J Biol,* 13**,** 135-143.

MONTENEGRO-MONTERO, A., GOITY, A. & LARRONDO, L. F. 2015. The bZIP transcription factor HAC-1 is involved in the unfolded protein response and is necessary for growth on cellulose in *Neurospora crassa*. *PloS one,* 10**,** e0131415.

MOOD, S. H., GOLFESHAN, A. H., TABATABAEI, M., JOUZANI, G. S., NAJAFI, G. H., GHOLAMI, M. & ARDJMAND, M. 2013. Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment. *Renewable and Sustainable Energy Reviews,* 27**,** 77-93.

MOORE, K. A. & HOLLIEN, J. 2012. The unfolded protein response in secretory cell function. *Annual review of genetics,* 46**,** 165-183.

MORI, K., SANT, A., KOHNO, K., NORMINGTON, K., GETHING, M. & SAMBROOK, J. 1992. A 22 bp *cis*-acting element is necessary and sufficient for the induction of the yeast *KAR2* (BiP) gene by unfolded proteins. *The EMBO Journal,* 11**,** 2583.

NAIK, S. N., GOUD, V. V., ROUT, P. K. & DALAI, A. K. 2010. Production of first and second generation biofuels: a comprehensive review. *Renewable and Sustainable Energy Reviews,* 14**,** 578-597.

NAVARRO-TAPIA, E., QUEROL, A. & PÉREZ-TORRADO, R. 2018. Membrane fluidification by ethanol stress activates unfolded protein response in yeasts. *Microbial biotechnology,* 11**,** 465-475.

NIGAM, P. S. & SINGH, A. 2011. Production of liquid biofuels from renewable resources. *Progress in energy and combustion science,* 37**,** 52-68.

OSLOWSKI, C. M. & URANO, F. 2011. Measuring ER stress and the unfolded protein response using mammalian tissue culture system. *Methods in enzymology.* Elsevier.

PAYNE, T., FINNIS, C., EVANS, L., MEAD, D., AVERY, S., ARCHER, D. & SLEEP, D. 2008. Modulation of chaperone gene expression in mutagenized *Saccharomyces cerevisiae* strains developed for recombinant human albumin production results in increased production of multiple heterologous proteins. *Applied and environmental microbiology,* 74**,** 7759-7766.

PENDIN, D., GREOTTI, E., LEFKIMMIATIS, K. & POZZAN, T. 2016. Exploring cells with targeted biosensors. *The Journal of general physiology***,** jgp. 201611654.

PEREIRA, S. C., MAEHARA, L., MACHADO, C. M. M. & FARINAS, C. S. 2015. 2G ethanol from the whole sugarcane lignocellulosic biomass. *Biotechnology for biofuels,* 8**,** 44.

PINCUS, D., CHEVALIER, M. W., ARAGÓN, T., VAN ANKEN, E., VIDAL, S. E., EL-SAMAD, H. & WALTER, P. 2010. BiP binding to the ER-stress sensor Ire1 tunes the homeostatic behavior of the unfolded protein response. *PLoS biology,* 8**,** e1000415.

RABEMANOLONTSOA, H. & SAKA, S. 2016. Various pretreatments of lignocellulosics. *Bioresource technology,* 199**,** 83-91.

RAO, L. V., GOLI, J. K., GENTELA, J. & KOTI, S. 2016. Bioconversion of lignocellulosic biomass to xylitol: An overview. *Bioresource technology***.**

RÜEGSEGGER, U., LEBER, J. H. & WALTER, P. 2001. Block of *HAC1* mRNA translation by long-range base pairing is released by cytoplasmic splicing upon induction of the unfolded protein response. *Cell,* 107**,** 103-114.

SAINI, J. K., SAINI, R. & TEWARI, L. 2015. Lignocellulosic agriculture wastes as biomass feedstocks for second-generation bioethanol production: concepts and recent developments. *3 Biotech,* 5**,** 337-353.

SAMALI, A., FITZGERALD, U., DEEGAN, S. & GUPTA, S. 2010. Methods for monitoring endoplasmic reticulum stress and the unfolded protein response. *International journal of cell biology,* 2010.

SAMBROOK, J. & RUSSELL, D. 2001. *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press.

SARKAR, N., GHOSH, S. K., BANNERJEE, S. & AIKAT, K. 2012. Bioethanol production from agricultural wastes: An overview. *Renewable Energy,* 37**,** 19-27.

SCHWIETZKE, S., KIM, Y., XIMENES, E., MOSIER, N. & LADISCH, M. 2009. Ethanol production from maize. *Molecular Genetic Approaches to Maize Improvement.* Springer.

SHUSTA, E. V., RAINES, R. T., PLÜCKTHUN, A. & WITTRUP, K. D. 1998. Increasing the secretory capacity of *Saccharomyces cerevisiae* for production of single-chain antibody fragments. *Nature biotechnology,* 16**,** 773-777.

SINDHU, R., BINOD, P. & PANDEY, A. 2016. Biological pretreatment of lignocellulosic biomass–An overview. *Bioresource technology,* 199**,** 76-82.

SUN, S., SUN, S., CAO, X. & SUN, R. 2016. The role of pretreatment in improving the enzymatic hydrolysis of lignocellulosic materials. *Bioresource technology,* 199**,** 49-58.

TANG, H., BAO, X., SHEN, Y., SONG, M., WANG, S., WANG, C. & HOU, J. 2015. Engineering protein folding and translocation improves heterologous protein secretion in *Saccharomyces cerevisiae*. *Biotechnology and bioengineering,* 112**,** 1872-1882.

TIAN, S.-Q., ZHAO, R.-Y. & CHEN, Z.-C. 2018. Review of the pretreatment and bioconversion of lignocellulosic biomass from wheat straw materials. *Renewable and Sustainable Energy Reviews,* 91**,** 483-489.

VALKONEN, M., PENTTILÄ, M. & SALOHEIMO, M. 2003. Effects of inactivation and constitutive expression of the unfolded-protein response pathway on protein production in the yeast *Saccharomyces cerevisiae*. *Applied and environmental microbiology,* 69**,** 2065-2072.

VAN RENSBURG, E., DEN HAAN, R., SMITH, J., VAN ZYL, W. H. & GÖRGENS, J. F. 2012. The metabolic burden of cellulase expression by recombinant *Saccharomyces cerevisiae* Y294 in aerobic batch culture. *Applied microbiology and biotechnology,* 96**,** 197-209.

VAN ZYL, J. 2015. *The effects of native Saccharomyces cerevisiae snare gene over expression on Heterologous cellulase secretion.* Stellenbosch: Stellenbosch University.

VAN ZYL, J. H. D., DEN HAAN, R. & VAN ZYL, W. H. 2014. Over-expression of native *Saccharomyces cerevisiae* exocytic SNARE genes increased heterologous cellulase secretion. *Applied microbiology and biotechnology,* 98**,** 5567-5578.

VAN ZYL, J. H. D., DEN HAAN, R. & VAN ZYL, W. H. 2016. Overexpression of native *Saccharomyces cerevisiae* ER-to-Golgi SNARE genes increased heterologous cellulase secretion. *Applied microbiology and biotechnology,* 100**,** 505-518.

VAN ZYL, W. H., GRANGE, D. C. & DEN HAAN, R. 2011. *Developing Organisms for Consolidated Bioprocessing of Biomass to Ethanol*, INTECH Open Access Publisher.

VAN ZYL, W. H., LYND, L. R., DEN HAAN, R. & MCBRIDE, J. E. 2007. Consolidated bioprocessing for bioethanol production using *Saccharomyces cerevisiae*. *Biofuels.* Springer.

VOLYNETS, B., EIN-MOZAFFARI, F. & DAHMAN, Y. 2017. Biomass processing into ethanol: pretreatment, enzymatic hydrolysis, fermentation, rheology, and mixing. *Green Processing and Synthesis,* 6**,** 1-22.

WALTER, P. & RON, D. 2011. The unfolded protein response: from stress pathway to homeostatic regulation. *Science,* 334**,** 1081-1086.

WANG, G., HUANG, M. & NIELSEN, J. 2017. Exploring the potential of *Saccharomyces cerevisiae* for biopharmaceutical protein production. *Current opinion in biotechnology,* 48**,** 77-84.

WANG, T.-Y. 2015. Engineering yeast for cellulosic ethanol production. *Austin Chem Eng,* 2**,** 1018.

YOSHIDA, H. 2007. ER stress and diseases. *Febs Journal,* 274**,** 630-658.

YOUNG, C. L., YURASZECK, T. & ROBINSON, A. S. 2011. Decreased secretion and unfolded protein response upregulation. *Methods in enzymology,* 491**,** 235.

YU, P., ZHU, Q., CHEN, K. & LV, X. 2015. Improving the secretory production of the heterologous protein in *Pichia pastoris* by focusing on protein folding. *Applied biochemistry and biotechnology,* 175**,** 535-548.

ZHANG, K. & KAUFMAN, R. J. 2006. The unfolded protein response A stress signaling pathway critical for health and disease. *Neurology,* 66**,** S102-S109.

ZHANG, W., ZHAO, H. L., XUE, C., XIONG, X. H., YAO, X. Q., LI, X. Y., CHEN, H. P. & LIU, Z. M. 2006. Enhanced secretion of heterologous proteins in *Pichia pastoris* following overexpression of *Saccharomyces cerevisiae* chaperone proteins. *Biotechnology progress,* 22**,** 1090-1095.

SUPPLEMENTARY INFORMATION

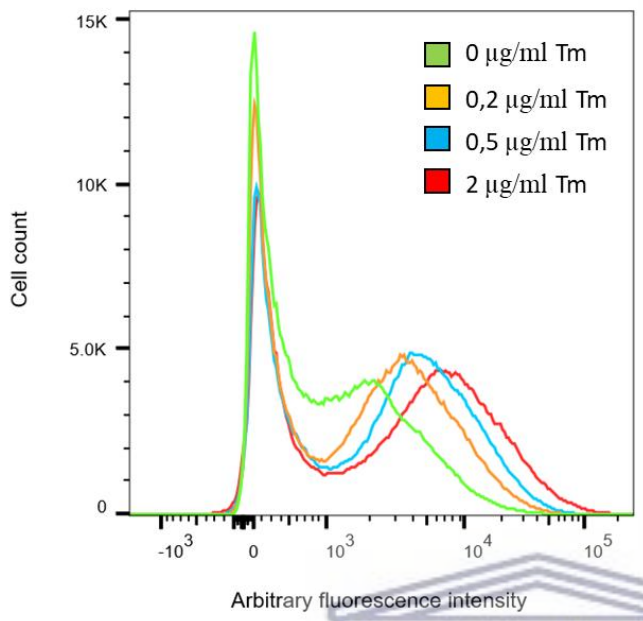

Figure S1: Distribution of cells in *S.c.*yENO1 transformants containing the pGC6 biosensor plasmid. Histogram of fluorescence distribution in *S.c.*yENO1 pGC6 transformants when induced by various concentrations of Tm.

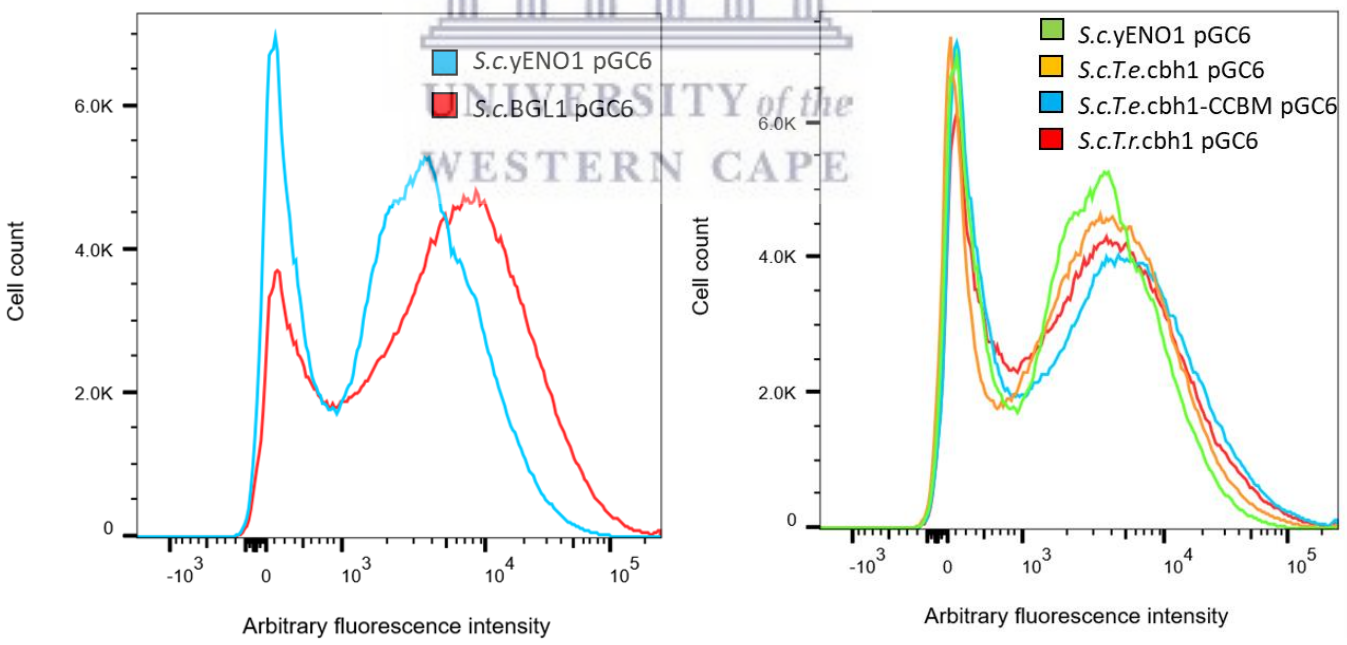

Figure S2: Distribution of cells in cellulase producing *S. cerevisiae* Y294 transformants containing the pGC6 biosensor plasmid. A) Histogram of fluorescence distribution in the BGL producing *S. cerevisiae* Y294 with pGC6. B) Histogram of fluorescence distribution of CBH producing *S. cerevisiae* Y294 strains with pGC6.