THE EFFECT OF BLOCKING SELECTED ENDOCYTIC MECHANISMS ON HETEROLOGOUS PROTEIN SECRETION IN THE YEAST *SACCHAROMYCES CEREVISIAE*

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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

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I declare that "The effect of blocking selected endocytic mechanisms on heterologous protein secretion in the yeast *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 I have used have been indicated and acknowledged by complete references.

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Signature:

Date: December 2018

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A. ACKNOWLEDGEMENTS

I would like to thank the following:

- Firstly, I would like to say shukran to the Almighty for granting me this opportunity and granting me the ability to come this far and achieving my goals.
- My mother, Melanie Freeman, for of the guidance, love and support she had given me throughout the years, for all the sacrifices and keeping me motivated even though she is not around anymore, my father, Andries Freeman, for helping me through my journey, supporting me thus far and for all the sacrifices made and lastly Mohamed Fazel Carrim for all the support, drive and for standing by me through my studies, with all the late night shifts keeping me awake.
- My supervisor, Dr Riaan Den Haan, for guiding me throughout my research, giving me the opportunity to be a part of an amazing group of people, supporting me, motivating me to stay focused and proof reading.
- My co-supervisor for allowing me the opportunity.
- My guardian, Karen Celento, for helping me get to University and for always being there.
- My colleagues in the yeast laboratory, Daylon, Bronwyn, Odwa, Michaela for the support and motivation through the hard times, especially Gillian and Jarryd for always keeping me calm and motivated.
- My friends and staff in the Biotechnology department.
- The National research foundation (NRF) for funding my research project and granting me the opportunity to further my studies.

B. LIST OF ABBREVIATIONS

1G	first generation	**ATP**	Adenine Triphosphate
2G	second generation	**ORF**	Open Reading Frame
BGL	β-glucosidase		
CBP	Consolidated bioprocessing		
CBH1	CellobiohydrolaseI		
CMC	Carboxylmethyl cellulose		
DCW	Dry cell weight		
EG2	EndogluconaseII		
ER	Endoplasmic reticulum		
GHG	Green house gas		
OD	Optical density		
PCR	Polymerase chain reaction		
pNP	*p*-nitrophenol		
pNPC	*p*-nitrophenyl-β-D-cellobioside		
RBB	Remazol brilliant blue		
SDS-PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis		
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor		
XYN2	Xylanase		
USA	United States of America		
YPD	Yeast extract, peptone, glucose		

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E. ABSTRACT

The yeast *Saccharomyces cerevisiae* is considered a good host used for heterologous protein production due to the organism's microbial safety, rapid growth and eukaryotic post-translational processing. As a fermentative organism, *S. cerevisiae* is thus not only a useful platform for the production of biopharmaceuticals and industrial enzymes, but also a promising organism for second-generation biofuel production. Substantial effort has been focused on alleviating the many bottlenecks in recombinant gene expression, as well as in the secretory pathway to enhance heterologous protein titres. It was recently shown that highly active endocytosis could decrease the overall secreted protein titre in the supernatant. In this study, we aimed to block endocytotic and vacuolar complexes to ultimately disrupt, or impair, the endocytotic and vacuolar mechanisms of proteolysis and test the effect that this would have on secreted heterologous protein titres. This was accomplished by knocking out various genes involved in endocytosis and transforming the strains with genes encoding various hydrolases including β-glucosidase (Bgl), xylanase (Xyn2), endoglucanase (Eg2) and cellobiohydrylase (Cbh1). Our study demonstrated that genetic blocking of endocytotic mechanisms as well as vacuolar complexes could theoretically improve heterologous protein secretion in *S. cerevisiae*. Endoglucanase (Eg2) titres displayed improvement of 26% and 30% in strains which had the *RVS161* and *VRP1* genes deleted and xylanase titres displayed an improvement of 71% and 143% in strains with the *END3* and *SSA4* gene deletions. Several of the gene knockouts tested improved Xyn2 and Eg2 titres but the effect of the different gene targets varied widely. A double knock-out strain with deletions in *CLC1* and *RVS161* secreted 104% more Eg2 than its parental control strain on a per dry cell weight basis, a significant synergistic improvement. Other double knock-out strains displayed additive or similar activities when compared to their controls. Cbh1 secretion could not be improved through the gene deletions tested in our study and Bgl activity could not be measured in our transformants. These results demonstrate the different relationships of various heterologous proteins with various components of the secretion machinery and may also imply how endocytosis as well as vacuolar complexes affect the level of secreted protein.

CHAPTER ONE

LITERATURE REVIEW

1.1. Bioenergy

1.1.1. Introduction to bioenergy

Due to worldwide economic development, there has been a worldwide increase in the demand for energy over the past few decades which has led to an increase in the demand for petroleum based fuels (Harun *et al,* 2011). The conventional fuels that provide the majority of our energy used are petroleum based fuels produced from fossil resources. These petroleum based fuels have become much more expensive and the price continues to grow with the resources becoming more limited (Bhattara, 2011). Fossil fuels make up 80% of primary energy which is consumed worldwide and approximately 58% of this is used by commuters in the transportation sector (Nigam and Singh, 2011). The long term environmental and economic concerns that are inherent to fossil fuel usage have thus resulted in a vast amount of research being targeted toward renewable sources of liquid fuels for the replacement of fossil fuels (Kumar *et al*, 2009). With the scarcity of these supplies on the rise, political risks also increase. Biofuels can thus play a key role in solving fundamental problems such as sustainability, energy security and environmental concerns.

The burning of fossil fuels, such as coal and oil, results in CO_2 emissions, (Kumar *et al*, 2009). These fossil resources cannot be replaced as fast as they are being used and are also a major contributor to greenhouse gas (GHG) emissions (Valdivia *et al*, 2016). This would ultimately result in climate change leading to receding glaciers with a concomitant rise in sea levels, including loss of biodiversity and global economic activity. With these fossil resources being rapidly depleted, the current energy supply is not renewable or sustainable enough to maintain a "business as usual" scenario in the future. Worldwide, countries are attempting to reduce their dependency on fossil fuels and finding energy alternatives (Paeralta-Yahya and Keasling, 2010).

Bioenergy has gained increasing recognition as an energy alternative, being not only environmentally friendly but also a renewable and potentially sustainable alternative to

conventional fossil fuels (Valdivia *et al*, 2016). Approximately 10% of world-wide primary energy supply is currently accounted for by bioenergy (Haberl *et al*, 2010). Bioenergy can be defined as the production of energy from organic non- fossil material which has a biological origin (Haberl *et al*, 2010). These can be organic substances which range from food derivatives to organic wastes such as agro- industrial wastes, agricultural residues and dedicated energy crops, which are termed feedstocks. Through a variety of processes these feedstocks can then be converted to liquids, such as bioethanol, solids or even gaseous biofuels such as bio-hydrogen (Sawatdeenarunat, 2016) (Fig 1.1).

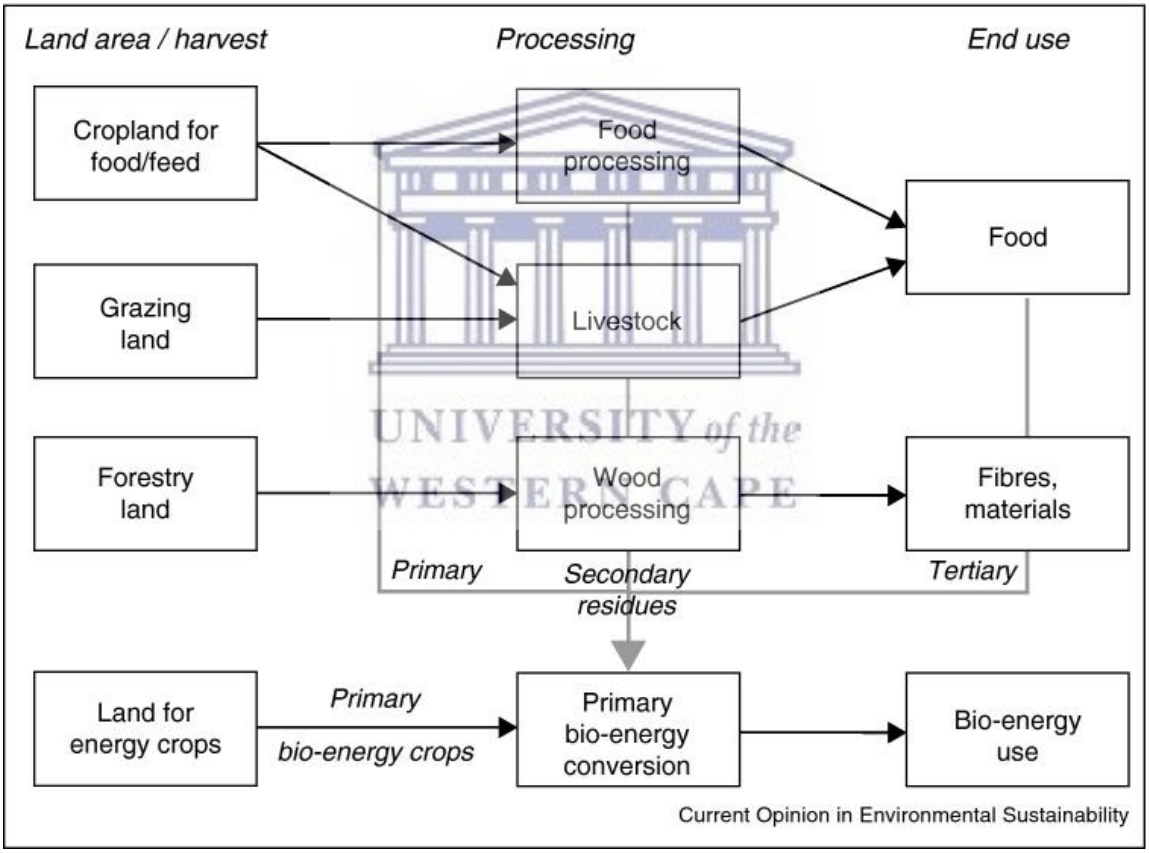


Figure 1.1: Description of land and biomass resources used for bioenergy production through primary as well as secondary energy conversions (Haberl *et al*, 2010).

1.2. Biofuels: a renewable fuel alternative

There are many renewable energy alternatives that have emerged for improved global energy security, such as biofuels, hydrogen, natural gas as well as syngas, wind and solar (Nigam and Singh, 2011). Biofuels have emerged as a way to make use of renewable biomass energy and have become an alternative to petroleum based liquid fuels (Solomon, 2010). This has led to a growing interest in the use of biofuels on a global scale to address the global challenges that have risen with the use of petroleum based methods (Nigam and Singh, 2011). Biofuels can be produced from the conversion of lignocellulosic biomass to a fuel which can be used in the transportation sector (Aro, 2016) (Fig. 1.2). Unlike fossil fuels, which make use of plant material that grew millions of years ago, biofuels makes use of plants that are grown currently, that can be re-planted yearly or as required. The worldwide production of oil is expected to peak in the coming decades as we have to find one new oil barrel for every four that is consumed to sustain the growing demand (Nigam and Singh, 2011). The use of alternative fuels, such as biofuels, will not only result in the decrease of vehicle emissions but also provide a security in the energy supply while providing a steady income in the agricultural industry (Parisutham *et al,* 2014).

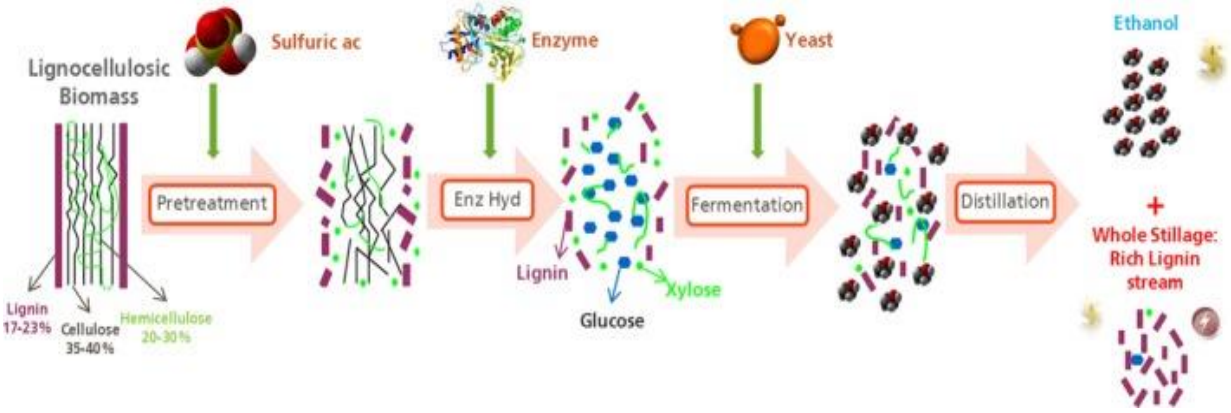


Figure 1.2: A schematic of the production of ethanol from lignocellulosic biomass. The breakdown of lignocellulosic biomass by sulphuric acid pre-treatment, hydrolysis and fermentation to a final product of bioethanol is shown (Valdivia *et al*, 2016).

Between 1980 and 2005, the production of biofuels increased to 50.1 billion litres from only 4.4 billion litres (Bhattara, 2011). In 2018, an estimated 143 billion litres of ethanol was produced with the 2019 estimate predicted as 149 billion litres (OECD-FAO Agricultural Outlook, 2018). This shows a remarkable increase in the production of biofuels in the last three decades. It can thus be seen that biofuels can make an effective contribution in partly alleviating some of the energy concerns that we are faced with.

1.2.1. Classification of biofuels

Biofuels are either gases such as biogas, liquids such as bioethanol, biodiesel, oils from pyrolysis or solids such as charcoal, fuel wood and wood pellets (Cherubini, 2010). Biofuels can be classified into two group's, namely primary and secondary biofuels, depending on the level of processing required (Bhatia *et al*, 2017). In addition, the production of ethanol can be either a first or second generation process, depending on the substrate used.

1.2.1.1. Primary and secondary biofuels

The primary group of biofuels usually represent the feedstocks in their natural form with little or no processing necessary (Fig. 1.3) (Nigam and Singh, 2011). Examples of these include pellets, firewood and wood chips (Clark, 2007). These are directly used for combustion in small and large scale industrial and domestic processing and can be used for heating and cooking. Secondary group of biofuels involves more complex processes and can be used for a wider range of applications. These include solids, liquids and gases. Most bioethanol currently used as fuels, is produced from the common grains (especially maize) as well as other cereals and sugarcane. This is known as first generation (1G) bioethanol (Lane *et al* 2018). Second generation (2G) biofuels are produced by means of processing of biomass, and have to undergo a form of modification in order to be used (Aro, 2016) (Fig. 1.3). These are then used to produce solid, liquid or gaseous biofuels such as charcoal, bioethanol and biogas (Jansen *et al* 2013). The products are used in the transportation sector and in many industrial processes. Although first generation biofuels are viewed positively as they are seen as renewable, there has been controversy (Valdivia *et al*,

2016). Questions have been raised with regards to the impact on food production such as the "food vs fuel" debate, the energy ratio, the security of energy supply, the costs that are involved as well as the impact on biodiversity (Harun *et al*., 2010b). Third generation biofuels refers to biofuels produced from algae (Carere *et al,* 2008). Although algae are capable of high yields with lower resource inputs, there are shortcomings that hinder the process in becoming a runway success.

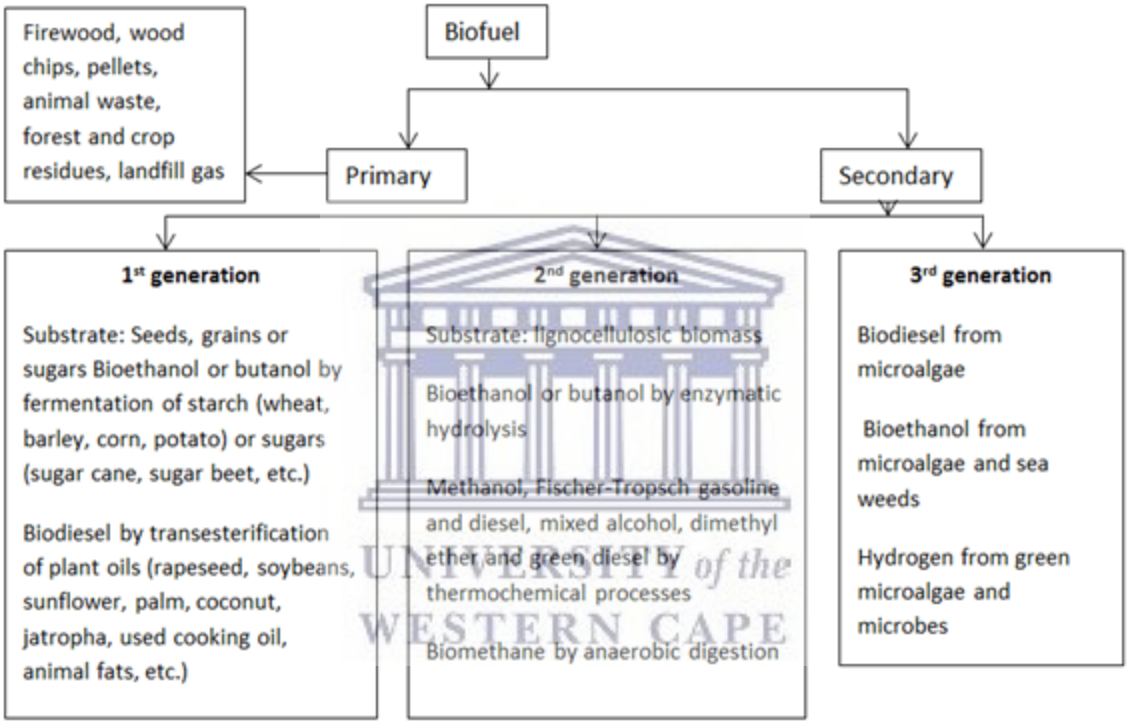


Figure 1.3: The classification of biofuels. First, second and third generation biofuels are grouped according to their feedstock source (Nigam and Singh, 2011).

1.3. Bioethanol

The most commonly used biofuel globally is bioethanol (Ramos *et al*, 2016). The ethanol produced in the United States alone accounts for 500 million barrels of replaced petroleum based fuels. The term bioethanol refers to the production of ethanol from a renewable feedstock, making the fuel not only renewable and sustainable but also more environmentally friendly (Vohra, 2014). Ethanol has been used in the engines of automobiles since the 1800's where it

was used on a wider scale before the petroleum industry developed. It is still being used today as a gasoline additive in some countries because of its ability to give engines an octane boost (Harun *et al*., 2010a). Bioethanol is a fuel component that is most commonly used in spark as well as compression ignition engines as an oxygenation compound (Malgorzata *et al,* 2016). This is due to the alcohol being able to positively influence some of the fuel properties while also reducing harmful gas emissions. Although ethanol has a lower energy density than petroleum based fuels, the octane rating ranges from 35- 40% which is much higher than that of petroleum based fuels, thus improving the thermal efficiency of fuel blends (Solomon, 2010). Upon mixing ethanol with petroleum, the octane level of the fuel is increased resulting in the reduction of carbon monoxide that is expelled from vehicles during combustion.

Bioethanol can either be blended with conventional petroleum based fuels or burned in its pure form in spark ignition engines that have been modified for this purpose (Paeralta-Yahya and Keasling, 2010). These mixtures are well known in Brazil where bioethanol based fuels have been used since 1975 for the substitution of petrol (Malgorzata *et al,* 2016). Brazil is one of the leading countries in the usage of biofuels and is also the only non- OPEC country to be energy independant, showing the positive impact biofuels can have. Brazil produces approximately 6.2 billion gallons of ethanol using sugarcane, making it the second largest ethanol producing country after the USA.

To move away from petroleum based fuels towards biofuels the latter must provide a net energy gain, have the ability to be produced on a large scale without disruption of food supply and also be economically competitive (Hill, *et al*, 2006). With this in mind, it can be seen that first generation bioethanol production does not fulfil these proposed requirements. However, in recent years, first generation biofuels have been subjected to improvements in the processes to make it more sustainable, though continuous improvements are still needed (Ramos, 2016). A promising feedstock material that may provide the sustainability and positive energy balance required is lignocellulosic biomass (Fig. 1.4) (Isikgor and Becer, 2015).

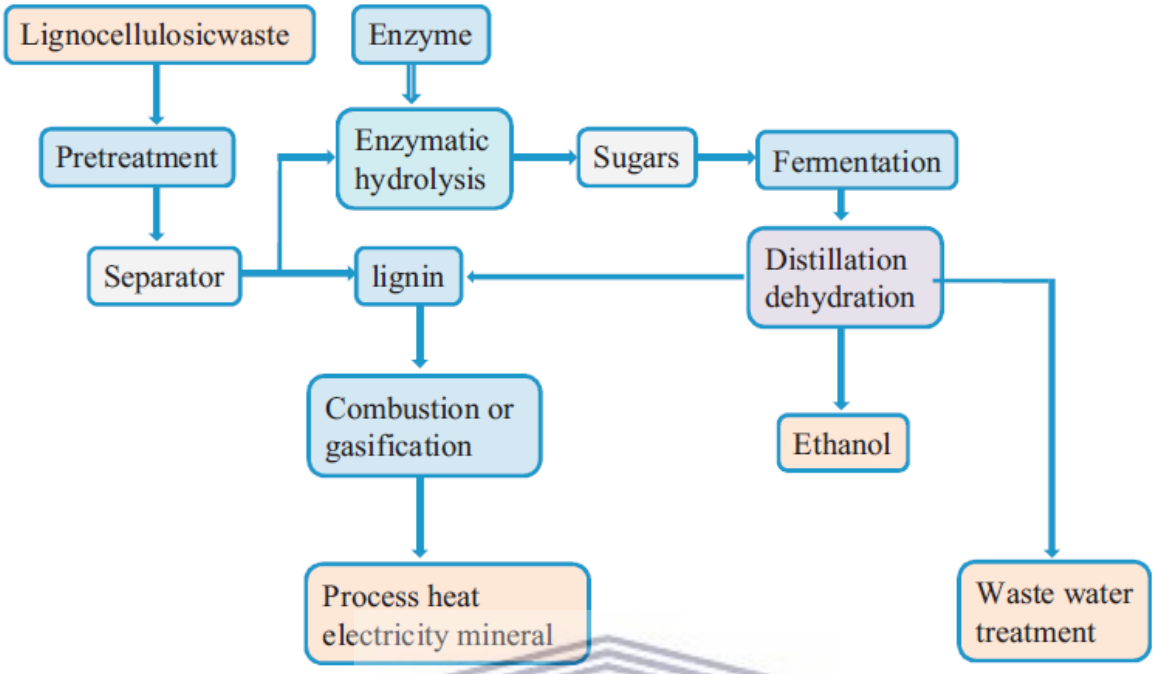


Figure 1.4: The production of ethanol from pre-treated biomass (Vohra *et al,* 2014).

1.3.1. Lignocellulosic biomass

Lignocellulosic biomass is considered a sustainable carbon source as it is generated through available CO_2, sunlight and water by means of photosynthesis (Isikgor and Becer, 2015). The principal fuels that can be produced with the use of biomass include ethanol, methanol, hydrogen as well as biodiesel. The fuel that has become of greatest interest in recent years, among these four, has been bioethanol. Lignocellulosic biomass is the most abundant raw material on earth and has sparked much interest for the production of fuels in a sustainable way (Hasunuma and Kondo, 2011; Isikgor and Becer, 2015).

Sources of lignocellulosic biomass include forestry, woody materials, agricultural wastes as well as grasses to name a few (Anwar *et al*, 2014). Lignocellulose consists of three main polymers namely cellulose, hemicellulose and lignin, along with smaller amounts of other components such as proteins, oils and ash (Fig. 1.5) (Lane *et al,* 2018). The major sugar components, cellulose and hemicellulose, are the feedstocks for 2G fuels (Chen, 2014). The three polymers are not uniformly distributed in the cell wall (Fig. 1.5). In most cases the distribution of the polymers in

lignocellulosic biomass are 40-55% of cellulose, 20-50% hemicellulose and 25-50% lignin, varying with the plant species (Den Haan *et al*, 2013).

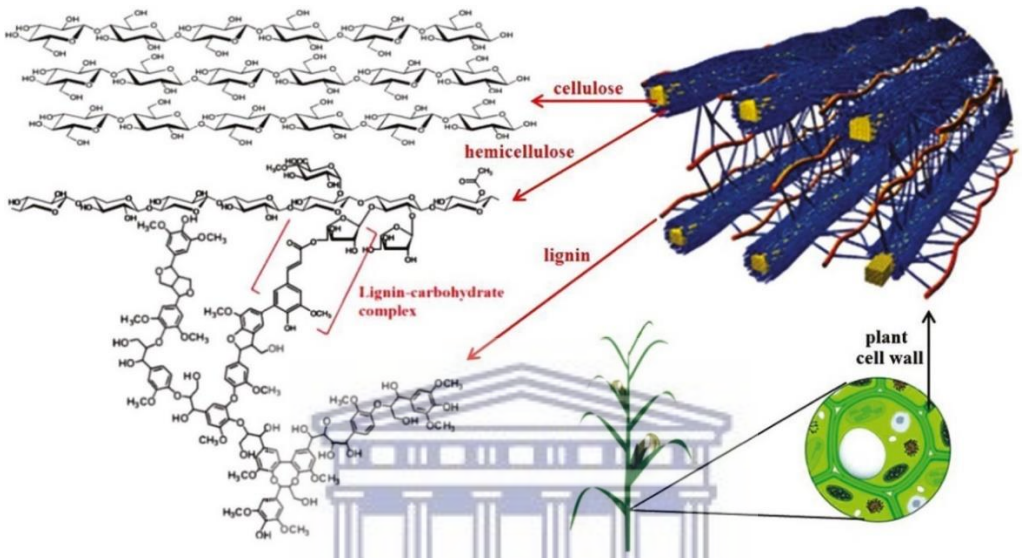


Figure 1.5: Illustration of the structure of lignocellulosic biomass consisting of cellulose, hemicellulose and lignin. The bonding patterns between the three polymers are also shown (Volynets *et al*, 2016).

Cellulose molecules arrange by gathering into bundles and thus determine the framework of the plant cell wall (Kumar *et al,* 2009*)*. The repeating unit in the cellulose chain is the disaccharide cellobiose which is two glucose residues connected with a β-1, 4-bond. Cellulose structure further consists of a hydrogen bonding network with intermolecular as well as intramolecular bonds. The second most abundant component, is hemicellulose. The structure of this polymer is quite different to that of cellulose and it consists of more random and amorphous heteropolymers. These heteropolymers are composed of various 5- and 6- carbon monosaccharide units, and include acetylated sugars. The bonds that make up this complex structure give structural strength to the plant cell wall by connecting cellulose fibres into micro fibrils. Lignin is composed of phenylpropanoid units which provide strength to the cell wall and protection against pathogens and insects (Kumar *et al,* 2009).

Biomass pre-treatment is a crucial step in the conversion of biomass to bioethanol (Harun *et al,* 2011c). This step provides the bases for the breakdown of the crystalline structure of cellulose for the release of the fermentable sugars (Fig. 1.6). There are many ways in which the process can be undertaken. The categories in which pre-treatment is divided into include mechanical, biological, chemical and physiochemical, with many variations done using combinations of these. Combinations include physical treatment, such as grinding or milling followed by ammonia fibre explosions or thermo-chemical methods, such as steam explosion (Alvira *et al*., 2010). The ultimate goal is to increase the surface area on the specific biomass which would enhance the possibility for enzyme access by opening its physical structure. This would allow for hydrolysis to take place more efficiently and effectively with greater sugar yields (Mosier *et al*, 2005). Unfortunately, the pre-treatment process causes the release of inhibitors, which ultimately interfere with the hydrolysis and the fermentation stages (Sun and Chen, 2002). Furthermore, this step remains costly and contributes the largest cost factor to the overall expenditure in bioconversion of biomass to bioethanol (Alvira *et al,* 2010).

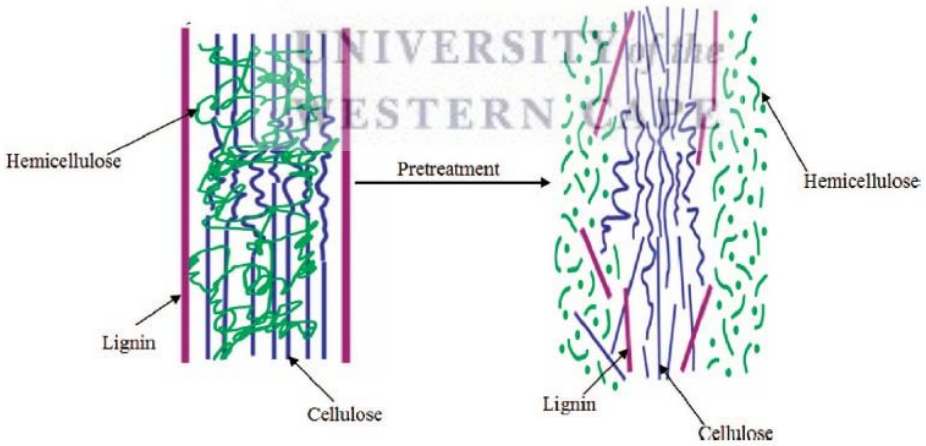


Figure 1.6: Schematic diagram of the role of pre-treatment in the conversion of biomass to liquid biofuels (Kumar *et al*, 2009). Pre-treatment breaks down the lignin component exposing cellulose to enzymes for breakdown to release fermentable sugars.

There are two main hydrolysis methods that are extensively used to produce the monomeric sugars for fermentation from lignocellulosic biomass (Saha *et al*, 2005). Acid hydrolysis involves dilute acids or concentrated acids dissolving the hemicellulose and disassembling the cellulose

components into fermentable sugars. Enzymatic hydrolysis in contrast uses enzymes for the release of fermentable sugars from the biomass (Wayman, 1996). Once sugars are released, fermentation can occur after which ethanol can be distilled from the broth.

For the hydrolysis of the cellulose fraction the activity of three major cellulases: endoglucanases (EGs), exoglucanases as well as β- glucosidases is required (Van Zyl *et al*. 2011). The endoglucanases hydrolyse the amorphous regions of the cellulose which breaks the β-1, 4 bonds and results in the formation of cellodextrins and new chain ends. Exoglucanases including cellobiohydrolases (CBHs) hydrolyse the crystalline regions of cellulose and thus yields cellobiose. The β-glucosidase (BGLs) then converts these products into glucose. The hemicellulose component requires a large complement of enzymes to hydrolyse it completely (Johnson and Martin, 2016). Xylan, the most abundant component of hemicellulose, requires xylanase (Xyn) and β- xylosidase to release D-xylose from its sugar backbone. In addition, it may also require esterases, arabinofuramosidases, α-glucuronidases and other side chain cleaving enzymes, depending on the type of hemicellulose involved.

1.4. Integrated technologies

Several technologies have been suggested for biological 2G ethanol production, which all require enzyme production, hydrolysis and fermentation of the different component sugars (Harun *et al,* 2011c). These technologies are used to help improve the production level of bioethanol while lowering the cost at the same time (Fig. 1.7). Currently, there are four conversion technologies which include separate hydrolysis and fermentation (SHF) where all biological processes are done separately, simultaneous saccharification and fermentation (SSF) where hydrolysis and fermentation of C-6 sugars are done at the same time, simultaneous saccharification and co-fermentation (SSCF) where hydrolysis and fermentation of all sugars take place at the same time and Consolidated bioprocessing (CBP), potentially the more cost effective option, which includes all four biological processes being done in a single reactor (Fig. 1.7).

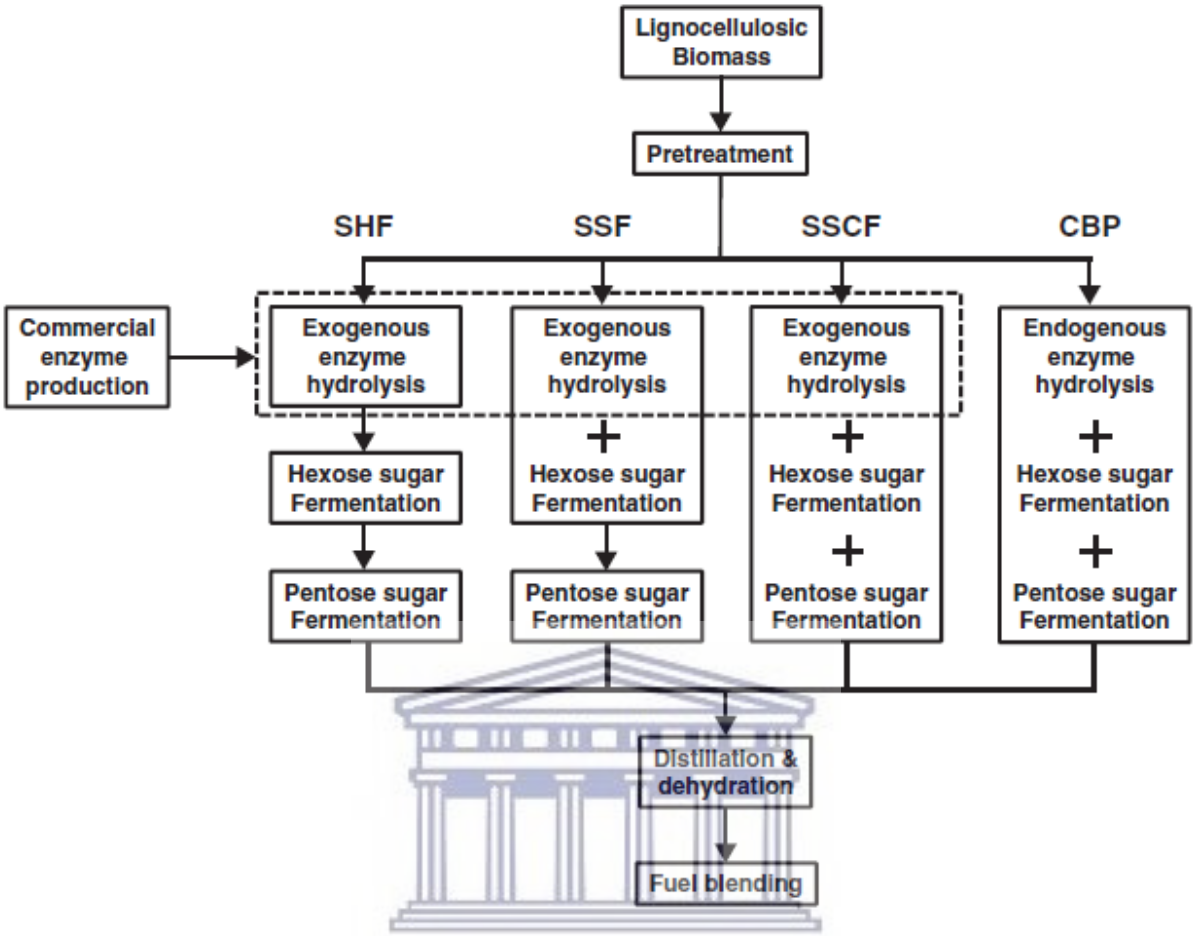


Figure 1.7: Depiction of the integrated technologies to convert pre-treated biomass to ethanol (Den Haan *et al*, 2015). The various integrated technologies that are used includes, separate hydrolysis and fermentation (SHF), simultaneous sachharification and fermentation (SSF), simultaneous sachharification and co- fermentation (SSCF) and consolidated bioprocessing (CBP).

SHF requires enzymatic hydrolysis being performed separately from fermentation (Tomas-Pejo *et al.,* 2008). The advantage of this technology is that the separate vessels allow for optimum conditions for each step, allowing enzymes and fermentation organisms to function at their respective optimal temperatures. However, one of the drawbacks with this technology is that there is an accumulation of hydrolysis product (glucose and cellobiose), leading to feedback inhibition, which ultimately reduces the rate of enzyme hydrolysis (Balat *et al,* 2008). SSF involves fermentation of C- 6 sugars and hydrolysis in the same reactor, which combats the feedback inhibition disadvantage of SHF, as the fermentation continually removes the sugars released (Lin and Tanaka, 2006). Although there are high ethanol yields produced, the disadvantage lies in

finding the temperature in which both of these reactions can occur simultaneously. SSCF is considered as an improvement over the technology of SSF, as the hydrolysis and fermentation are combined in a one reactor, and hexose and pentose fermentations are combined. All of these technologies make use of enzymes produced outside of the reactor, making these processes costly (Olsen *et al,* 2012). SHF and SSF are the technologies currently applied in the 2G bioethanol industry (Lynd *et al*, 2017).

1.4.1. Challenges in converting biomass to biofuels

There are many features of cellulosic plant biomass that make it desirable as a feedstock for biofuels, however, several problems have been identified in the conversion process (Harun *et al,* 2011c). These include the main challenge that the process is costly, partly due to the separation of steps. An ideal process to combat this is consolidated bioprocessing (CBP) which involves pre-treated cellulosic biomass being fermented to a desired product without the need for the addition of externally produced enzymes (Jansen *et al.* 2017). CBP has gained interest as a potential breakthrough for low cost biomass processing (Van Zyl *et al*, 2007), as it combines enzyme production, hydrolysis and fermentation of hexose and pentose sugars in a single reactor (Fig 1.8) (den Haan *et al,* 2015).

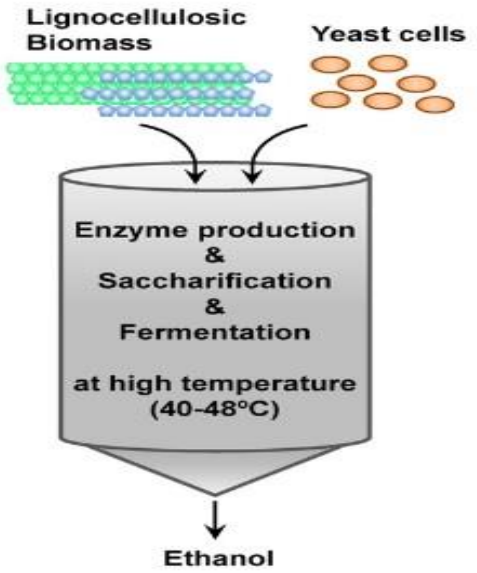


Figure 1.8: Consolidated bioprocessing is an integrated technology which involves four biological processes in a single reactor. Temperatures used will vary with the choice of fermentation organisms (Hasunuma and Kondo, 2012)

The drawback of the CBP process is that there is no known organism that exists which can perform all of these processes in one biological step at the rates and titres required by industry (Vohra *et al*, 2014). The microorganism for CBP must be able to do such processes, meaning, the microbe has to solubilize a biomass substrate and produce the desired product with a very high yield under the industrial conditions.

1.5. CBP organism

Microorganisms which exhibit a combination of substrate utilization as well as desirable product formation properties are required for CBP (Van Zyl *et al*., 2007). The production of cellulolytic enzymes continues to be a major objective and cost factor in biofuel production (Olsen *et al,* 2012, Lynd *et al*, 2017). There are currently two categories which are being pursued by researchers to engineer microbes for CBP. These include (i) the engineering of microorganisms that naturally hydrolyse cellulose to improve their product related properties by increasing yields and titres and (ii) the recombinant cellulolytic strategy that involves the engineering of non-

cellulolytic organisms which show great promise in exhibiting high yields of product to ultimately express a heterologous cellulase system enabling cellulose utilization (Lynd *et al*., 2005). *Saccharomyces cerevisiae* is the world's foremost industrial ethanol production microorganism and a promising organism for consolidated bioprocessing (Den Haan *et al*, 2013). However, *S. cerevisiae* lacks the ability to perform fermentation of pentose sugars, and cannot produce enzymes to hydrolyse cellulose and thus requires significant engineering to be considered as a CBP organism (Fig. 1.9).

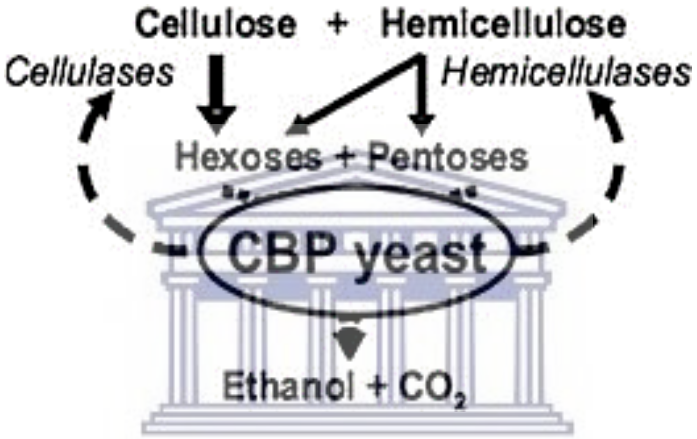


Figure 1.9: Consolidated bioprocessing using recombinant *S. cerevisiae* (Van Zyl *et al*, 2007).

1.5.1. *Saccharomyces cerevisiae* as heterologous protein production and CBP host

S. cerevisiae is an excellent eukaryotic platform for biofuels and chemical production and a model organism used for basic research, which enables the discovery of functional relationships between sequence and gene products in fungi and higher organisms (Hou *et al,* 2012b). For thousands of years, yeasts have been used in the production of alcoholic beverages. *S. cerevisiae* is the most common yeast that is used in industry for ethanol production as it can be cultivated at low cost and can tolerate a wide range of pH and ethanol concentrations (Vohra *et al*, 2014). While a single perfect host does not exist for every heterologous protein, several protein expression systems have been established which include the yeast *S. cerevisiae* (Mattanovich *et al,* 2011). *S. cerevisiae* has also been used as a platform for biopharmaceutical production with

almost 20% of approved pharmaceutical peptide products currently being synthesized by this host (Delic *et al*, 2013, Rodríguez-Limas *et al*, 2015). Although bacteria are efficient protein producers, they cannot perform several of the required post translational modifications (Mattanovich *et al,* 2011). Yeasts are able to perform modifications including glycosylation, phosphorylation as well as removal of introns. They are fast growing having a doubling time of over 90 minutes and reproduce via budding to high cell density on inexpensive media, with the ability to be easily genetically manipulated.

There have been reports of the expression of cellulase encoding genes in *S. cerevisiae* to either study the enzymes themselves or to enable the yeast to hydrolyze cellulosic substrates (Den Haan *et al,* 2015). These follow strategies such as free enzymes that are secreted into the media and cell wall tethered enzymes (Lane *et al,* 2018; Hasunuma and Kondo, 2014). Each strategy comes with advantages and disadvantages. The free enzyme strategy is limited by the amount of enzyme which can be secreted but is not affected by surface display limitations. However, secreted free enzymes diffuse away from the cell that produced them, resulting in less enzymes being recycled. In contrast, enzymes which are immobilized to the cell surface are maintained along with their catalytic products (Lane *et al,* 2018). However, cell immobilization could also lead to diminished crystalline cellulose conversion as the immobilized systems may suffer from inefficiency of their processive enzymes which are the CBHs.

Recombinant *S. cerevisiae* strains have thus been created to have the ability to convert cellobiose, amorphous cellulose and crystalline cellulose to ethanol (Lane *et al,* 2018; Feng *et al,* 2015). However, a general feature among these reports where the low heterologous CBH secretion titres severely limiting conversion efficiency (Den Haan *et al.* 2013; Kroukamp *et al,* 2018). There have been very few reports that have concentrated on ratios of surface- displayed cellulase enzymes because regulating the enzyme ratio in non-complexed cellulase systems is very challenging (Lui *et al*, 2017). Recently optimization of cellulose ratios by a cocktail δ-intergration method was achieved by screening transformants on cellulose as a substrate. This produced strains that achieved relatively high ethanol yield from pre-treated rice straw in the

absence of exogenous cellulase. It was shown that partially cellulolytic strains could significantly decrease the amount of exogenous enzymes that had to be added to achieve conversion of cellulosic substrates to ethanol (Feng *et al,* 2015). In addition to utilizing all available sugar substrates, CBP yeasts should also thrive in a hostile fermentation environment that will include pre-treatment derived inhibitors and eventually high ethanol concentrations. While yeast are comparatively robust, further engineering may be required to enhance its environmental responses to enable it as a CBP organism (Desparis *et al,* 2017).

S. cerevisiae has been used as a host for the production of heterologous cellulases with the goal of enabling CBP (Den Haan *et al*, 2015). However, it was shown that heterologous cellulase secretion levels were too low to sustain the growth of yeast on crystalline cellulose feedstocks. Recent studies explored strain engineering options to improve heterologous cellulase secretion by *S. cerevisiae* (Kroukramp *et al,* 2018). Overexpression of foldases, chaperones and vesicle fusion proteins were explored as options to improve secretion (Van Zyl *et al,* 2014; Van Zyl *et al,* 2016; Kroukamp *et al,* 2018). While improvement in secretion of all three classes of cellulases were reported, protein-specific results were always seen with no option yielding uniformly improved secretion. In this study we investigated the roles of proteolysis and more particularly of endocytosis to see if manipulation of these factors can be used to improve secretion titres. This information can then be used to create better *S. cerevisiae* CBP strains.

1.6. Proteolysis

1.6.1. Introduction to proteolysis

Proteolysis is an essential part of life as it plays an important role in nitrogen metabolism, protein turnover and the cellular response to stress (Hilt and Wolf, 1992). The intracellular concentration of a particular protein is dependent on the rates of its synthesis and degradation and as such both events must be tightly regulated (Zhang *et al*, 2007). Proteolysis which occurs within the cell creates a risk of undesired cleavage of active or functional proteins. As such, selectivity is of importance to sort out which proteins have to remain and which proteins have to be hydrolysed.

Selective proteolysis is implemented in the regulation of the rate-limiting steps involved in biosynthetic pathways.

1.6.2. Protein secretion pathway

Protein sorting or targeting is the transport of newly synthesized proteins to specified cellular destinations and includes two initial processes which may be involved (Lodish *et al*, 2000). The first process occurs after translation and involves the targeting of proteins to intracellular organelle membranes. The second process involves proteins which are targeted to the endoplasmic reticulum and subsequently transported through the cellular membranes to specific organelles or the plasma membrane, known as the protein secretion pathway (Fig. 1.10) (Delic *et al*, 2013). Protein secretion is of importance for all living organisms (Zhang *et al*, 2007). For bacteria, secreted proteins have functions either in the cell wall or membrane or in the exterior space. Eukaryotes on the other hand produce many proteins which are then targeted to the various intracellular compartments of the cell where they function. The pathway is initiated by the translocation of a newly translated protein through the endoplasmic reticulum (ER) membrane, to the ER and then to the Golgi apparatus (Fig. 1.10).

In yeast, proteins enter the secretion pathway by post translational translocation into the ER lumen where they are then folded into their native structure under quality control (Anelli and Sitia, 2008). They are bound by chaperone proteins which facilitate protein folding into native structures (Delic *et al*, 2013). Misfolded proteins bind to a complex called the BiP, encoded by *KAR2,* which acts as a quality control system that then redirects these proteins to the cytoplasm. This process goes by the name of ER-associated protein degradation. The proteins which were folded in the correct manner are transported to the Golgi apparatus with the help of special soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) vesicles for further modification and transport (Idris *et al,* 2010). The recycling of proteins which have retention signals is then done and is accomplished by the retrograde transport by protein complex I vesicles. The proteins going toward the membrane are sorted by clathrin coated vesicles (Mellman and Warren, 2000). It can thus be said that the Golgi apparatus plays a maturing role in the transportation and maturation of proteins to the secretion vesicles. The

proteases which are part of the secretory pathway are located mainly in the Golgi apparatus and in the plasma membrane and act by processing of precursors to one or more secreted peptides (Hilt and Wolf, 1992).

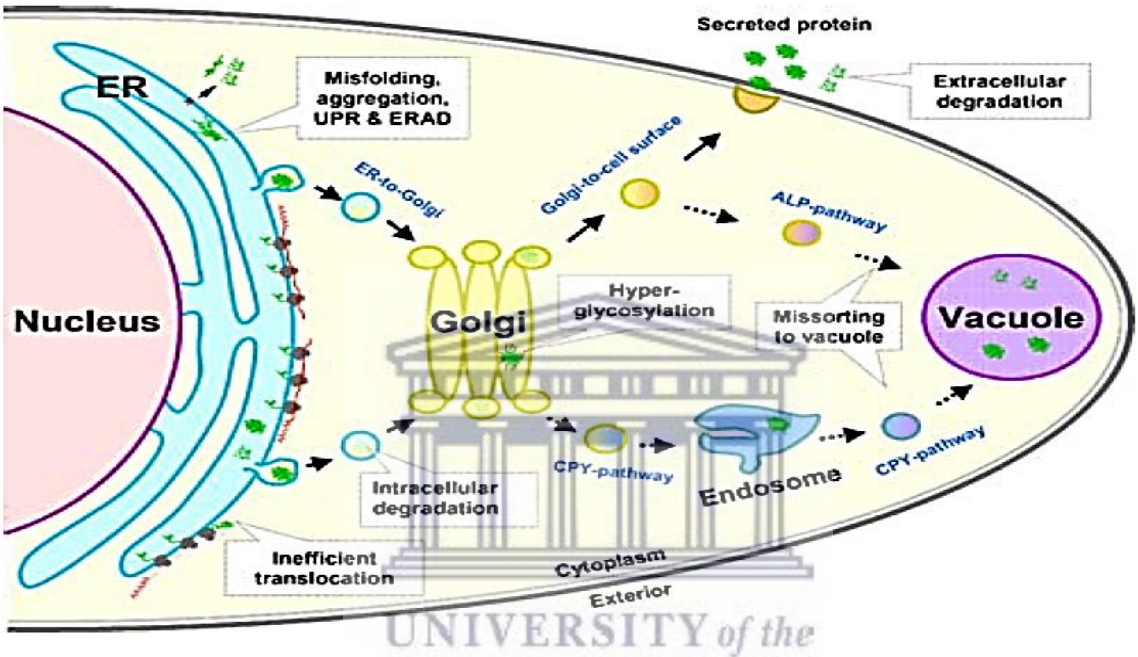


Figure 1.10: A representation of the yeast secretion pathway detailing steps of secretion as well as the major bottlenecks of heterologous protein secretion in yeast (Idris *et al,* 2010).

1.6.3. Heterologous protein secretion

Yeasts provide a platform for production of many of heterologous proteins used in various industries (Lui, 2013). However, heterologous protein secretion is not as efficient in yeast as in other systems such as filamentous fungi. There are many potential bottlenecks in the secretion pathway (Fig 1.10) (Idris *et al*, 2010). Protein folding in the ER can become a major bottleneck due to the strict quality control (QC) systems (Gasser *et al*, 2009). Recombinant protein secretion involves many limiting factors and include the translocation of the proteins to the ER, the incorrect folding of the proteins and QC in the ER, incorrect glycosylation in the ER as well as in

the Golgi apparatus and missorting of proteins to the vacuole leading to an increase in the degradation of proteins. This leads to the lowering of the titre of heterologous protein secretion and may also cause stress in the cell (Hou *et al*, 2012a). As such, a variety of studies have been aimed at improving protein production as well as secretion in *S. cerevisiae*. There are many additional factors that affect the titre of heterologous protein in yeast including (i) properties of the target protein, (ii) the host strain and (iii) the cultivating systems. This has resulted in many efforts focused on increasing protein secretion and alleviating these problematic factors (Den Haan *et al,* 2013; Kroukamp *et al,* 2018). Endocytosis is one aspect that was investigated as a possible negative factor to the goal of increasing the titre of protein secretion in *S. cerevisiae*.

1.7. Endocytosis

1.7.1. Introduction to Endocytosis

There are two forms of bulk transport whereby molecules are transported either into or out of the cell which results in the use of energy in the form of Adenine Triphosphate (ATP) (Cooper, 2000). These transportation systems are known as endocytosis and exocytosis (Fig. 1.11). Exocytosis is the term given to the process whereby a vesicle is released from a cell (Battey *et al,* 1999). It is the final step in the secretory pathway. Endocytosis however, is a process that involves vesicle recovery (Park *et al,* 2016). In mammalian and *S. cerevisiae* cells, internalization and degradation of membrane proteins are a major function of endocytosis (Wu *et al*, 2016). The term endocytosis was given as it involves ingestion of molecules as well as the uptake of fluids. The process involves capturing material from the cell surface and the transportation of the material into the cytoplasm (Casem, 2016). The material is taken into the cell inside membrane-bound vesicles formed by the phospholipid bilayer of the plasma membrane (Pastan and Willingham, 1985). Plasma membrane surrounds the molecule that is ingested which buds off during the process inside the cell and forms a vesicle.

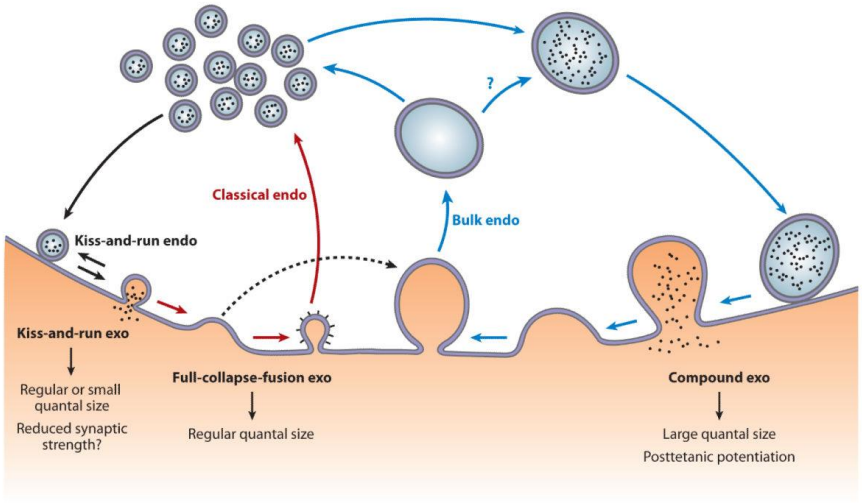


Figure 1.11: A schematic diagram showing endocytosis and exocytosis (Wu *et al,* 2016). For endocytosis it can be seen that the cell takes up the molecule forming a capsule as the molecule enters the cell while exocytosis shows the molecule leaving the cell with the vesicle being ruptured.

This process was discovered over a century ago in white blood cells, which are known as the body's "professional phagocytes" (Cooper, 2000). Endocytosis is a process which has a wide range of functions involved in nutrient absorption, drug transportation cellular homeostasis and receptor signalling regulation. As such, malfunctions in this process can cause a number of fatal diseases. Endocytosis involves a carrier or a channel that facilitates the movement or transport of macromolecules such as proteins or polysaccharides through a phospholipid bilayer into the cell from the surrounding medium (Fig 1.12). Endocytosis can be classified into three types. These types include phagocytosis, pinocytosis and receptor-mediated endocytosis.

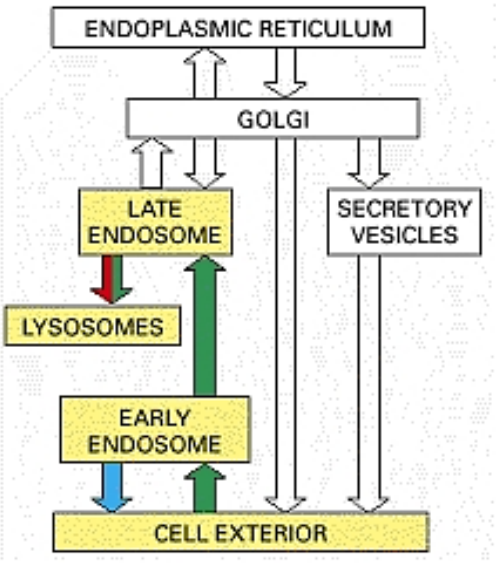


Figure 1.12: The different stages of endocytosis (Alberts *et al*, 2002). The stages of endocytosis showing the movement from the cell exterior to the endoplasmic reticulum.

1.7.2. Phagocytosis

Phagocytosis is known as the cell eating type of endocytosis (Cooper, 2000). Cells make use of the process of phagocytosis to engulf large particles such as cell debris as well as bacterial cells and intact cells, in the case of white blood cells (Fig 1.13). The process begins by the binding of particles to receptors which are on the surface of a phagocytic cells which triggers pseudopodia to be extended. Pseudopodia are an actin based movement of the cell surface. The particle is then surrounded by the pseudopodia and the vesicles start fusing, resulting in the formation of an intracellular vesicle known as a phagosome. These then produce phagolysosomes where the phagosome fuses with the lysosome, resulting in the recycling of membrane proteins by maturation of the phagolysosomes.

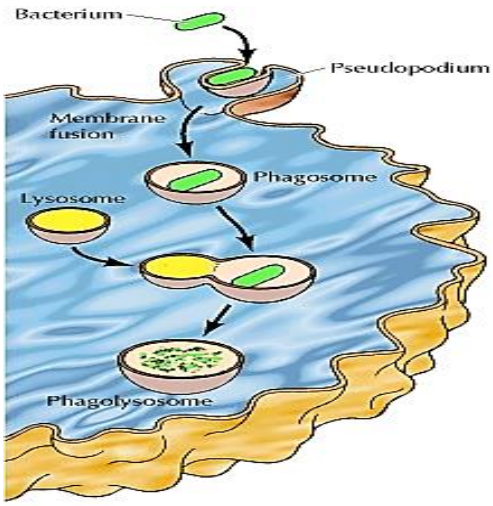


Figure 1.13: Phagocytosis is a form of endocytosis where the cell takes up solid particles from the exterior (Cooper, 2000).

1.7.3. Pinocytosis

Pinocytosis has specialized roles and is common amongst eukaryotes (Cooper, 2000). The process is referred to as cell drinking as solutes are taken into the cell. The rate of pinocytosis varies between cells. The cell surface area and volume remains unchanged as what is added by endocytosis is extracted by exocytosis (Alberts *et al*, 2002). Unlike the receptor-mediated endocytosis and phagocytosis, pinocytosis is relatively non-specific about molecules that are taken into the cell.

1.7.4. Receptor- Mediated Endocytosis

Receptor- mediated endocytosis is a process whereby a mechanism is provided for specific molecule selection for uptake (Cooper, 2000). The specific macromolecules which are about to be internalized are first bound to receptors on the cell surface (Fig 1.14). The receptors are concentrated in regions known as clathrin-coated pits on the plasma membrane. These then bud from the membrane internally and form vesicles which contain not only the bound macromolecules but also the receptors. These vesicles then fuse with endosomes where the

contents are subsequently sorted for transportation to the lysosomes or recycled to the plasma membrane.

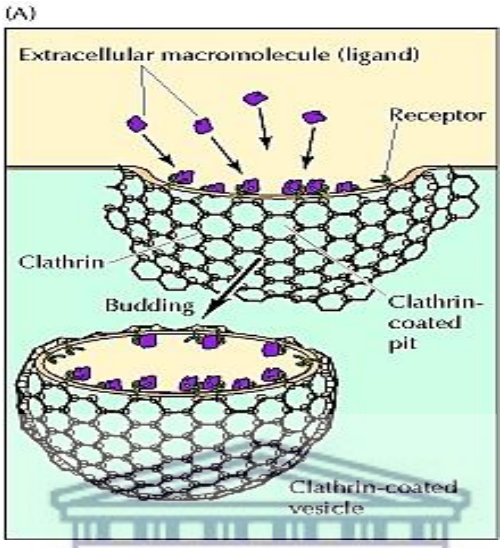


Figure 1.14: Receptor- mediated endocytosis requires various receptors that aid the take up of molecules from the exterior of the cell (Cooper, 2000).

1.7.5. Blocking Mechanisms of Endocytosis

As stated earlier, *S. cerevisiae* is not only a useful platform for protein production of biopharmaceuticals and industrial enzymes, but is also useful in the production of biofuels (Rodríguez-Limas *et al,* 2015; Kroukamp *et al* 2018). To date, substantial effort has focused on alleviating several bottlenecks in the expression and the secretory pathway to improve heterologous cellulase secretion in this yeast (Idris *et al,* 2010; Kroukamp *et al*, 2018). Endocytosis is a very complex process which involves many different proteins (Kaksonen and Roux, 2018). These proteins play roles in the different stages of endocytosis. Recently, it has been shown that highly active endocytosis could decrease the overall heterologous protein titres in the yeast supernatant (Rodríguez-Limas *et al*, 2015). Endocytosis can potentially cause newly secreted heterologous cellulases to be immediately taken up and degraded.

A study showed that blocking certain endocytotic mechanisms improved heterologous protein production in *S. cerevisiae* (Rodríguez-Limas *et al*, 2015). Mutant strains with *END3* and *RVS161* knockouts produced 1.95- and 3.12- fold more heterologous α-amylase compared to the control strains after cultured adaptation of the strains. As per the study mentioned, *END3* and *RVS161* are directly involved in the process whereby *END3* is part of the initiating step. This suggests a strategy to increase the heterologous secretion titre by blocking endocytosis genes. In this study, the effects of blocking endocytosis by the knockout of genes involved in the process was evaluated. We expressed various cellulase encoding genes in strains where a range of genes involved in endocytosis were knocked out. This yielded clues as to the importance of endocytosis in heterologous cellulase secretion. Increased secreted cellulase titres would have major benefits for the biofuel industry, as it could improve the efficiency of CBP yeast strains.

Thirteen gene knock- outs were selected for this study and their products are associated with different complexes. The genes that made the most impact in this study were *RVS161* and *END3. RVS161* encodes amphiphysin- like lipid protein involved in actin cytoskeleton polarization which regulates endocytosis and is involved in cell fusion, cell polarity and the viability which follows osmotic stress or starvation (Lombardi and Riezman, 2001; Rondriquez- Limas *et al*, 2015). *END3* encodes a protein that is needed in the actin cytoskeleton organization and forms part of the initializing step of endocytosis. *VPS45* is needed for fusion of Golgi- derived vesicles with pre-vacuolar compartment and thus forms an important part of vacuolar protein sorting (Bryant and James, 2001). *CLC1* is involved in endocytosis and the intracellular protein transport forming part of the major coat protein (Silveira *et al*, 1990). *VRP1* encodes a protein that is involved in cytokinesis and cytoskeletal organization (Donnelly *et al*, 1993). *YPT7* is involved in the trafficking via the late endosome to the vacuole after the alkaline phosphatase pathway (Schimmoller and Riezman, 1993). *VSM1* encodes a DNA damage- inducible v- SNARE binding protein and plays a role in suppressing protein secretion (Lustgarten and Gerst, 1999). *VTA1* is a multivesicular protein which is involved in endosomal protein sorting and promotes oligomerization by regulating the VPS4P protein. *VPS10* encodes a type I transmembrane sorting receptor used for multiple vacuolar hydrolases. *MNN10* encodes a membrane protein that mediates elongation of the polysaccharide mannan backbone along with manosyltransferases (Bartkevičiūtė and

Sasnauskas, 2004). SSA4 is a heat shock protein which is induced upon stress. UTH1 is known to be implicated in cell wall biogenesis and is a mitochondrial inner membrane protein.

1.8. Objectives of this study

In this study we evaluated the effects of blocking endocytosis by the knockout of relevant genes involved in the process on heterologous lignocellulase production in yeast. This would yield clues as to the importance of endocytosis in heterologous cellulase secretion. The increase of secreted cellulase titre would have major benefits for the biofuel industry. The objectives of the study included the following:

- Receive 13 yeast strains from the EUROSCARF strain collection with knockouts in endocytosis related genes.
- Transform each of the 13 strains, respectively, inclusive of a reference strain, with 4 expression plasmids encoding 3 cellulase and one xylanase gene.
- Confirmation of these transformants with PCR and plate assays.
- Testing the activity and secretion levels of the yeast transformants while noting changes in ethanol and temperature tolerance and monitoring growth.
- Construction of double gene knock-outs using the best possible mutations which had positive effects on the secretion of the heterologous cellulase.
- Testing the activity and secretion levels of the double gene knock-out mutants
- Noting changes in ethanol and temperature tolerance while monitoring growth of the mutants.

The following chapter will cover the materials and methods which were used during this study. That will then be followed by the results and discussion, chapter three. Lastly there will be a final chapter with a summative discussion and an outlook on the future direction of the study.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Media and culturing conditions

For plasmid propagation, *E. coli* DH5α was used. Bacterial cultivation was done using Terrific Broth which contained 100 µg/ml ampicillin. Cultures were incubated overnight at 37°C on a rotary wheel. *S. cerevisiae* strains were obtained from the EUROSCARF strain collection (IWBT, Stellenbosch University) and transformed with plasmids detailed below. The *S. cerevisiae* strains were cultivated in YPD (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose) medium at a temperature of 30°C shaking at 180 rpm, unless stated otherwise. *S. cerevisiae* strains were alternatively grown on selective SC^{-URA} agar (1.7 g/L yeast nitrogen base with amino acids (Difco), 1.5 g/L amino acid dropout pool without uracil (Sigma), 20 g/L glucose, 5 g/L ammonium sulphate and 20g g/L agar) plates when required. The initial screening of Eg2 and Xyn2 transformants started by streaking strains onto SC^{-URA} agar plates containing either 1% CMC (Sigma) or 0.2% Remazol Brilliant Blue (RBB)-xylan (Sigma) and incubating the plates at 30˚C for 24 hours. Enzyme assays were performed following cultivation of yeast strains for 72 hours in 100 ml flasks which contained 10 mL SC^{-URA} medium for selection. For stress related assays, strains were cultured in 10 mL SC^{-URA} media plates that were supplemented with ethanol (10%, 20% or 30%) and, for heat stress cultures, were grown at 30 and 35°C.

2.2. Recombinant yeast strain construction

Genotypes of the strains and plasmids used in this study are summarised in Table 1. Four previously constructed episomal plasmids were used in this study namely pRDH180 containing *Trichoderma reesei eg2* (endoglucanase), pRDH182 containing *T. reesei xyn2* (xylanase), ySFI containing *Saccharomycopsis fibuligera bgl1* (β-glycosidase encoding gene, also named *cel3A*) and pMI529 containing *Talaromyces emersonii cbh1*-CCBM (Table 1). The yeast transformations of the BY4742 and mutant strains were carried out with the use of a LiOAc/DMSO protocol (Hill *et al*., 1991). A pMU1531 vector that contained no heterologous gene was transformed to

create strains that could be used for negative controls. For positive controls, the parental *S. cerevisiae* BY4742 strain containing no endocytosis-related mutations was transformed with each of the above mentioned plasmids.

Table 2.1 List of vectors and yeast strains used in this study

Constructed yeast strains/plasmids	Abbreviated name	Relevant genotype	Reference
Plasmids:			
pMI529		*bla Shble URA3 ENO1p-T.e.cbh1*	(Ilmen *et al* 2011)
pRDH180_ EG2		*bla Shble URA3 ENO1p-Tr.EG2--ENO1t*	(Brevnova *et al*., 2011)
pRDH182_XYN2		*bla Shble URA3 ENO1p-Tr.XYN2--ENO1t*	(Brevnova *et al*., 2011)
pMU1531		*bla Shble URA3 ENO1p-ENO1t*	(Brevnova *et al*., 2011)
ySFI		*bla Shble URA3 PGK1p-bgl-PGK1t*	(Van Rooyen *et al*. 2005)
***S. cerevisiae* strains:**			
***S. cerevisiae* BY4742**		*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	(Brachmann *et al*., 1998)
BY4742 + pMU1531	Ref	*bla ura3/URA3 Shble ENO1p-ENO1t*	This work
BY4742+ pRDH180	Parental Eg2	*bla ura3/URA3 Shble ENO1p-Tr.EG2- ENO1t*	This work
BY4742 ::VRP1 + pRDH180	BY*Δvrp1*-Eg2	*bla VRP1Δ:: kanMX ura3/URA3 Shble ENO1p-Tr.EG2- ENO1t*	This work
BY4742 ::YPT7 + pRDH180	BY*Δypt7*-Eg2	*bla YPT7Δ:: kanMX ura3/URA3 Shble ENO1p-Tr.EG2- ENO1t*	This work
BY4742 VPS45 + pRDH180	BYΔ*vps45*-Eg2	*bla VPS45Δ:: kanMX ura3/URA3 Shble ENO1p-Tr.EG2- ENO1t*	This work
BY4742 RVS161 + pRDH180	BYΔ*rvs161*-Eg2	*bla RVS161Δ:: kanMX ura3/URA3 Shble ENO1p-Tr.EG2- ENO1t*	This work
BY4742 END3 + pRDH180	BYΔ*end3*-Eg2	*bla END3Δ:: kanMX ura3/URA3 Shble ENO1p-Tr.EG2- ENO1t*	This work

BY4742 CLC1 + pRDH180	BYΔ*clc1*-Eg2	*bla CLC1Δ:: kanMX ura3/URA3 Shble ENO1p-Tr.EG2- ENO1t*	This work
BY4742 ::MNN10 + pRDH180	BYΔ*mnn10*-Eg2	*bla MNN10Δ:: kanMX ura3/URA3 Shble ENO1p-Tr.EG2- ENO1t*	This work
BY4742 ::VPS10 + pRDH180	BYΔ*vps10*-Eg2	*bla VPS10Δ:: kanMX ura3/URA3 Shble ENO1p-Tr.EG2- ENO1t*	This work
BY4742 ::SSA4 + pRDH180	BY*Δssa4*-Eg2	*bla SSA4Δ:: kanMX ura3/URA3 Shble ENO1p-Tr.EG2- ENO1t*	This work
BY4742 ::UTH1 + pRDH180	BYΔ*uth11*-Eg2	*bla UTH1Δ:: kanMX ura3/URA3 Shble ENO1p-Tr.EG2- ENO1t*	This work
BY4742 ::VTA1 + pRDH180	BYΔ*vta1*-Eg2	*bla VTA1Δ:: kanMX ura3/URA3 Shble ENO1p-Tr.EG2- ENO1t*	This work
BY4742 ::VSM1 + pRDH180	BYΔ*vsm1*-Eg2	*bla VSM1Δ:: kanMX ura3/URA3 Shble ENO1p-Tr.EG2- ENO1t*	This work
BY4742 ::HAC1 + pRDH180	BYΔ*hac1*-Eg2	*bla HAC1Δ:: kanMX ura3/URA3 Shble ENO1p-Tr.EG2- ENO1t*	This work
BY4742 + pRDH182	Parental Xyn2	*bla ura3/URA3 Shble ENO1p-Tr.xyn2- ENO1t*	This work
BY4742 ::VRP1 + pRDH182	BYΔ*vrp1*-Xyn2	*bla VRP1:: kanMX ura3/URA3 Shble ENO1p-Tr.xyn2- ENO1t*	This work
BY4742 ::YPT7 + pRDH182	BYΔ*ypt7*-Xyn2	*bla YPT7 ura3/URA3 Shble ENO1p- Tr.xyn2- ENO1t*	This work
BY4742 ::VPS45 + pRDH182	BYΔ*vps45*-Xyn2	*bla VPS45Δ:: kanMX ura3/URA3 Shble ENO1p-Tr.xyn2- ENO1t*	This work
BY4742 ::RVS161 + pRDH182	BYΔ*rvs161*-Xyn2	*bla RVS1611Δ:: kanMX ura3/URA3 Shble ENO1p-Tr.xyn2- ENO1t*	This work
BY4742 ::END3 + pRDH182	BYΔ*end3*-Xyn2	*bla END3Δ:: kanMX ura3/URA3 Shble ENO1p-Tr.xyn2- ENO1t*	This work
BY4742 ::CLC1 + pRDH182	BYΔ*clc1*-Xyn2	*bla CLC1Δ:: kanMX ura3/URA3 Shble ENO1p-Tr.xyn2- ENO1t*	This work
BY4742 + pRDH182	Parental Bgl	*bla ura3/URA3 Shble PGK1p-Tr.XYN2- PGK1pt*	This work
BY4742 ::MNN10+ pRDH182	BYΔ*mnn10*-Xyn2	*bla MNN10Δ:: kanMX ura3/URA3 Shble ENO1p-Tr.xyn2- ENO1t*	This work
BY4742 ::VPS10 + pRDH182	BYΔ*vps10*-Xyn2	*bla VPS10Δ:: kanMX ura3/URA3 Shble ENO1p-Tr.xyn2- ENO1t*	This work

BY4742 ::SSA4 + pRDH182	BYΔ*ssa4*-Xyn2	*bla SSA4Δ:: kanMX ura3/URA3 Shble ENO1p-Tr.xyn2- ENO1t*	This work
BY4742 ::UTH1 + pRDH182	BY*Δuth1*-Xyn2	*bla UTH1Δ:: kanMX ura3/URA3 Shble ENO1p-Tr.xyn2- ENO1t*	This work
BY4742 ::VTA1 + pRDH182	BYΔ*vta1*-Xyn2	*bla VTA1Δ:: kanMX ura3/URA3 Shble ENO1p-Tr.xyn2- ENO1t*	This work
BY4742 ::VSM1 + pRDH182	BYΔ*vsm1*-Xyn2	*bla VSM1Δ:: kanMX ura3/URA3 Shble ENO1p-Tr.xyn2- ENO1t*	This work
BY4742 ::HAC1 + pRDH182	BYΔ*hac1*-Xyn2	*bla HAC1Δ:: kanMX ura3/URA3 Shble ENO1p-Tr.xyn2- ENO1t*	This work
BY4742 ::VRP1 + ySFI	BYΔ*vrp1*-Bgl	*bla VRP1Δ:: kanMX ura3/URA3 Shble PGK1p-S.f.bgl1- PGK1pt*	This work
BY4742 ::YPT7 + ySFI	BYΔ*ypt7*-Bgl	*bla YPT7Δ:: kanMX ura3/URA3 Shble PGK1p-S.f.bgl- PGK1t*	This work
BY4742 ::VPS45 + ySFI	BYΔ*vps45*-Bgl	*Bla VPS45Δ:: kanMX ura3/URA3 Shble PGK1p-S.f.bgl- PGK1t*	This work
BY4742 ::RVS161+ ySFI	BYΔ*rvs161*-Bgl	*bla RVS161: KanMX ura3/URA3 Shble PGK1p-S.f.rbgl- PGK1t*	This work
BY4742 ::END3 + ySFI	BYΔ*end3*-Bgl	*bla END3Δ:: kanMX ura3/URA3 Shble PGK1pp-S.f.bgl- PGK1t*	This work
BY4742 ::CLC1 + ySFI	BY*Δclc1*-Bgl	*bla CLC1Δ:: kanMX ura3/URA3 Shble PGK1p-S.f.bgl-PGK1t*	This work
BY4742 ::MNN10 + ySFI	BYΔ*mnn10*-Bgl	*bla MNN10Δ:: kanMX ura3/URA3 Shble PGK1pp-S.f.bgl- PGK1t*	This work
BY4742 ::VPS10 + ySFI	BYΔ*vps10*-Bgl	*bla VPS10Δ:: kanMX ura3/URA3 Shble PGK1pp-S.f.bgl- PGK1t*	This work
BY4742 ::SSA4 + ySFI	BYΔ*ssa4*-Bgl	*bla SSA4Δ:: kanMX ura3/URA3 Shble PGK1pp-S.f.bgl- PGK1t*	This work
BY4742 ::UTH1 + ySFI	BYΔ*uth1*-Bgl	*bla UTH1Δ:: kanMX ura3/URA3 Shble PGK1pp-S.f.bgl- PGK1t*	This work
BY4742 ::VTA1 + ySFI	BYΔ*vta1*-Bgl	*bla VTA1Δ:: kanMX ura3/URA3 Shble PGK1pp-S.f.bgl- PGK1t*	This work
BY4742 ::VSM1 + ySFI	BYΔ*vsm1*-Bgl	*bla VSM1Δ:: kanMX ura3/URA3 Shble PGK1pp-S.f.bgl- PGK1t*	This work

BY4742 ::HAC1 + ySFI	BYΔ*hac1*-Bgl	*bla HAC1Δ:: kanMX ura3/URA3 Shble PGK1pp- S.f.bgl- PGK1t*	This work
BY4742 + pMI1529	cbh1 Parental	*bla ura3/URA3 Shble ENO1p- Trbgl - ENO1t*	This work
BY4742 ::VRP1 + pMI529	BYΔ*vrp1*-Cbh1	*bla VRP1Δ:: kanMX ura3/URA3 Shble ENO1p- T.e. cbh1- ENO1t*	This work
BY4742 ::YPT7 + pMI529	BYΔ*ypt7*-Cbh1	*bla YPT7Δ:: kanMX ura3/URA3 Shble ENO1p- T.e. cbh1- ENO1t*	This work
BY4742 ::VPS45 + pMI529	BYΔ*vps45*-Cbh1	*bla VPS45Δ:: kanMX ura3/URA3 Shble ENO1p – T.e. cbh1- ENO1t*	This work
BY4742 ::RVS161 + pMI529	BYΔ*rvs161*-Cbh1	*bla RVS161Δ:: kanMX ura3/URA3 Shble ENO1p- T.e. cbh1 - ENO1t*	This work
BY4742 ::END3 + pMI529	BYΔ*end3*-Cbh1	*bla END3:: kanMX ura3/URA3 Shble ENO1p – T.e. cbh1- ENO1t*	This work
BY4742 ::CLC1 + pMI529	BY*Δclc1*-Cbh1	*bla CLC1:: kanMX ura3/URA3 Shble ENO1p- T.e. cbh1- ENO1t*	This work
BY4742 ::MNN10 + pMI1529	BYΔ*mnn10*-Cbh1	*bla MNN10Δ:: kanMX ura3/URA3 Shble ENO1p- T.e. cbh1- ENO1t*	This work
BY4742 ::VPS10 + pMI529	BYΔ*vps10*-Cbh1	*bla VPS10Δ:: kanMX ura3/URA3 Shble ENO1p- T.e. cbh1- ENO1t*	This work
BY4742 ::SSA4 + pMI529	BYΔ*ssa4*-Cbh1	*bla SSA4Δ:: kanMX ura3/URA3 Shble ENO1p- T.e. cbh1- ENO1t*	This work
BY4742 ::UTH1 + pMI529	BY*Δuth1*-Cbh1	*bla UTH1Δ:: kanMX ura3/URA3 Shble ENO1p- T.e. cbh1- ENO1t*	This work
BY4742 ::VTA1 + pMI529	BYΔ*vta1*-Cbh1	*bla VTA1Δ:: kanMX ura3/URA3 Shble ENO1p- T.e. cbh1- ENO1t*	This work
BY4742 ::VSM1 + pMI529	BYΔ*vsm1*-Cbh1	*bla VSM1Δ:: kanMX ura3/URA3 Shble ENO1p- T.e. cbh1- ENO1t*	This work
BY4742 ::HAC1 + pMI529	BY*Δhac1*-Cbh1	*bla HAC1Δ:: kanMX ura3/URA3 Shble ENO1p- T.e. cbh1- ENO1t*	This work
Double knock-out strains:			
BY4742 ::CLC1+ RVS161+ pRDH180	BYΔ*clc1+rvs161*-Eg2	*bla CLC1+ RVS161Δ:: kanMX ura3/URA3 Shble ENO1p- Tr.EG2 ENO1t*	This work
BY4742 ::VRP1+ RVS161+ pRDH180	BYΔ*vrp1+rvs161*-Eg2	*bla VRP1+ RVS161Δ:: kanMX ura3/URA3 Shble ENO1p- Tr.EG2 ENO1t*	This work
BY4742 ::RVS161+ END3 pRDH180	BYΔ*rvs161+end3*-Eg2	*bla RVS161+ END3Δ:: kanMX ura3/URA3 Shble ENO1p- Tr.EG2 ENO1t*	This work

BY4742 ::VTA1+ END3 pRDH182	BYΔ*vta1+end3* -Xyn2	*bla VTA1+ END3Δ:: kanMX ura3/URA3 Shble ENO1p- Tr.xyn2 ENO1t*	This work
BY4742 ::RVS161+ END3 pRDH182	BYΔ*rvs161+end3*-Xyn2	*bla RVS161+ END3Δ:: kanMX ura3/URA3 Shble ENO1p- Tr.xyn2 ENO1t*	This work
BY4742 ::UTH1+ RVS161 pMI529	BYΔ*uth1+ rvs161*- Cbh1	*bla UTH1+RVS161Δ:: kanMX ura3/URA3 Shble ENO1p- T.e cbh1- ENO1t*	This work

Table 2.2 Primers used in this study.

Primer name	Oligonucleotide sequence (5' 3')
RVSdelHYG-L	ATGAGTTGGGAAGGTTTTAAGAAAGCTATCAACAGAGCTGactagtttttcgacactggatggcg
RVSdelHYG-R	TTATTTTATCCCGAGCGCACAAATATCTAGGCTTGTCATTGCGGCCGCGTTTAGCTTGC
ENDdelHYG-L	ATGCCCAAGTTGGAACAATTTGAAATAAAAAAATACTGGCactagtttttcgacactggatggcg
ENDdelHYG-R	TCAATTGATTTCTGCTTGTAATGCTTGCAATTCGTGTCTCGCGGCCGCGTTTAGCTTGC
END3-L	ATGCCCAAGTTGGAACAATTTG
END3-R	TCAATTGATTTCTGCTTGTAATGCTTG
RVS-L	ATGAGTTGGGAAGGTTTTAAG
RVS-R	TTATTTTATCCCGAGCGC
ENOpromoter-L	GTAACATCTCTCTTGTCCCTTATTCCTTCTAGC
ENOpromoter-R	CAATTTAATTATATCAGTTATTACCC

2.3. Enzyme Assays

Yeast strains were inoculated in triplicate into 10 ml of SC^{-URA} in 125- mL Erlenmeyer flasks and then cultivated for 72 h on a rotary shaker at 180 rpm at 30°C for each of the enzyme activities. To evaluate the β-glucosidase secretion capabilities of the recombinant strains, enzyme assays were performed in triplicate at a 72 h interval on the extracellular cell fractions of each of the relevant *S. cerevisiae* BY4742 strains. Assays were carried using *p*-nitrophenyl-β-D-glucopyranoside (pNPG, Sigma) as substrate, according to a previously described methodology (Van Zyl *et al*. 2014). The extracellular cellobiohydrolase activity of the recombinant BY4742

strains were evaluated using *p*-nitrophenyl-β-D-cellobioside (pNPC, Sigma—St. Louis, USA) as substrate according to Van Zyl *et al* (2014). The DNS assay was used, as described previously by La Grange *et al*. (2001) and Kroukamp *et al*. (2013) to test for activity of Xyn2 and Eg2 either using 1% beechwood xylan (Sigma) or 1% CMC as a substrate. Standard curves were set using D-glucose (CMC-assay) D-xylose (xylan-assay) or pNP (pNPG and pNPC assays) as previously described. One unit was defined as the amount of enzyme required to produce one µmol of reducing sugar or equivalent in one minute under the assay condition. All spectrophotometric readings for the enzymatic assays were taken at 400 nm (pNPG and pNPC), or 540 nm (for DNS) on a SPECTROstar (BMG LAB TECH) and media blanks were included. A reference strain (with no heterologous encoding gene) was included in all assays to serve as negative control. The OD_{600} readings of all cultures were taken at the 72 hour cultivation time for all of the respective mutant and transformant strains and these values were used to calculate the DCW for all strains (Van Zyl *et al*. 2014).

2.4. SDS- PAGE analysis of secreted proteins

Secretion of the heterologous enzymes in the supernatant was investigated using polyacrylamide gel electrophoresis. Strains were cultivated for 72 hours after which the OD_{600} reading of each culture was recorded. These were used to obtain standardized protein levels per supernatant against the OD values of each of the cultures, so that the differences in culture densities had no effect on the comparison of the proteins from the different strains on the gel. The extracellular protein fractions were then analysed using either an 8% or 10% sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE), according to the method of Laemmli (1970). The separated protein bands were visualized using silver staining (Kroukamp *et al*., 2013).

2.5. Double knock-out strains

To obtain double gene knock- outs of selected genes in selected strain backgrounds, the *HYG*^{R} gene encoding the Hygromycin resistance marker was amplified with PCR. The phusion polymerase (Thermo Scientific) was used as instructed by the manufacturer to amplify the *HYG*^{R} gene from the plasmid pHK211 (received from H. Kroukamp, Macquerie University, Australia).

Primers were designed to contain a 40-bp overhang homologous to the first and last 40 bases of either of the *S. cerevisiae END3* (primers ENDelHYG-L/-R) or *RVS161* (primers ENDelHYG-L/-R) genes open reading frames (Table 2.2). The amplicons obtained were used to knock out either the *END3* or *RVS161* genes in the relevant strains through heterologous recombination. Strains were transformed with the PCR product and transformants were selected on SC^{-URA} medium with 100 µg/ml Hygromycin (Sigma). Knock- outs were confirmed using primers END3-L and END3-R for *END3* knock- outs or RVS-L and RVS-R for *RVS161* with NEB Taq polymerase (New England Biolabs) as instructed by the manufacturer. Transformants detailed in Table 2.1 were confirmed by PCR analysis after total DNA extraction (Hoffman and Winston, 1987). Primers ENOpromoter-L and ENOpromoter-R were designed to amplify any gene cloned in between the *S. cerevisiae* (Table 2.2) ENO_1 promoter and terminator such as those on pRDH180, pRDH182, and pMI529. These primers were thus used with the NEB Taq polymerase to confirm the presence of the heterologous genes in the transformants.

CHAPTER THREE

RESULTS AND DISCUSSION

3.1. Creating Endocytosis deletion mutant strains expressing various hydrolases

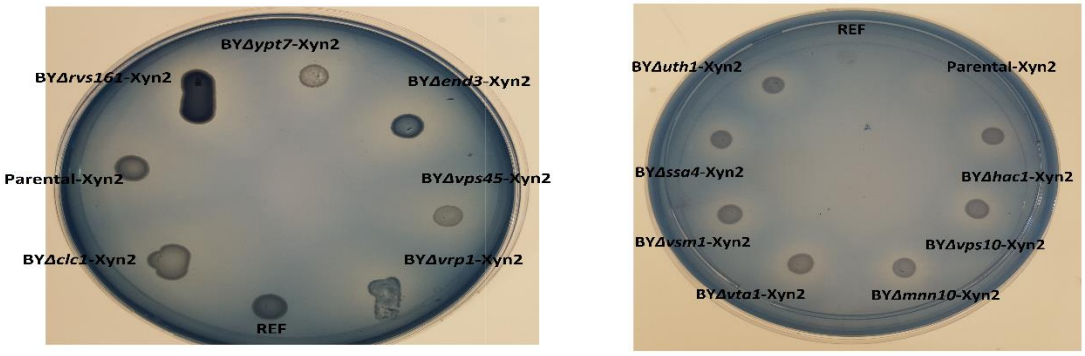
The deletion strains that were used in this study originated from the EUROSCARF (European *Saccharomyces cerevisiae* archive for functional analysis) strain collection (Brachman *et al*, 1998). The EUROSCARF yeast strain collection originated as an output from the "*Saccharomyces* Genome Deletion Project" that was aimed at generating a complete set of possible yeast deletion strains with the goal of ascribing function to open reading frames (ORFs) by phenotypic analysis of mutants. We obtained the mutant strains used in this study from the repository at the Stellenbosch University Institute for Wine Biotechnology. Strains were selected on the basis of mutations in genes important to various aspects of endocytosis. In addition, other gene knock-outs that were previously shown to have a positive effect on heterologous protein secretion (*Δhac1, Δmnn10, Δvps10, Δssa4, Δuth1, Δvta1, Δvsm1*) were also included. These strains were each transformed with plasmids containing reporter genes that are detailed in table 2.1. The confirmation of these transformants were done with the use of plate assays for Xyn2 and Eg2 and using PCR for Cbh1 and Bgl1 transformants. The study aimed to test the cell specific activity of each of the enzymes in the various yeast backgrounds and determine whether the mutation that was present in that strain *(Δvrp1, Δypt7, Δvps45, Δrvs161, Δend3*, *Δclc1*, *Δhac1, Δmnn10, Δvps10, Δssa4, Δuth1, Δvta1, Δvsm1*) had an effect on the cell specific activity and secretion of the various reporter proteins (endoglucanase (Eg2), xylanase (Xyn2), β-glucosidase (Bgl1) or cellobiohydrolase (Cbh1) compared to an unmutated positive control strain. Details of the functions of the various mutant genes as described by the *Saccharomyces* genome database (www.yeastgenome.org) can be found as an appendix at the end of this thesis.

3.2. Heterologous xylanase production in the mutant strains

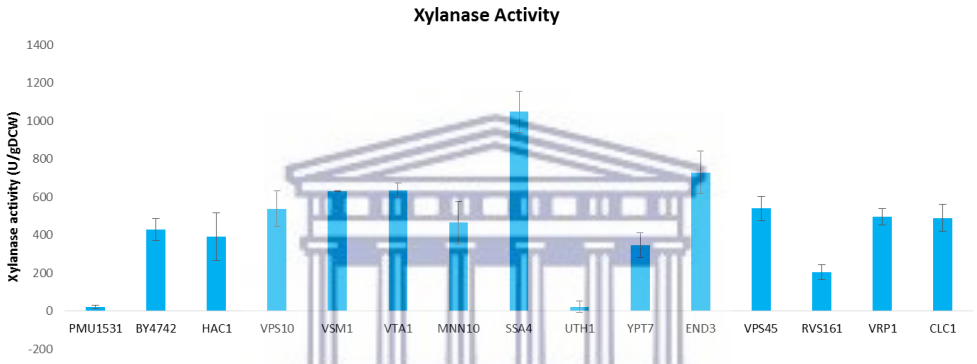
The RBB-xylan plate assay was done to confirm the presence of the *xyn2* gene insert in transformants and the strains that showed activity were represented by a clear halo

surrounding the colonies (Fig. 3.1 A). All of the transformant strains can be seen as positive for the *xyn2* gene transformation excluding the negative control reference strain. The extracellular Xyn2 activity was subsequently quantified after a 72 hour cultivation in SC $^{-URA}$ medium. Overall, the mutant strain with the best activity performance per dry cell weight was BYΔ*ssa4*. The mutant strain with the *SSA4* deletion yielded a 143% increase in activity for Xyn2 (Fig. 3.1 B), compared to the positive control and was followed by the mutant strain with the deletion in *END3* with a 71% increase in secreted activity (Fig. 3.1 B). These strains also had comparatively lower OD values so it could be assumed that they had somewhat impaired growth rates. Deletions in *VSM1* and *VTA1* yielded an almost 50% increase in secreted Xyn2 activity. There were three deletions namely *UTH1*, *YPT7* and *RVS161* which displayed decreased activity for those particular mutant strains in comparison with the positive reference strain (Fig. 3.1 B) and so these knockouts had a negative effect on the secretion of the enzyme Xyn2. These strains also yielded among the highest OD values amongst the strains and thus grew rapidly. When looking at the SDS-PAGE gel (Fig. 3.1 C) for the Xyn2 expressing strains, it could be seen that there were four lanes which contained significantly more proteins overall compared to the BY4742 reference and positive control strains namely those of the *Δend3, Δrvs161, Δvrp1* and *Δhac1* mutants. The Xyn2 band can be seen just below the 25 kDa mark, as confirmed by previous studies using a zymogram (La Grange *et al*, 1996). The mutant strain with the *END3* deletion yielded the brightest band on the gel, confirmed by densitometry analysis using Image J software, thus confirming that more secreted Xyn2 protein was produced by this strain than the control strain. The mutant with the *RVS161* deletion yielded a lot of protein in its lane, however, the xylanase band was significantly lighter compared to that of the mutant strain with the *END3* deletion and as such corresponds to the decreased activity observed for the BYΔ*rvs161*-Xyn2 strain (Fig. 3.1 B). The BYΔ*clc1*-Xyn2 and BYΔ*vrp1*-Xyn2 strains yielded Xyn2 bands similar in brightness which thus corresponds with their activity which is similar to that of the Xyn2 positive control strain.

A.



B.



C.

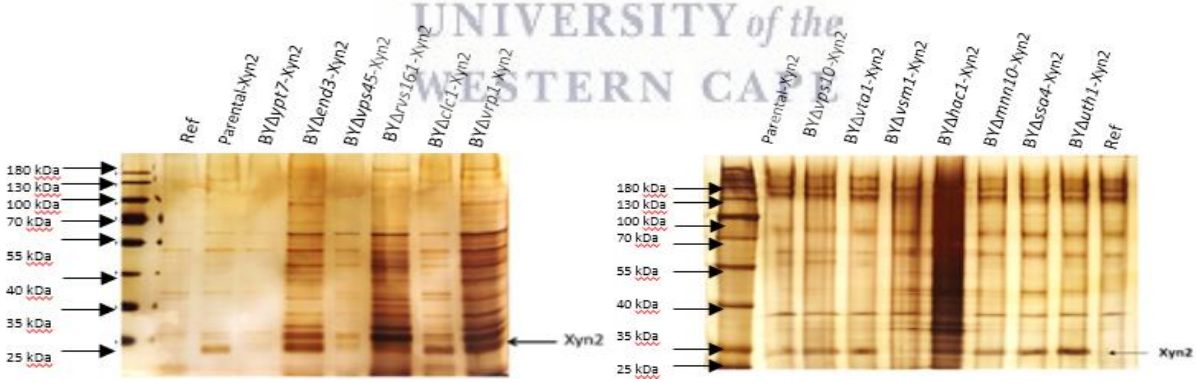
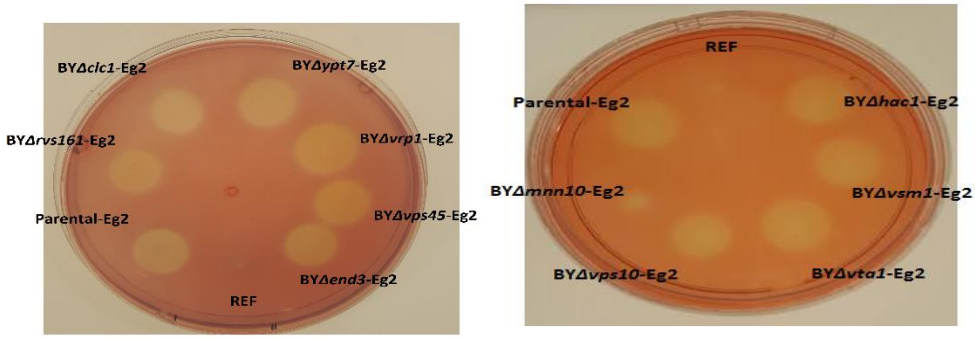


Figure 3.1. Xylanase production by of the transformed mutant strains. (A) RBB-xylan plate assay showing activity of Xyn2 represented by a clear halo surrounding the colonies. Plates were incubated at 30˚C for 3 days. (B) Activity of Xyn2 producing strains on Beechwood xylan, after 72 h of cultivation. Values given are the mean values of enzyme assays conducted in biological triplicate normalised with dry cell weight. Error bars indicate standard deviation from the mean value. For simplicity, mutant strains producing Xyn2 are indicated using only the name of the gene knocked out in that strain. (C) SDS–PAGE analysis of the proteins secreted by *S. cerevisiae* BY4742 transformants. 10% silver stained SDS–PAGE gel with supernatants of Xyn2 producing strains diluted according to OD_{600} values to allow direct comparison.

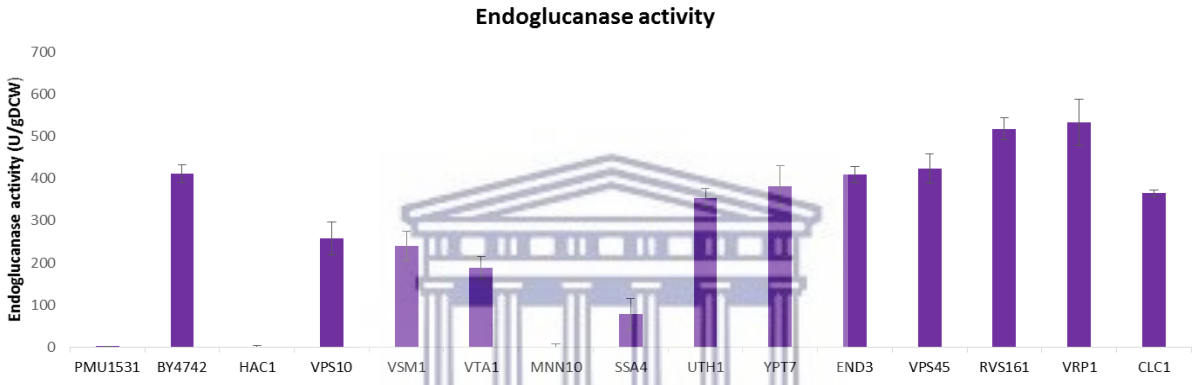
3.3. Heterologous endoglucanase production in the mutant strains.

A CMC plate assay was used for the confirmation of the *eg2* gene insert into the transformed strains (Fig. 3.2 A). Positive strains displayed a clear halo surrounding colonies after the plate was washed and stained. All of the transformed strains were positive for Eg2 activity excluding the negative control reference strain. Eg2 activity was quantified after 72 hours of cultivation. The mutant strain with the *VRP1* deletion yielded a 30% increase in activity for Eg2 (Fig. 3.2 B), followed by the mutant strain with the deletion in *RVS161* with a 26% increase in activity. These two genes are directly involved in endocytosis and therefore the impact was greater by the process being hindered. The mutant strains with deletions in *VPS45, YPT7*, *END3* or *CLC1* appeared to have slightly increased activity compared to the positive control but these were determined not to be significant according to the T-test. These strains also had high OD_{600} values, suggesting a rapid growth similar to the positive control strain. The SDS-PAGE gel for the Eg2-expressing strains (Fig. 3.2 C) indicated that there were significantly more proteins secreted by the mutant strains with the *RVS161* and *SSA4* deletions compared to the control strain. Therefore more proteins were secreted in general from these mutants even though this did not translate to more secreted Eg2. The endoglucanase band can be seen just below the 55 kDa mark as a smear-like band, as confirmed by previous studies with zymogram analysis (Den Haan *et al.* 2013). The mutant strains with the *VRP1* and *RVS161* deletions yielded the brightest bands on the gel, corresponding with the higher Eg2 activity measured for these strains which can therefore be said to secrete more of the enzyme. The *Δrvs161* mutant strain again displayed a lot of secreted protein when compared to the other lanes (Fig. 3.2 C), similar to the observation for the xylanase producing strains. The observation that BY*Δssa4*-Eg2 strain yielded almost no secreted activity (Fig. 3.2 B) but produced a significant band on the SDS-PAGE (Fig. 3.2 C) was unexpected. It is possible that the secreted protein was misfolded and inactive, as was previously reported for *T. reesei* Cbh1 secretion in yeast (Ilmen *et al*, 2011). A zymogram analysis of this protein would show if it was active.

A.



B.



C.

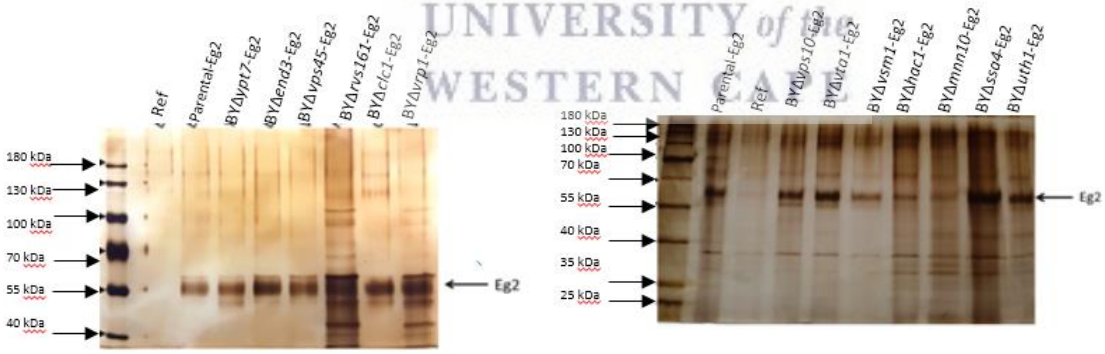
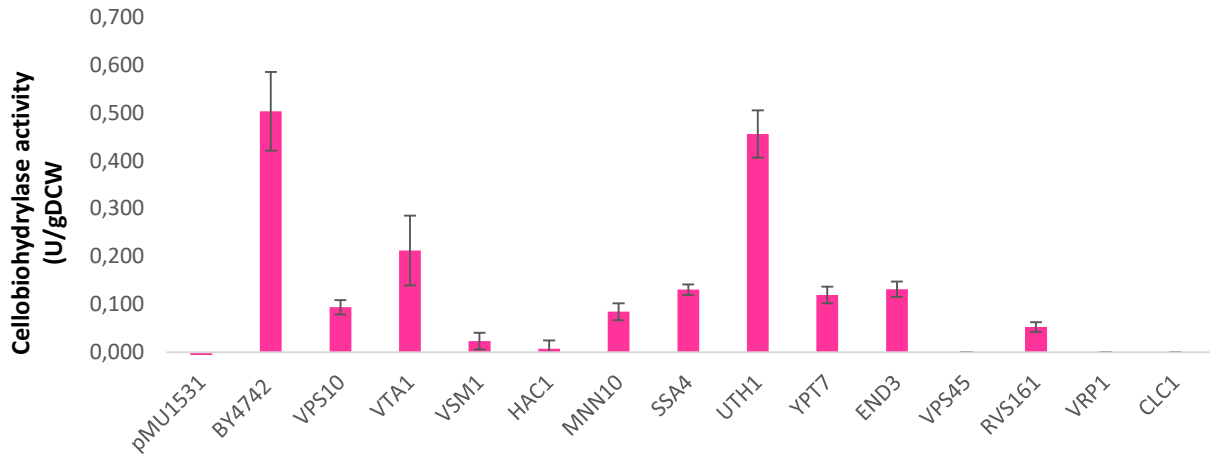


Figure 3.2. Endoglucanase production of the transformed mutant strains. (A) Congo red stained CMC plate assays showing activity of Eg2 represented by a clear halo. Plates were incubated at 30˚C for 3 days. (B) The activity of Eg2 producing strains on CMC after a 72 h cultivation. Values obtained were normalized with the dry cell weight of each specific yeast strain. Values given are the mean values of enzyme assays conducted in biological triplicate. Error bars indicate standard deviation from the mean value. For simplicity, mutant strains producing Eg2 are indicated using only the name of the gene knocked out in that strain. (C) SDS–PAGE analysis of the proteins secreted by *S. cerevisiae* BY4742 transformants. 10% silver stained SDS–PAGE gel with supernatants of Eg2 producing strains diluted according to culture OD_{600} values to allow direct comparison.

3.4. Screening of the Cellobiohydrolase production in the mutant strains.

Yeast transformants were confirmed with PCR (not shown). Cbh1 activity was measured after a 72 hour cultivation time. No statistically significant increase in secreted Cbh1 activity could be measured for any of the mutant strains in comparison to the parental strain (Fig. 3.3 A). Furthermore, mutants with deletions in *VPS45*, *VRP1* and *CLC1* had no detectable Cbh1 activity while the mutants with the deletions in *END3*, *RVS161* and *YPT7* had a decreased level of activity when compared to the BY4742- Cbh1 control strain. SDS-PAGE analysis (Fig. 3.3 B) for the Cbh1-producing strains, revealed that the mutant strain with the *RVS161* deletion again secreted the most total protein, although this did not translate into more Cbh1 being produced. The Cbh1 band can be seen just below the 70 kDa mark, as confirmed by previous studies (Ilmen *et al.* 2011, Den Haan *et al*. 2013). Even though the mutant strain with the *RVS161* deletion yielded quite a lot of protein in its lane, the Cbh1 band was lighter when compared to that of the mutant strain with the *END3* deletion and as such corresponds to the quantitative activity difference observed (Fig. 3.3 A). The mutants with deletions in *VRP1*, *VPS45* and *CLC1* produced little to no band for Cbh1 (Fig. 3.3 B), corresponding their lack of measureable activity (Fig. 3.3 A).

A.



B.

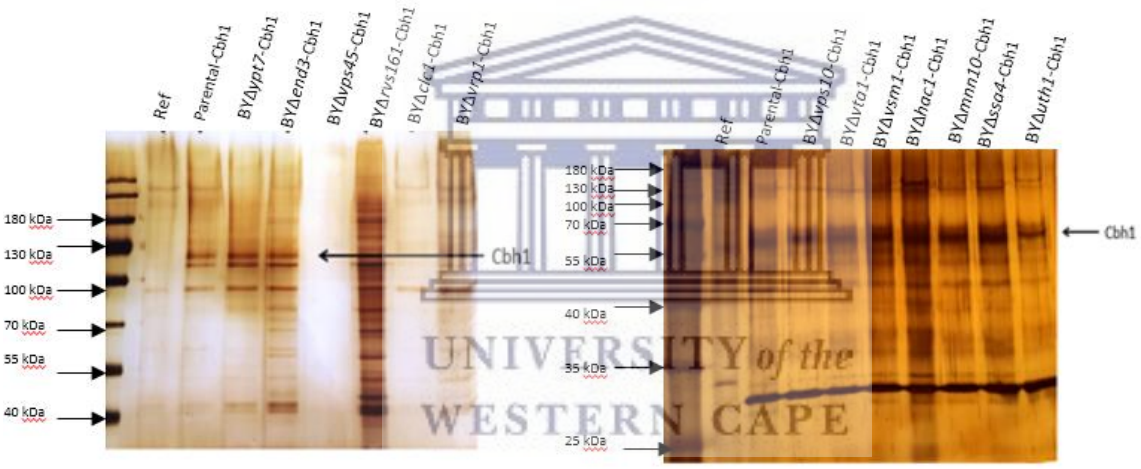


Figure 3.3. Enzyme activity profiles of constructed strains after 72 h of cultivation. (A) Activity of Cbh1 producing strains on pNPC after 72 h cultivation. A reference strain was cultured and assayed in the same manner. Values given are the mean values of enzyme assays conducted in biological triplicate. Error bars indicate standard deviation from the mean value. For simplicity, mutant strains producing Cbh1 are indicated using only the name of the gene knocked out in that strain. (B) SDS–PAGE analysis of the proteins secreted by *S. cerevisiae* BY4742 transformants. 8% silver stained SDS–PAGE gel with supernatants of Cbh1 producing strains diluted according to OD_{600} values to allow direct comparison.

3.5. The effect of single gene knock- outs on heterologous protein secretion

The *Saccharomycropsis fibuligera* β-glucosidase encoding gene *S. f. bgl1* was transformed to EUROSCARF deletion strains in a manner similar to the other three hydrolase encoding genes just discussed. Despite showing the presence of the heterologous gene in the transformants (Table 2.1) with PCR, no Bgl activity could be measured for these strains. While BY4742 based strains have been previously been endowed with Bgl activity (Lui *et al*. 2015), it was also shown that heterologous Bgl activity of these strains was exceptionally low when strains were cultivated in selective SC medium (Davison *et al.* 2016). For this reason, we did not pursue the effect of endocytosis on Bgl secretion further.

It was recently shown that endocytosis could hinder and decrease secreted protein levels (Rodríguez-Limas *et al*., 2015). However, that study focused only on the use of one enzyme, namely α-amylase. In this study, four enzymes which are required for the breakdown of lignocellulosic biomass for second generation bioethanol production were expressed in BY4742 mutant yeast strains where genes involved in endocytosis or other aspects previously shown to influence secretion, had been knocked out to determine whether this had an effect on the secreted protein titres of these enzymes. A total of 60 strains were made which included a negative and a positive control for each of the corresponding enzymes. It is clear that no single endocytosis mutant among the candidates tested in our study had a positive effect on all of the reporter genes used (Table 3.1). All of the mutants that positively affected one candidate had a detrimental effect on at least one other gene. This is in line with the reporter protein specific secretion enhancement effects of rational secretion improvement in yeast that was previously reported (Van Zyl *et al*. 2014, Van Zyl *et al*. 2016; Kroukamp *et al*. 2018).

Table 3.1 Summary of the effect of endocytosis related gene deletions on the expression of the three reporter genes. Only genes that had a statistically significant effect as determined using the T-test (two-tailed and assuming unequal variance) on at least one of the genes are indicated. Percentage of improvement (green blocks) or impairment (red blocks) of activity compared to the relevant parental control strains are indicated.

	Eg2	Xyn2	Cbh1
Δ*vps10*	-37	ND	-81
Δ*vsm1*	-42	47	-95
Δ*vta1*	-54	48	-58
Δ*uth1*	ND*	-95	ND
Δ*ssa4*	-81	143	-74
Δ*end3*	ND	71	-74
Δ*rvs161*	26	-52	-89
Δ*vrp1*	30	ND	NA**
Δ*clc1*	-11	ND	NA

* Not significantly different according to the T-test

**No activity detected

According to the T- test analysis (Table 3.1), it was seen that for Xyn2 there were a few genes that showed a significant difference. The mutant strain with the *SSA4* gene deletion had the greatest change in secreted activity, increasing by 143%. The mutants with the gene deletions *END3, VSM1* and *VTA1*, had significant increases of 71%, 47% and 48 %, respectively but there were others yielding significant decreases. For Eg2, there were two mutants that showed an increase in activity namely the mutants of the *RVS161* and *VRP1* genes which had an increase of 26% and 30%, respectively. For Cbh1 there was no significant increases detected and in most of the strains, the activity was significantly decreased or abolished completely.

It was seen that the effect of the gene deletions resulted in different cell specific activities as well as secreted titres of the various reporter proteins when compared to each other. For example, the mutant strain with the deleted *RVS161* gene produced comparatively less Xyn2 but produced 26% more Eg2 activity than the control strain. As such, it can be said that these

deletions might be advantageous to certain enzymes but deleterious in the case of others. For the Cbh1 data it was observed that the strains with the *SSA4* and *END3* deletions had decreased activity compared to the reference strain but the same mutations showed an increase of 143% and 71%, respectively, for Xyn2 activity. Overall it was shown that the genes involved in endocytosis which were deleted most positively affected the secretion of the Xyn2 and Eg2 enzymes, however most had a negative effect on Cbh1 secretion. The increased titres found in the mutant strains with the *END3* and *RVS161* deletions tied in with the previous study on the secretion of α-amylase where these also had a positive effect (Rodríguez-Limas *et al*., 2015). These results give just a small indication of the impact of endocytosis on secreted protein titres of various types of enzymes in *S. cerevisiae.*

Using these strains and the information gained from them double gene knock- outs, were subsequently created from selected strains to ascertain the feasibility and effect of mutating two endocytosis related genes on the secreted protein titres and the enzyme activities of the mutant strains. This would then give more insight into which genes are actually needed by the yeast cells and which are likely to have a negative effect on cell growth and performance.

3.6. Double gene knock- out analysis

Double gene knockout strains were created using some of the best candidates for each reporter in terms of both enzyme activity and growth. These were done using *RVS161* and *END3* genes, where primers were synthesized that would allow gene replacement of either of these genes with the Hygromycin selection marker gene (Table 2.2). The cultivations and assays were performed as stated above in addition to SDS- PAGE analysis for protein secretion determination.

3.6.1. Xylanase production in the double knockout mutant strains

The activity of the constructed strains were measured at 48 and 72 h cultivation intervals (Fig 3.4 A). To test if double knock-outs of endocytosis- related genes would further improve xylanase secretion, the *END3* gene of the BY*Δvta1*–Xyn2 and BY*Δrvs161*-Xyn2 strains were knocked out through replacement of the gene with the Hygromycin resistance marker. Knock-outs were confirmed with PCR analysis (not shown). Despite the negative effect of the *RVS161* deletion on

xylanase secretion (Fig 3.1 B), we observed a high level of protein on the SDS-PAGE analysis and wanted to test the effect of the double knock-out in this background strain. The double knock-out strain showing the best improvement was Δ*vta1+end3*-Xyn2*,* with an increase of 56% at 72 h incubation when compared to the parental strain. The double deletion of *RVS161* and *END3* had a negative effect on the xylanase producing strain, showing a significant drop in activity when compared to that of the parental. When looking at the SDS-PAGE gel for these Xyn2- producing strains (Fig 3.4 B), it was seen that the Δ*vta1+end3*-Xyn2 strain produced notably more xylanase (band at 25 kDa). This corresponds directly to the amount of activity which can be seen from the enzyme assays and suggests that the enzyme secreted was more than that of the parental showing that improved activity was due to improved secretion titre and not a changed specific activity. Lane 4 had a faint band which also corresponded to the decreased activity in the enzyme assay for the Δ*rvs161+end3*-Xyn2 and suggests that very little enzyme was secreted in comparison to the parental. As *END3* and *VTA1* deletion had the effect of improving Xyn2 activity by 71% and 48% as single knockouts, the improvement seen for the double knock-out was smaller than expected.

A. **B.**

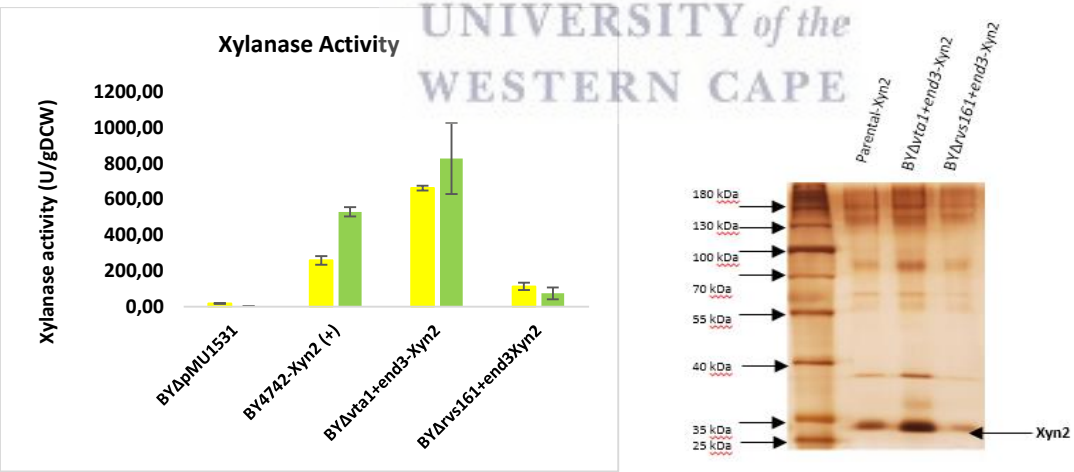


Figure 3.4. Enzyme activity profiles of double knock-out xylanase producing strains after 48 and 72h of cultivation (A) Activity of Xyn2 producing strains on Beechwood xylan. Values given are the mean values of enzyme assays conducted in biological triplicate. Error bars indicate standard deviation from the mean value. The yellow represents 48 h incubation and the green represents the 72h incubation. (B) SDS–PAGE analysis of the proteins secreted by *S. cerevisiae* BY4742 transformants. (B) 10% silver stained SDS–PAGE gel with supernatants of Xyn2 producing strains diluted according to OD_{600} to allow direct comparison.

3.6.2. Endoglucanase production in the double knockout mutant strains

Double gene knockouts were constructed for Eg2 producing strains using the genes *END3* and *RVS161*. Strains BY*Δrvs161*- Eg2, BY*Δvrp1*- Eg2 and BY*Δclc1*- Eg2 were transformed with Hygromycin resistance markers flanked with homologous sequences to allow gene replacement as described, to create strains BY*Δvrp1+ rvs161*- Eg2, BY*Δrvs161+ end3*- Eg2 and BY*Δclc1+ rvs161*- Eg2. The activity of the constructs were measured after 48 and 72 hour cultivation intervals (Fig 3.5 A). Strains Δ*vrp1+rvs161*-Eg2 and Δ*clc1+rvs161*-Eg2*,* displayed significant increases of 46% and 104% at the 72 hour cultivation period, respectively, when compared to the parental control strain. Δ*rvs161+end3*-Eg2 displayed no improvement over the parental, but there was also no negative effect on Eg2 production in this double knock-out, which indicates that the strain likely did not need these gene products for the production of Eg2 in comparison to the xylanase, where the activity was abolished for this double knock-out.

The *rvs161-vrp1* double knockout strain produced 46% more activity compared to the parental control strain which is close to an additive amount of improvement compared to the effects of the individual gene knock- outs (Table 3.1). Surprisingly, the *rvs161-clc1* double knockout strain produced 104% more activity compared to the parental control strain, despite the negative effect that the *clc1* mutation had in isolation on the Eg2 activity (Fig 3.5 A). This may point to epistatic effects of the genes involved in endocytosis as was also described for other multi-gene dependant traits in yeast (Swinnen *et al* 2012; Kroukamp *et al*. 2017).

A. B.

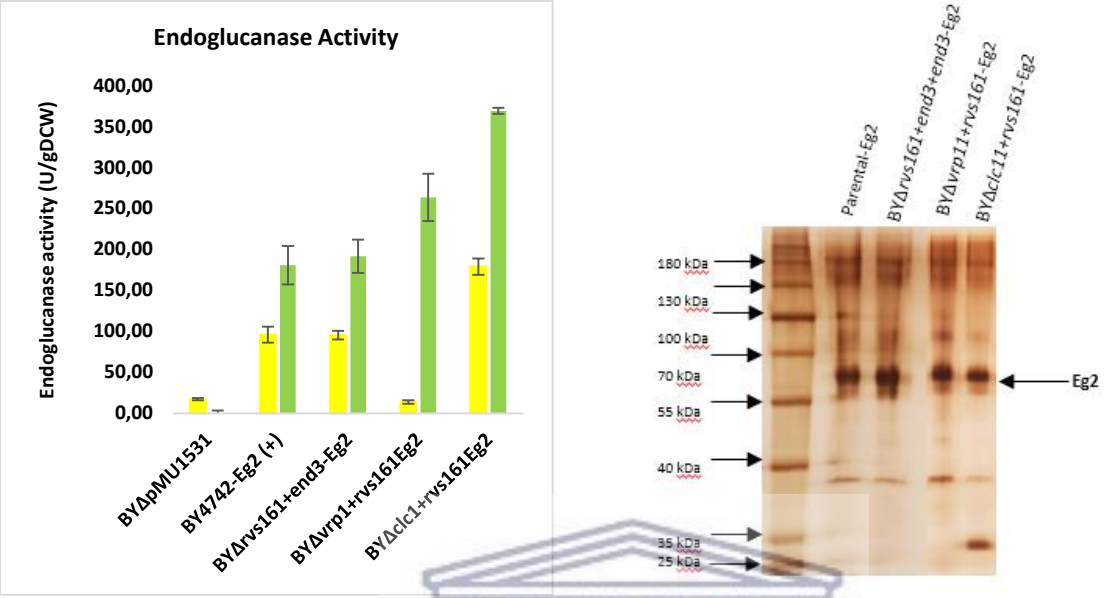


Figure 3.5. Enzyme activity profiles of double knock-out endoglucanase producing strains after 48 and 72 h of cultivation. (A) Activity of Eg2 producing strains on CMC. A reference strain was cultured and assayed in the same manner on all substrates which expressed no heterologous genes. Values given are the mean values of enzyme assays conducted in biological triplicate. Error bars indicate standard deviation from the mean value. The yellow represents 48 h incubation and the green represents the 72h incubation. (B) SDS–PAGE analysis of the proteins secreted by *S. cerevisiae* BY4742 transformants. 10% silver stained SDS–PAGE with supernatants of Eg2 producing strains diluted according to culture OD_{600} to allow direct comparison.

3.6.3. Cellobiohydrolase production in the double knockout mutant strains.

Cbh1 producing double knock-out strains were cultivated over 48 and 72 hour periods after which activity was measured as before. There was no statistically significant increase or decrease in activity in comparison to the parental strain, and therefore the double gene knock-out had no measurable impact (Fig 3.6 A). For the SDS–PAGE analysis, it was observed that the level of secreted enzyme for the mutant strain was similar to that of the parental which corresponded to the enzyme assay where no significant change in activity was seen. The generally negative impact of the mutants of the endocytosis-related genes on Cbh1 production might point toward the importance of these proteins for functional secretion of this enzyme. In future, effects of

overexpression of these genes on Cbh1 secretion should be ascertained to shed light on this question.

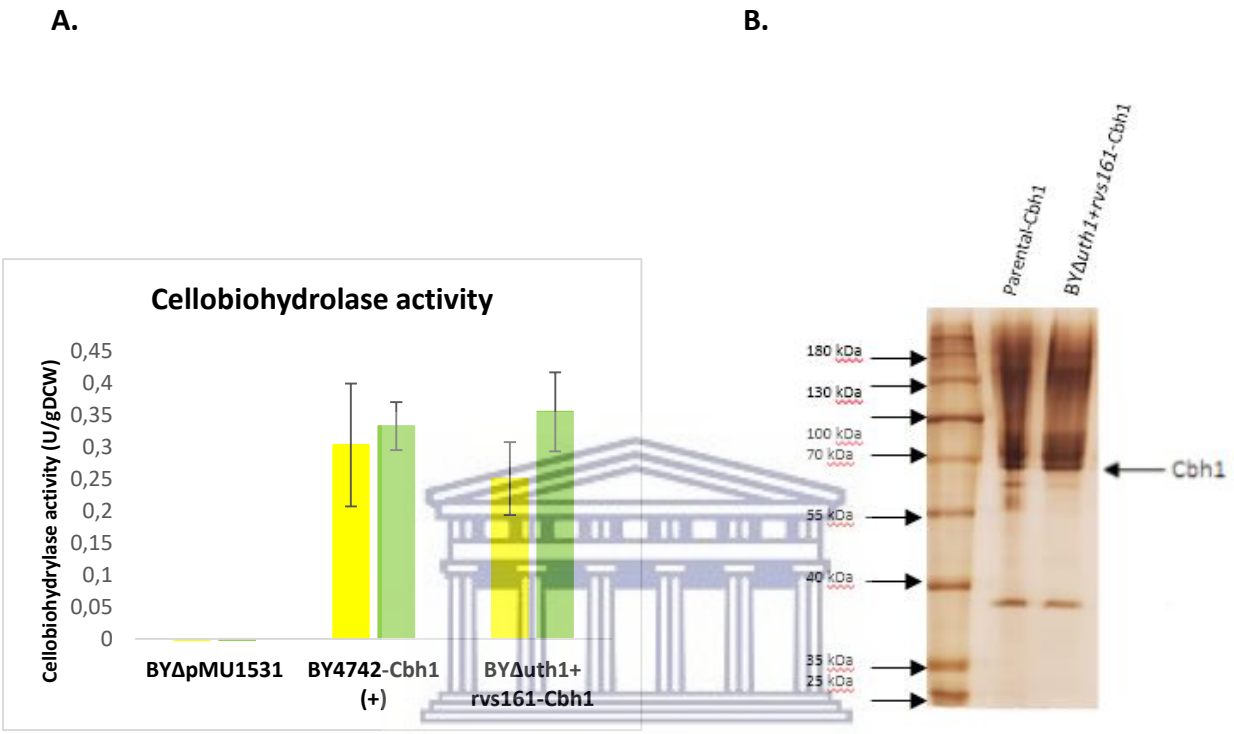


Figure 3.6. Enzyme activity profiles of double knock-out Cbh1 producing strains strains after 48 and 72h of cultivation. (A) Activity of Cbh1 producing strains on pNPC. A reference strain was cultured and assayed in the same manner on all substrates which expressed no heterologous genes. Values given are the mean values of enzyme assays conducted in biological triplicate. Error bars indicate standard deviation from the mean value. The yellow represents 48 h incubation and the green represents the 72h incubation. (B) SDS–PAGE analysis of the proteins secreted by *S. cerevisiae* BY4742 transformants. 10% silver stained SDS–PAGE gel with supernatants of Cbh1 producing strains diluted according to culture OD_{600} to allow direct comparison.

3.7. Stress Plate Assays

There are many stress factors associated with the fermentation process which could be contributing factors in the way yeast cells grow and ferment (Kaufman, 1999, Bauer and Pretorius, 2000). Any intervention used to increase heterologous secretion of cellulases by CBP yeasts should not compromise the ethanol yield or productivity or robustness of the organism.

As such, a series of growth stress tests were done on the EUROSCARF mutant strain transformants in order to determine the survival of the yeast under these stress conditions and how heterologous protein production impacted on the robustness of these strains under the stress conditions. The yeast strains were first cultivated in liquid SC^{-URA} medium at 30°C to an OD_{600} of 1. Following this, ten- fold serial dilutions of the cultures were made up to 10^{-5} and 3 µl amounts were used to spot plate on SC^{-URA} media with the appropriate stress factor. In this study, the tolerance capabilities of the strains were tested using increased temperature and various ethanol concentrations as stress factors. A control plate was also included with SC^{-URA} media and incubated with no stress factor for demonstration and comparison. Throughout the growth analysis, no colony growth changes were observed for the control plates under no stress (Fig. 3.8 A).

3.7.1. Ethanol stress plate assays for endocytosis mutants

Ethanol concentration as well as high temperature stress both yielded increased cellular sensitivity. Ethanol and temperature stress, lead to alteration of the cellular membrane properties by increasing membrane permeability as well as changing membrane fluidity (Bauer and Pretorius, 2000). As the ethanol concentration increases, the cell growth decreased as a result of stress in the cell.

For the ethanol plate assays containing 20%- 30% ethanol, growth was completely abolished for most strains (not shown). For the 10% ethanol plates (Fig. 3.7), notable differences were seen among all of the plates. Xylanase producing strains were relatively unaffected in growth under 10% ethanol compared to the control (Fig. 3.8 A), except for BY*Δuth1*-Xyn2 where almost no growth was observed. The plates containing Eg2 and Cbh1 producing strains showed that growth was poor across most of the strains tested. This suggests that there is a notable difference between the stress experienced by the strains producing different heterologous proteins and the ethanol posed as an additional stress factor. Eg2 and Cbh1 production are thus bigger stress factors on their own than Xyn2 production and as such, the additional stress contributed to the strains struggling to grow. For Xyn2 however, some of the knock-outs did have a greater negative

effect than others. Knock- outs strains *SSA4*, *UTH1* and *END3* struggled to grow in the lower dilutions. The *UTH1* mutant grew especially poorly.

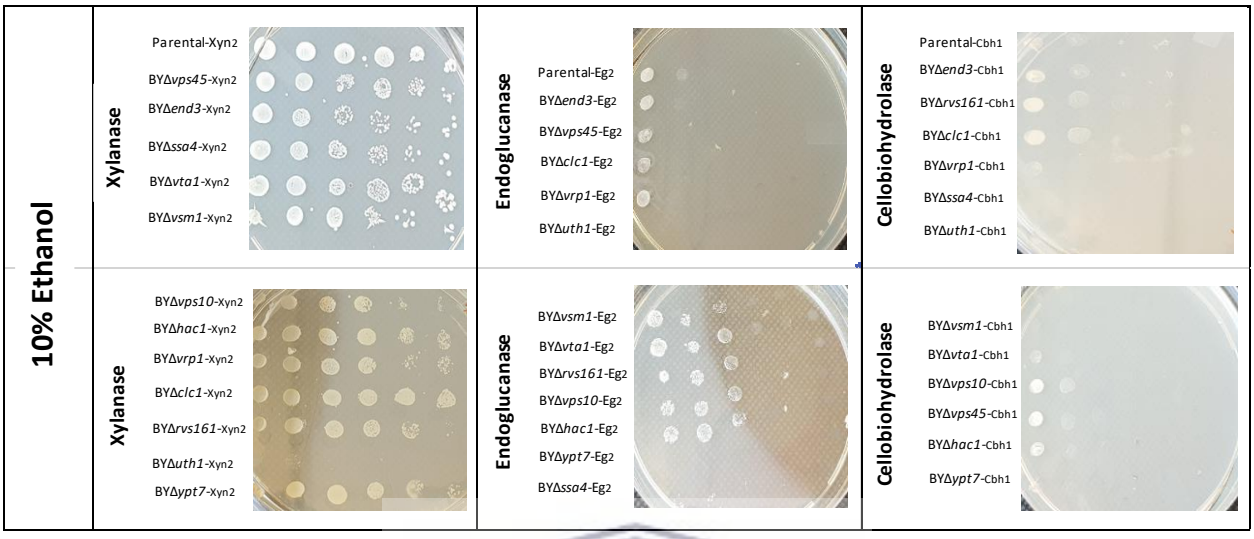


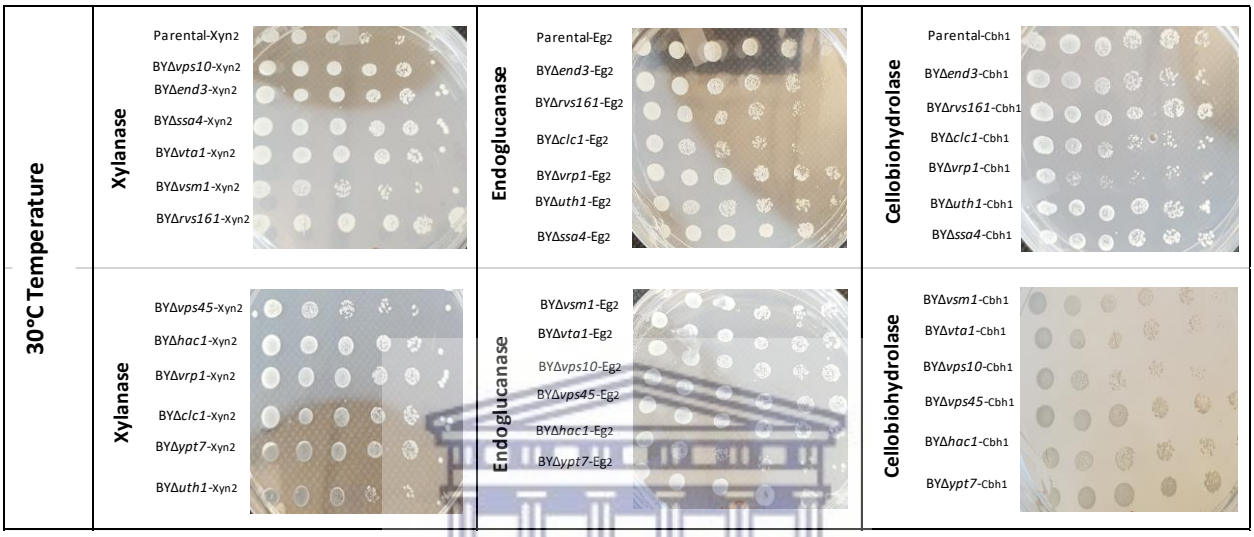
Figure 3.7. Stress plate assays of selected strains after 72 h cultivations. Plates containing 10% ethanol were spotted with each strain at increasing dilutions for the different reporter genes indicated.

3.7.2. Temperature stress plate assays for endocytosis mutants

Temperature stress plate assays were done at 35°C using SC^{-URA} media, and we included 30˚C growth, as a control condition. Plates were incubated over a period of 3 days at the appropriate temperature for each of the mutant strains with the respective plasmids (Fig 3. 8 B). Upon the exposure to the different temperatures for a period of 3 days, various tolerances were observed across all of the plasmid bearing mutant strains. Compared to the control plates, it was seen that mild heat stress had a similar effect as ethanol on the strains. While most strains were unaffected by the change in temperature conditions, it was noted that Eg2 and Cbh1 producing strains generally grew poorer than Xyn2 producing strains at 35˚C, similar to the ethanol stress results. This result coincides with previous observations that Eg2 and Cbh1 production lead to greater metabolic burden and secretion stress in recombinant *S. cerevisiae* strains (Van Rensburg *et al,* 2012, Ilmen *et al,* 2011). The stress tolerance tests show that we need to test the impact of any strain engineering done to improve heterologous protein secretion on strain fitness so that we

do not produce strains that are negatively affected in industrially important parameters such as growth vigour, ethanol productivity, yield or tolerance.

A.



B.

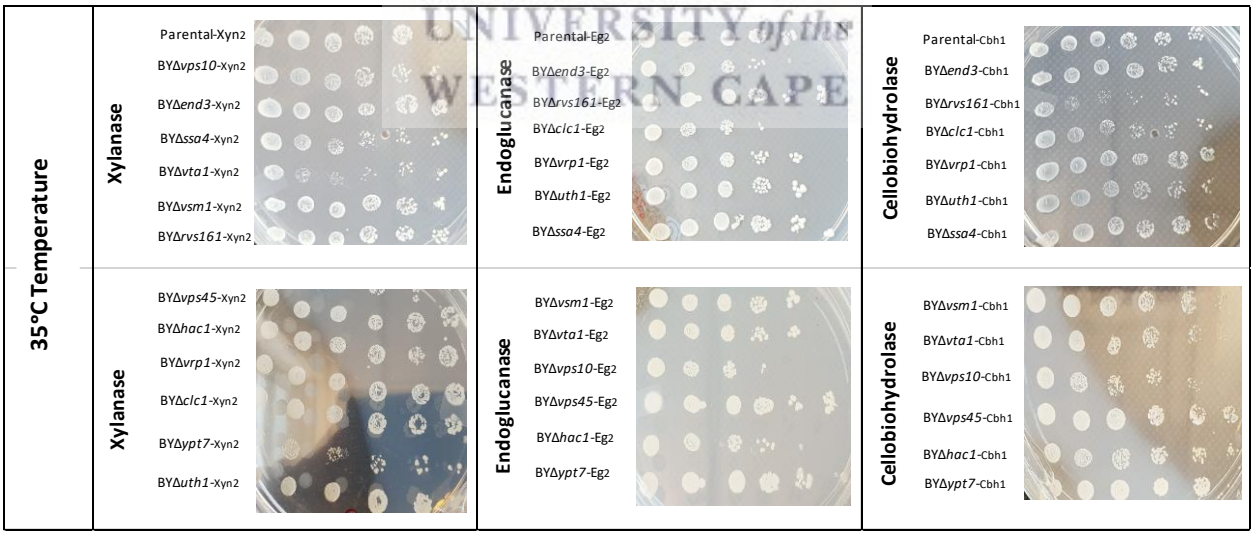


Figure 3.8. Stress plate assays of selected strains after 72 h cultivations. Temperature stress plate assays were done at (A) 30˚C and (B) 35 ˚C incubation for each strain for the different reporter genes.

CHAPTER FOUR

SUMMARY AND CONCLUDING REMARKS

The growing energy crisis has resulted in the need for sustainable and renewable energy alternatives for the future. Many potential energy alternatives have emerged to improve global energy security and one such alternative is bioenergy which is potentially sustainable and environmentally friendly if applied correctly (Nigam and Singh, 2011). As such, biofuels will play a major role in providing liquid transport fuels in a more sustainable way. Lignocellulosic biomass is a renewable carbon source which can be used for sustainable production of fuels and chemicals and does not have to compete with food production (Galbe and Zacchi, 2012). Lignocellulose consists of three main components namely cellulose, hemicellulose and lignin and has to be pre-treated in order to be easily broken down into simple sugars by enzyme hydrolysis to undergo fermentation. The fermentation process can subsequently be done by the yeast *S. cerevisiae*. The enzyme hydrolysis stage however, currently represents up to 20% of the entire conversion cost (Sánchez and Cardona, 2008). As such, consolidated bioprocessing of pre-treated biomass to ethanol has been considered to not only reduce cost but also reduce production time.

Consolidated bioprocessing (CBP) is a biomass to ethanol conversion process that has gained interest as it provides a potential breakthrough for low cost biomass processing (Van Zyl *et al*, 2007). CBP involves the consolidation of the four biological biomass conversion steps in one reactor that are alternatively performed separately making them not only time consuming but also expensive (Njokweni *et al*, 2012). However, no known organism exists that can perform all these processes in one step while producing a desirable product at the rates and titres required by industry (Olson *et al*, 2012; Den Haan *et al*, 2015). Development of the yeast *S. cerevisiae* for CBP in second generation ethanol production would not only lower fuel production cost but also save processing time, resulting in more biofuels being produced. There have been reports where the three classes of cellulases: (i) endoglucanases (EGs), (ii) exoglucanases, including cellobiohydrolases (CBHs) and cellodextrinases, as well as (iii) β-glucosidase (BGL) have been produced by yeast (Van Zyl *et al*, 2011; Lane *et al,* 2018). However the amount of cellulase

secretion from *S. cerevisiae* is limited and this has resulted in a major bottleneck towards engineering of yeast as a CBP organism.

Several strain engineering strategies have been attempted to improve yeast heterologous protein secretion (Idris *et al,* 2010). These include overproduction of native chaperones and foldases as well as SNARE proteins and others, as was recently reviewed by Kroukamp *et al* (2018). Strain engineering strategies tended to have varying reporter protein specific effects on secretion titres. It was recently shown that highly active endocytosis decreased the overall protein titre in the supernatant (Rodríguez-Limas *et al*., 2015). Endocytosis and exocytosis are two forms of bulk transportation where molecules are transported either into or out of the cell with the use of ATP (Cooper, 2000). Endocytosis has a wide variety of functions in the cell and includes a carrier or a channel which facilitates the movement or transport of molecules or polysaccharides via a phospholipid bilayer into the cell from the surrounding medium (Park *et al*, 2016). Blocking endocytosis mechanisms may be a promising strain engineering option that can be used to increase cellulase secretion. The knock- out of genes involved in this process was thus investigated in this study to determine if an increase in secreted heterologous protein titre of enzymes required in biomass conversion could be attained.

In this study we have used *S. cerevisiae* strains disrupted in genes involved in the vacuolar sorting and endocytosis pathways to ascertain their influence on the heterologous secretion of xylanase, endoglucanase, β-glucosidase and cellobiohydrolase in *S. cerevisiae.* Specific knock- outs were obtained using the EUROSCARF strain collection and further double knockouts were made using some of the best mutants identified in the screening of single knock- outs. We observed that there was no mutant that had a general secretion enhancement and that all changes to activity levels were reporter protein specific. For example, *SSA4* and *END3* mutations enhanced xylanase secretion but inhibited Eg2 and Cbh1 secretion (Table 3.1). Furthermore, *RVS161* deletion enhanced Eg2 secretion but decreased Xyn2 and Cbh1 secretion. Finally, no mutant allowed for increased Cbh1 secretion, though this may indicate the importance of these genes for Cbh1 secretion. The effect of overexpressing these genes on the level of Cbh1 secretion should be tested in future.

This shows the varying effects the different knock- outs have on the diverse reporter genes. Xyn2, even though it is glycosylated, it is a small protein of about 20kDa, whereas Eg2 is more than double the size. This simple size factors plays an important role in the differences in secretion. The reporter proteins used also have various amounts of cysteine bridges that have to be folded correctly by the yeast, which is unaccountable for in our study, especially in Cbhs and Bgls that are also much larger proteins. With this said, some interventions such as those attempted in this study might not help all of the reporter genes in the same way. Xyn2 being a smaller protein with less cysteine bridges might not have much trouble folding while the Cbh with more cysteine bridges has more potential for errors in folding. This would thus account for the various differences in the effects of the interventions for the various reporter genes tested.

There were many endocytosis mutant strains, namely BYΔ*rvs161*-Eg2, BYΔ*vrp1*-Eg2, BYΔ*ssa4*-Xyn2 and BYΔ*end3*-Xyn2 that yielded significantly higher extracellular enzyme activity for the various enzymes tested. *RVS161* is an amphiphysin- like lipid protein involved in actin cytoskeleton polarization which regulates endocytosis is involved in cell fusion, cell polarity and the viability which follows osmotic stress or starvation (Lombardi and Riezman, 2001; Rondriquez- Limas *et al*, 2015). *END3* is needed in the actin cytoskeleton organization and forms part of the initializing step of endocytosis. This shows the importance of these genes directly in endocytosis (Benedetti *et al*, 1994). Thus, by performing the knock- out of the genes we showed that hindering the process does have an effect on the overall protein titre in the supernatant. Similarly, a study done on α- amylase where mutants with these gene knock- outs showed an increase in α- amylase (Rondriquez- Limas *et al*, 2015). This led us to create specific double knock- outs to test if there was an additive or synergistic effect when more than one of the positive affecting genes were mutated. For the double gene knock- outs, the BYΔ*clc1+rvs161*-Eg2, had the greatest increase in activity and indicated that there could be great value in the investigating a wider range of double knock-outs. The difference in secretion of protein in the supernatant could clearly be seen with SDS-PAGE analysis, showing that the differences in activity were due to the impact on secretion and not altered specific activity.

The tolerance of stress in the cell is of utmost importance as this would affect the fermentation efficiency, ultimately affecting ethanol productivity (Bauer and Pretorius, 2000). Results of the

spot plate assays showed that there were differences in ethanol stress tolerance in the respective plasmid bearing strains. The strains producing Xyn2 showed the best results for ethanol tolerance when compared to Cbh1 and Eg2 producing strains. For the temperature assays, overall, all of the plasmid producing strains showed similar results and managed to grow despite the increase in temperature. We could therefore conclude that the different reporter genes also lead to different amounts of stress on the cell which impact their tolerance of ethanol and heat stress. In closing, we have shown that manipulating endocytic mechanisms can make a positive contribution in creating CBP capable strains of the yeast *S. cerevisiae,* if the appropriate candidate yeast gene(s) can be identified and manipulated for the cellulases selected.

4.1. FUTURE PROSPECTS

Future work on this topic should include:

- Further analysis should be done to purify the enzymes produced by these mutants in order to confirm that there were no changes in specific activity.
- Growth curve analysis and proteomic analysis of these mutants would help understand the mechanism by which these mutants confer higher/lower secretion titres.
- Further growth analysis to ascertain the impact of the mutations on strain fitness, stress tolerance and ethanol productivity is also required.
- Overexpression of a number of genes identified in this study in Cbh1 bearing strains for comparative analysis.
- Testing how different strain backgrounds may influence the effect of endocytosis.

Future research could also combine the gene candidates shown here to have a positive effect with other genes that were previously shown to improve secretion.

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6. APPENDIX

6.1. Table 6.1. Description of genes used in this study according to the Saccharomyces cerevisiae genome database (www.yeastgenome.org).

GENE K/OS	DESCRIPTION
YPT7	Rab family GTPase; GTP-binding protein of the rab family; required for homotypic fusion event in vacuole inheritance
END3	EH domain-containing protein involved in endocytosis
VPS45	Protein of the Sec1p/Munc-18 family; essential for vacuolar protein sorting; required for the function of Pep12p
VRP1	Verprolin, proline-rich actin-associated protein; involved in cytoskeletal organization and cytokinesis; promotes actin nucleation and endocytosis
RVS161	Amphiphysin-like lipid raft protein; N-BAR domain protein that interacts with Rvs167p and regulates polarization of the actin cytoskeleton, endocytosis
CLC1	Clathrin light chain; subunit of the major coat protein involved in intracellular protein transport and endocytosis; regulates endocytic progression
UTH1	Mitochondrial inner membrane protein; role in mitophagy is disputed; implicated in cell wall biogenesis, the oxidative stress response, life span during starvation, and cell death; SUN family member; UTH1 has a paralog, NCA3, that arose from the whole genome duplication
VSM1	DNA damage-inducible v-SNARE binding protein; role in suppression of protein secretion; may play a role in S-phase checkpoint control
VTA1	Multivesicular body (MVB) protein; involved in endosomal protein sorting; regulates Vps4p activity by promoting its oligomerization
HAC1	Basic leucine zipper (bZIP) transcription factor (ATF/CREB1 homolog); regulates the unfolded protein response, via UPRE binding
VPS10	Type I transmembrane sorting receptor for multiple vacuolar hydrolases; cycles between the late-Golgi and prevacuolar endosome-like compartments.
MNN10	Subunit of a Golgi mannosyltransferase complex; complex mediates elongation of the polysaccharide mannan backbone; membrane protein of the mannosyltransferase family
SSA4	Heat shock protein that is highly induced upon stress; plays a role in SRP-dependent cotranslational protein-membrane targeting and translocation; member of the HSP70 family; cytoplasmic protein that concentrates in nuclei upon starvation; SSA4 has a paralog, SSA3, that arose from the whole genome duplication